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ESSENTIALS OF CHEMICAL PHYSIOLOGY

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CHEMICAL PHYSIOLOGY

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THE ESSENTIALS
OF
CHEMICAL PHYSIOLOGY

FOR THE USE OF STUDENTS

BY

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EIGHTH EDITION

With Coloured Plate

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PREFACE

TO

THE EIGHTH EDITION

THE few years which have elapsed since the publication of the seventh edition of this book have witnessed great advances in all branches of Bio-chemistry; increase of our knowledge has been mainly due to the introduction of new methods of research. The multiplication of accurate analytical processes has, in fact, been somewhat of an embarrassment to me in the work of revision, but I trust the selection I have ultimately made will be found a judicious one.

Although I have not increased the number of the lessons, it will be found that most of them have been somewhat lengthened. They can, however, be easily subdivided by the teacher according to the time at his disposal. In cases where numerous methods are described, having one object in common, it is not intended that the student should necessarily be acquainted with each. It is far better, for instance, that a student should know thoroughly one method for estimating sugar, than have an imperfect smattering of several. The selection of what methods are to be actually performed in class must depend upon the judgment of the individual teachers.

I shall not attempt here an enumeration of the alterations and additions I have made, for hardly a page has escaped revision, but will merely call attention to a change in the type used for the practical instructions. It is hoped that the heavy type adopted will impress the students with the importance of their practical work.

I have to acknowledge with thanks help and valuable suggestions from Mr J. Barcroft, F.R.S., especially in the sections which deal with Respiration, and the methods which he has introduced for investigating this subject. I am also indebted to my two colleagues at King's College, Dr O. Rosenheim and Mr C. F. Myers-Ward, for similar assistance in other portions of the book. Both of them have also rendered me invaluable service in the reading of the proof-sheets, and Mr Myers-Ward is responsible for the Index.

W. D. HALLIBURTON.

King's College,
London, 1913.

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ESSENTIALS OF CHEMICAL PHYSIOLOGY

INTRODUCTION

Chemical Physiology is a branch of physiological science which deals with the chemical composition of the body and the part played by the various substances found there in carrying out the phenomena of life. It thus differs from **Physiological Chemistry**, which is a branch of organic chemistry, and treats of the chemical composition and reactions of physiological substances. These two subjects are closely interwoven, and this book really deals with both, although special prominence will be given to their physiological aspect.

The substances found in the body are numerous, and in most cases complex; the majority of the foods from which the body is built up are equally elaborate, for animals do not possess to such an extent as plants do the power of building up complex from simple materials.

The **elements** found in the body are carbon, hydrogen, nitrogen, oxygen, sulphur, phosphorus, fluorine, chlorine, iodine, silicon, sodium, potassium, calcium, magnesium, lithium, iron, and occasionally manganese, copper, and lead.

Of these very few occur in the free state. Oxygen and nitrogen (to a small extent) are found dissolved in the blood-plasma; hydrogen is formed by putrefaction in the alimentary canal. With some few exceptions such as these, the elements enumerated above are found combined with one another to form compounds.

The **compounds** found in the body are divided into—

(1) Mineral or inorganic compounds, such as water and salts.
(2) Organic compounds, or compounds of carbon.

The time-honoured classification of the organic principles into

Proteins
Carbohydrates
Fats

is a little amplified in the following table:—

i. Nitrogenous:
    (*a*) *Proteins*, *e.g.* albumin, myosin, gelatin.
    (*b*) *Nitrogenous lipoids*, *e.g.* the fat-like substance known as lecithin.
    (*c*) *Products of protein cleavage*, *e.g.* amino-acids, urea, ammonia.
ii. Non-nitrogenous:
    (*a*) *Fats*, *e.g.* butter, fats of adipose tissue.
    (*b*) *Carbohydrates*, *e.g.* sugar, starch.
    (*c*) *Non-nitrogenous lipoids*, *e.g.* cholesterol.
    (*d*) *Simpler organic substances*, mainly products of the break-down of those previously enumerated, *e.g.* glycerol, fatty acids, lactic acid.

Living material or **protoplasm** is the substance of which the body cells are built. It is in a continual state of unstable chemical equilibrium, building itself up on the one hand, breaking down on the other; the term used for the sum-total of these intra-molecular rearrangements is **metabolism.**

The chemical substances in the protoplasm which are the most important from this point of view are the complex nitrogenous compounds called **Proteins** and the group of substances known as the **Lipoids.** So far as is at present known, proteins and lipoids are never absent from living substance, and up to the present time they have not been synthesised by laboratory processes.

The chemical structure of protoplasm can only be investigated after the protoplasm has been killed. The substances it yields are (1) **Water**; protoplasm is semi-fluid, and at least three-quarters of its weight, often more, are due to water. (2) **Proteins.** These are the most constant and abundant of the solids. A protein or albuminous substance contains the elements carbon, hydrogen, nitrogen, oxygen, with sulphur and phosphorus in small quantities only. In *nuclein*, a protein-like substance obtained from the nuclei of cells, phosphorus is more abundant. The protein obtained in greatest abundance from the cell-protoplasm is *nucleo-protein*: that is, a compound of protein with varying amounts of *nuclein.* White of egg is a familiar instance of an albuminous substance or protein, and the fact (which is also familiar) that this sets into a solid on boiling will serve as a reminder that the greater number of the proteins found in nature have a similar tendency to coagulate under the influence of heat and other agencies. (3) **Lipoids**, substances which resemble fats in their solubilities; they play an important part in metabolism; as instances of these we may mention **lecithin**, a fat-like substance containing phosphorus and nitrogen, and **cholesterol**, a monohydric alcohol. (4) **Inorganic salts**, especially phosphates and chlorides of calcium, sodium, and potassium.

The fats and carbohydrates are not essential constituents of protoplasm, but they are found within many cells (cell-contents) and are mainly utilised for combustion, whereby heat and other forms of energy are liberated.

ELEMENTARY COURSE

LESSON I

THE ELEMENTS CONTAINED IN PHYSIOLOGICAL COMPOUNDS

1. Take a fragment of meat about the size of a pea and place it in a porcelain crucible over a Bunsen flame. Note that it chars, showing the presence of carbon, and that it gives off the unpleasant odour of burning flesh, which is due to the fact that it contains the nitrogenous substances called proteins. In course of time the organic material is completely burnt up, and a small amount of white ash or inorganic material is left behind.

2. Repeat the experiment with a pure organic substance such as sugar. Note that no ash is left. Charring, as before, indicates the presence of carbon, but there is no characteristic smell of burning nitrogenous substances (absence of nitrogen).

3. The tests for carbon depend on the fact that when this element is oxidised it gives rise to carbon dioxide ; the test for hydrogen depends on the fact that when this element is oxidised it gives rise to water. If all the carbon dioxide and water formed by oxidation from a weighed amount of any organic substance under examination are collected and estimated, the amount of carbon and hydrogen respectively which it contains can be easily calculated. The following exercises, however, deal only with the qualitative detection of these elements.

4. TESTS FOR CARBON.—The following tests can be carried out with sugar.

(a) When burnt in the air it chars and subsequently the carbon entirely disappears, passing off in combination with oxygen as carbon dioxide (carbonic acid gas).

(b) Mix some of the powdered sugar in a dry mortar with about ten times the quantity of cupric oxide (which has been freed from water by previous heating); place the mixture in a dry test-tube provided with a rubber cork perforated by a bent glass tube which dips into either lime water or baryta water. Heat the tube over a Bunsen flame, and as the carbon of the sugar becomes oxidised carbon dioxide comes off and causes a white precipitate of calcium or barium carbonate, as the case may be.

5. TEST FOR HYDROGEN.—In the experiment just described (4 b) note that drops of water due to oxidation of hydrogen condense in the upper colder part of the test-tube.

6. TESTS FOR NITROGEN.—The greater number of tests for this element are due to the circumstance that on the breaking up of organic substances which contain it, it is given off as ammonia. If the ammonia is all collected and estimated, the amount of nitrogen can be easily calculated. Kjeldahl's method for carrying out this quantitative analysis is described ~~in the Appendix~~. The following exercises, however, are qualitative only.

XXII

(a) The characteristic odour of burning flesh, horn, hair, feathers, etc., has been already noted, and, though only a rough test, is very trustworthy.

(b) Take a little dried albumin and mix it thoroughly in a mortar with about twenty times the amount of soda-lime and heat in a test-tube over a Bunsen flame. Ammonia comes off in the vapours produced, and may be recognised by (i) its odour; (ii) it turns moistened red litmus paper (held over the mouth of the tube) blue; (iii) it gives off white fumes with a glass rod (held over the mouth of the tube) which has been dipped in hydrochloric acid.

(c) Mix some dried albumin with about ten times its weight of a mixture of equal parts of magnesium powder and anhydrous sodium carbonate. A small quantity of the mixture is then carefully heated in a dry test-tube and finally heated more strongly for about half a minute to red heat. Dip the tube whilst still glowing into a beaker containing about 10 c.c. of distilled water; the tube will break and its contents mix with the water. Filter and label the filtrate A; divide it into two parts; to one part add one or two drops of cold saturated solution of ferrous sulphate and a drop of ferric chloride solution. Warm the mixture, then cool and acidify with hydrochloric acid. The fluid becomes bluish-green, and gradually a precipitate of Prussian blue separates out. This test is due to the fact that some of the nitrogen is fixed as sodium cyanide, and this gives the Prussian blue reaction with the reagents added.

7. TESTS FOR SULPHUR.—(a) In the foregoing test (6 c) the sulphur of the albumin combines with the sodium to form sodium sulphide. This may be detected by taking the other part of the filtrate A and adding freshly prepared solution of sodium nitro-prusside; a reddish-violet colour forms.

(b) **TEST FOR LOOSELY COMBINED SULPHUR.**—Add 2 drops of a neutral lead acetate solution to a few c.c. of caustic soda solution. The precipitate of lead hydroxide which is first formed soon dissolves. Heat a small portion of the albumin with this alkaline solution. The mixture turns black in consequence of the formation of lead sulphide, part of the sulphur present in albumin having been split off from it by the caustic soda as sodium sulphide.

(c) Take some dried albumin and fuse with a mixture of potash and potassium nitrate. Cool; dissolve in water and filter. The filtrate will give the following test for sulphates:—Acidulate with hydrochloric acid and add barium chloride; a white precipitate of barium sulphate is produced.

8. **TEST FOR PHOSPHORUS.**—The test just described (7 c) may be repeated with some substance (such as caseinogen, nucleo-protein, or lecithin) which contains phosphorus in organic combination; or the organic matter may be more conveniently destroyed by Neumann's method, which consists in heating it with a mixture of sulphuric and nitric acids. The resulting fluid in each case gives the following test for phosphoric acid:—Mix it with half its volume of nitric acid; add ammonium molybdate in excess and boil; a yellow crystalline precipitate of ammonium phospho-molybdate falls.

SYMBOLS AND ATOMIC WEIGHTS OF THE PRINCIPAL ELEMENTS

Aluminium	Al	27·1	Magnesium	Mg	24·32
Antimony	Sb	120·2	Manganese	Mn	55·0
Arsenic	As	75·0	Mercury	Hg	200·0
Barium	Ba	137·37	Nickel	Ni	58·7
Bismuth	Bi	208·0	Nitrogen	N	14·01
Boron	B	11·0	Osmium	Os	191·0
Bromine	Br	79·92	Oxygen	O	16·0
Cadmium	Cd	112·4	Phosphorus	P	31·0
Calcium	Ca	40·1	Platinum	Pt	195·0
Carbon	C	12·0	Potassium	K	39·10
Chlorine	Cl	35·46	Silver	Ag	107·88
Copper	Cu	63·57	Silicon	Si	28·3
Fluorine	F	19·0	Sodium	Na	23·00
Gold	Au	197·2	Strontium	Sr	87·6
Hydrogen	H	1·008	Sulphur	S	32·07
Iodine	I	126·92	Tin	Sn	119·0
Iron	Fe	55·85	Tungsten	W	184·0
Lead	Pb	207·1	Zinc	Zn	65·37

The above atomic weights are taken on the basis that O = 16.

The practical exercises of the foregoing lesson show, in the first instance, how the substances with which we have to deal fall under the two main categories of organic and inorganic. In some of the tissues of the body, such as bone and tooth, the inorganic or mineral material is in excess, but in the softer portions of the organism the organic compounds are in great preponderance.

Organic chemistry is sometimes defined as the chemistry of the carbon compounds; carbon is in all cases present, and is usually the most abundant element.

The most important of the nitrogenous substances are the proteins, as already explained in the introductory chapter, and the detection and estimation of nitrogen are thus exercises of the highest interest.

All the proteins contain a small amount of sulphur; keratin, or horny material, contains more than most of them do.

Phosphorus is another element of considerable importance, being present in nuclein and nucleo-proteins, and also in certain complex lipoids, of which lecithin may be taken as a type. Iodine occurs united to protein material in the colloid substance of the thyroid gland; iron in the pigment of the blood called hæmoglobin; sodium, calcium, potassium, and other metals in the inorganic substances of the body. It would, however, lead us too far into the regions of pure chemistry to undertake exercises for the detection of these and other elements which might be mentioned, and have been already commented upon. The teacher of physiological chemistry is bound to assume that the students who come before him have already passed through a course of ordinary chemistry.

The main interest of the exercises selected as types lies in their physiological application. As a rule an element is detected by breaking up or oxidising the more or less complex molecule in which it occurs into substances of simpler nature, and then performing tests for these simpler products. Thus carbon is identified by the formation of carbon dioxide, nitrogen by the formation of ammonia, and so forth.

A great many reactions which can be performed in the test-tube imitate those which are performed in the body. Reactions *in vitro* and *in vivo*, to use the technical phrases, often, though not always, run parallel. Life, from one point of view, is a process of combustion or oxidation; the fuel is supplied by the food; this is incorporated by the tissues and is then burnt up by the oxygen brought to it by the blood-stream, giving rise to animal heat and other manifestations of energy; and finally the simple products of oxidation or chemical breakdown are carried to the organs of excretion (lung, skin, kidney, etc.), where they are discharged from the body.

A candle consists principally of carbon and hydrogen; when it is burnt the products are carbonic acid gas and water; the former may be detected by means of lime water, the latter, by holding a dry beaker upside down for a few moments over the burning candle, when the moisture will condense on the cold glass.

The body is more complex than a candle, but so far as its carbon and hydrogen are concerned the final products of combustion are the same. The carbon dioxide is discharged by the expired air, as may be proved by blowing it into lime water. The water finds an outlet by several channels, lungs, skin, and kidneys. The presence of nitrogen in the body is perhaps the most striking chemical distinction between it and a candle, and here again the process of metabolism runs a course analogous in some degree to our experiments *in vitro*; for the most important and abundant substance which contains the waste nitrogen is the simple material ammonia; but ammonia is only discharged as such to a very small extent in health. It unites with carbon and oxygen to form the substance called urea (CON_2H_4), which finds its way out of the body *viâ* the urine. The urine also contains the sulphates, due to the oxidation of the sulphur of the proteins, and the phosphates due to the similar oxidation of the phosphorus of such substances as lecithin and nuclein. Some of the salts of the urine, however, in particular the chlorides, come directly from the food. This we shall discuss at the proper place when we come to the study of the urine.

LESSON II

SOME TYPICAL ORGANIC COMPOUNDS

1. ALCOHOL (*ethyl alcohol*).—**The following reactions are to be carried out with 96 per-cent. alcohol or with the distillate obtained from an alcoholic liquid. The use of a distilling apparatus should de demonstrated.**

(a) Add 10 drops of alcohol to a small quantity of sodium acetate crystals in a test-tube. Allow about 20 drops of concentrated sulphuric acid to fall on the mixture and warm gently. *Acetic ester* **(ethyl acetate) is formed, and is recognised by its characteristic smell:**

$$C_2H_5OH + CH_3COOH = CH_3.COOC_2H_5 + H_2O.$$

(b) Mix a few drops of alcohol with an equal amount of benzoyl chloride and add excess of strong potash. On continuous shaking the irritant smell of benzoyl chloride disappears and is replaced by the fruit-like odour of *benzoic ester* **:—**

$$C_2H_5OH + C_6H_5COCl = C_6H_5.COOC_2H_5 + HCl.$$

(c) Heat 3 drops of alcohol with 2 drops of concentrated sulphuric acid. After cooling notice the smell of *ether* **(ethyl ether).**

(d) Warm 10 drops of alcohol with 5 drops of concentrated sulphuric acid. After cooling neutralise with potash or soda. Potassium or sodium-ethyl sulphate is thus obtained; add a few drops of sodium sulphide. On warming, *ethyl mercaptan* **is formed and may be recognised by its garlic-like smell:—**

$$C_2H_5.SO_4Na + NaSH = C_2H_5.SH + Na_2SO_4.$$

(e) Add 1 drop of alcohol to about 5 c.c. water. Make alkaline with strong potash and add iodine solution until the fluid remains yellow. On warming a yellow crystalline precipitate of iodoform (CHI_3) forms, and its characteristic smell is noticed (Lieben's reaction):—

$$C_2H_5.OH + 6NaOH + 4I_2 = CHI_3 + H.COONa + 5NaI + 5H_2O.$$

2. ALDEHYDE (*acetaldehyde*).—**Warm a few drops of alcohol with a solution of potassium bichromate and dilute sulphuric acid. The solution**

turns green, owing to reduction. *Aldehyde* is formed and recognised by its penetrating and irritant smell :—

$$CH_3.CH_2.OH + O = CH_3.CHO + H_2O.$$

Perform the following reactions with the solution of aldehyde supplied.

(a) Acetaldehyde solution *reduces Fehling's solution* on boiling, and a yellowish-red precipitate of cuprous oxide is formed :—

$$CH_3CHO + 2CuO + NaOH = CH_3.COONa + Cu_2O + H_2O.$$

(b) Add a few drops of ammonia to silver nitrate until the white precipitate just redissolves, and add a few drops of dilute aldehyde solution. Then add a little potash, place the tube in a cold-water bath and heat to boiling. A mirror of *metallic silver* is formed.

$$Ag_2O + CH_3.CHO = Ag_2 + CH_3.COOH.$$

(c) On warming an aldehyde solution with caustic alkalis a brown resinous substance (aldehyde resin) is formed ; this is insoluble in water, but easily soluble in alcohol or ether.

(d) Add a small amount of an aldehyde solution to a solution of phenylhydrazine hydrochloride and sodium acetate. An oily *hydrazone* is formed :—

$$C_6H_5.NH.NH_2 + CH_3.CHO = C_6H_5.NH.N : CH.CH_3 + H_2O.$$

3. FORMIC ACID.—(a) Sodium formate, dissolved in a little water, is acidified with dilute sulphuric acid and warmed. Formic acid comes off and is recognised by its smell.

(b) Warm a concentrated solution of sodium formate with concentrated sulphuric acid. An odourless gas is developed which burns with a blue flame (*Carbon monoxide*) :—

$$H.COOH = CO + H_2O.$$

(c) Add a few drops of alcohol and a few drops of concentrated sulphuric acid to some dry sodium formate in a test-tube and warm the mixture. Notice the smell of *formic ester*.

(d) Add silver nitrate to a dilute solution of sodium formate and warm. A deposit of black *metallic silver* is formed :—

$$2H.COOAg = Ag_2 + H.COOH + CO_2.$$

(e) Heat a solution of mercuric chloride with a solution of sodium formate. *A white precipitate of mercurous chloride* is formed :—

$$2H.COONa + 2HgCl_2 = Hg_2Cl_2 + CO_2 + CO + 2NaCl + H_2O.$$

(f) A solution of formate gives a red solution on the addition of ferric chloride. On boiling a *brownish-red precipitate* is formed (a basic iron formate) which dissolves on the addition of hydrochloric acid.

4. **ACETIC ACID** is prepared from alcohol by oxidising it with potassium permanganate and sulphuric acid, and then distilling it over. It is recognised by—

(a) The characteristic taste and smell of vinegar.

(b) Neutralise a few drops of glacial acetic acid with potash or soda and add ferric chloride; the red colour of ferric acetate appears; on boiling a brownish-red precipitate of basic ferric acetate is formed.

5. **OXALIC ACID.**—(a) Heat a few crystals of oxalic acid on platinum or nickel foil. It volatilises without charring or separation of carbon.

(b) Dissolve a few crystals of oxalic acid in a few c.c. of concentrated sulphuric acid and warm. Carbon monoxide and carbon dioxide are evolved:—

$$COOH.COOH = CO_2 + CO + H_2O.$$

(c) Acidify a solution of oxalic acid with dilute sulphuric acid and add potassium permanganate. The colour of the permanganate solution is discharged:—

$$COOH.COOH + O = 2CO_2 + H_2O.$$

(d) Calcium chloride (or barium chloride) gives a white precipitate with oxalic acid solution. These oxalates are not soluble in acetic acid, but are easily soluble in hydrochloric acid.

6. **PHENOL** (*carbolic acid*).—(a) Add a few drops of dilute ferric chloride to a phenol solution: a purple colour is formed, which is discharged by hydrochloric acid:—

$$3C_6H_5OH + FeCl_3 = (C_6H_5O)_3Fe + 3HCl.$$

(b) On adding bromine water to a dilute solution of phenol, a yellowish-white flocculent precipitate of tribromophenol is produced even in very dilute solutions:—

$$C_6H_5OH + 3Br_2 = C_6H_2Br_3.OH + 3HBr.$$

(c) Boil a dilute phenol solution with nitric acid. A bright yellow solution results (picric acid), which turns into a brownish-yellow on adding alkali:—

$$C_6H_5OH + 3HNO_3 = C_6H_2(NO_2)_3OH + 3H_2O.$$

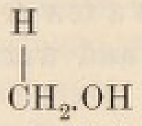
(d) Millon's reagent gives a deep red colour on warming with even very dilute phenol solutions.

It is necessary to study first some of the simpler organic compounds in order that we may understand the nature of the more elaborate substances which are found in the body.

Hydrocarbons.—These are compounds of hydrogen and carbon, and form the basis of classification in organic chemistry. The simplest hydrocarbon known is *methane*, or marsh gas; it has the formula CH_4; if one of its hydrogen atoms is replaced by hydroxyl OH, we obtain the simplest alcohol; thus:—

$$\begin{array}{c} H \\ | \\ CH_3 \end{array}$$

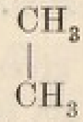
[methane]



$$\begin{array}{c} H \\ | \\ CH_2.OH \end{array}$$

[methyl alcohol]

The next alcohol in the series is formed in a similar way from the next hydrocarbon C_2H_6 (*ethane*); thus:—



$$\begin{array}{c} CH_3 \\ | \\ CH_3 \end{array}$$

[ethane]

$$\begin{array}{c} CH_3 \\ | \\ CH_2.OH \end{array}$$

[ethyl alcohol]

and so on.

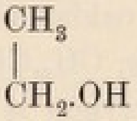
Alcohols.—We may take ordinary or *ethylic* alcohol as a type of that important class of organic substances known as the alcohols. These are substances the knowledge of which may be regarded as the starting point of many other organic compounds. Ethylic alcohol has the formula C_2H_6O; one of its hydrogen atoms is replaceable by metals such as sodium or potassium, and we may therefore write the formula C_2H_5OH, the last hydrogen atom being the one which is replaceable by other monad elements. This atom is united to oxygen to form the atomic group called hydroxyl (OH). The hydroxyl can be replaced by chlorine by treating alcohol with phosphorus pentachloride, and a substance with the formula C_2H_5Cl is obtained. The atomic group C_2H_5 which is unchanged and united to OH in alcohol, and to Cl in ethyl chloride, is called *ethyl*.

There are other alcohols in which the place of ethyl is taken by other organic radicals, or *alkyls*. Thus if the radical called *methyl* (CH_3) is united to hydroxyl we obtain *methylic* alcohol, $CH_3.OH$. These two alcohols, methylic and ethylic, form the first two members of a group of alcohols, which are termed monohydric; all of these contain only one hydroxyl group, and the following is a list of the first six members of this group with their formulæ:—

$CH_3.OH$	methyl alcohol
$C_2H_5.OH$	ethyl ,,
$C_3H_7.OH$	propyl ,,
$C_4H_9.OH$	butyl ,,
$C_5H_{11}.OH$	amyl ,,
$C_6H_{13}.OH$	hexyl ,,

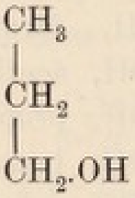
Each differs from the preceding by CH_2.

Aldehydes and Ketones.—In the alcohols, there are possibilities for *isomerism* to occur; by this one means that although two (or more) substances may have the same empirical formula, the arrangement of the atoms or atomic groups within the molecule is different. A *primary* alcohol is one in which the hydroxyl (OH), and *two* hydrogen atoms are attached to the same carbon atom; it therefore contains the group $CH_2.OH$. Thus the formula for common alcohol (primary ethyl alcohol) may be written:—



$$\begin{array}{l} CH_3 \\ | \\ CH_2.OH \end{array}$$

and the formula for the next alcohol of the series (primary propyl alcohol) is:—



$$\begin{array}{l} CH_3 \\ | \\ CH_2 \\ | \\ CH_2.OH \end{array}$$

If a primary alcohol is oxidised, two atoms of hydrogen are removed, and the oxidation product so formed is called an *aldehyde* (the name is derived from "alcohol dehydrogenatum"); thus methyl alcohol yields *formaldehyde*, ethyl alcohol yields *acetic aldehyde*, and propyl alcohol yields *propionic aldehyde*, as shown in the following equations:—

$$\underset{\text{[methyl alcohol]}}{H.CH_2.OH} + O = \underset{\text{[formaldehyde]}}{H.CHO} + H_2O$$

$$\underset{\text{[ethyl alcohol]}}{CH_3.CH_2OH} + O = \underset{\text{[acetic aldehyde]}}{CH_3.CHO} + H_2O$$

$$\underset{\text{[propyl alcohol]}}{CH_3.CH_2.CH_2OH} + O = \underset{\text{[propionic aldehyde]}}{CH_3.CH_2.CHO} + H_2O$$

The typical group of the aldehydes (CHO) is not stable, but is easily oxidisable to form the group COOH, which is called *carboxyl.* The readiness with which aldehydes are oxidisable renders them powerful reducing agents, and tests for their detection mainly depend upon this fact.

A secondary alcohol is one in which the OH group and *one* hydrogen atom is attached to the same carbon atom—thus secondary propyl alcohol (the first case where isomerism is possible) has the formula

$$CH_3.CHOH.CH_3.$$

The typical group is therefore the divalent radical $>CHOH$, and when this is oxidised, the first oxidation product is called a *ketone*, thus:—

$$\underset{\text{[secondary propyl alcohol]}}{CH_3.CHOH.CH_3} + O = \underset{\text{[propyl-ketone]}}{CH_3.CO.CH_3} + H_2O$$

It therefore contains the group $>CO$. Propyl-ketone is usually called acetone, the tests for which we shall have to consider in our study of diabetic urine.

In the next alcohol (*butyl alcohol*), four isomers are possible, one of which is a tertiary alcohol, *i.e.* it contains the trivalent radical $\equiv C - OH$, which breaks down on oxidation into smaller molecules.

The Fatty Acids.—These form a series of acids derived from the monohydric alcohols by oxidation. Thus to take ordinary ethyl alcohol $CH_3.CH_2OH$, the first stage in oxidation is the removal of two atoms of hydrogen to form an aldehyde, $CH_3.CHO$, as we have just seen; on further oxidation an atom of oxygen is added to form the acid called acetic acid, $CH_3.COOH$. A similar acid can be obtained from the other alcohols of the series, thus:—

From	methyl	alcohol	$CH_3.OH$,	formic	acid	$H.COOH$	is obtained.
,,	ethyl	,	$C_2H_5.OH$,	acetic	,,	$CH_3.COOH$	,,
,,	propyl	,,	$C_3H_7.OH$,	propionic	,,	$C_2H_5.COOH$	,,
,,	butyl	,,	$C_4H_9.OH$,	butyric	,,	$C_3H_7.COOH$	,,
,,	amyl	,,	$C_5H_{11}.OH$,	valeric	,,	$C_4H_9.COOH$	,,
,,	hexyl	,,	$C_6H_{13}.OH$,	caproic	,,	$C_5H_{11}.COOH$	,,

and so on.

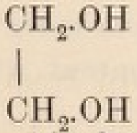
Or in general terms:—

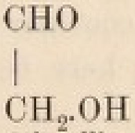
From the alcohol with formula $C_nH_{2n+1}.OH$ the acid with formula $C_{n-1}H_{2n-1}COOH$ is obtained. The above series of acids constitutes that known as the *fatty acid* series. Just as their parent alcohols contain one OH group, so do the acids contain one carboxyl group (COOH); they are therefore termed *mono-carboxylic acids.*

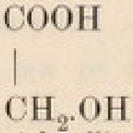
In addition to the monohydric alcohols, there are other series of alcohols which differ from these in containing more than one OH group. Those which, like glycol $[C_2H_4.(OH)_2]$, contain two OH groups are called dihydric; those which, like glycerol $[C_3H_5(OH)_3]$, contain three OH groups are called trihydric, there are also tetrahydric, pentahydric, hexahydric, etc., series of alcohols, containing respectively four, five, six, etc., hydroxyl groups; and the hexahydric alcohols are particularly interesting to the physiologist as they are the parent substances of the chief carbohydrates.

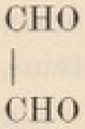
The aldehydes or ketones obtained as the first stages in the oxidation of these alcohols are correspondingly complex; and by still further oxidation organic acids are produced. Thus *oxalic acid* is an instance of an acid obtained by the oxidation of a dihydric alcohol; it is therefore a *di-carboxylic acid*, as it contains two carboxyl (COOH) groups, just as the alcohol (glycol) from which it is derived contains two hydroxyl groups.

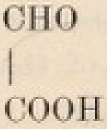
We may give the formulæ for glycol and its derivatives as an example of a dihydric alcohol, but it will not be necessary at this point to go into further details of other more complex alcohols:—

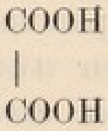

$$\begin{array}{c} CH_2.OH \\ | \\ CH_2.OH \end{array}$$
[glycol]


$$\begin{array}{c} CHO \\ | \\ CH_2.OH \end{array}$$
[glycollic aldehyde]


$$\begin{array}{c} COOH \\ | \\ CH_2.OH \end{array}$$
[glycollic acid]


$$\begin{array}{c} CHO \\ | \\ CHO \end{array}$$
[glyoxal]


$$\begin{array}{c} CHO \\ | \\ COOH \end{array}$$
[glyoxylic acid]


$$\begin{array}{c} COOH \\ | \\ COOH \end{array}$$
[oxalic acid]

Ethers are obtained by abstracting water from two molecules of an alcohol, and may be regarded as the anhydrides of alcohols. Ethyl ether, usually called *ether* simply, is obtained by heating alcohol with concentrated sulphuric acid, and the reaction occurs in the two stages represented by the following equations:—

$$\underset{\text{[ethyl alcohol]}}{C_2H_5.OH} + H_2SO_4 = \underset{\text{[ethyl sulphuric acid]}}{C_2H_5.SO_3.OH} + H_2O$$

$$\underset{\text{[ethyl alcohol]}}{C_2H_5.OH} + \underset{\text{[ethyl sulphuric acid]}}{C_2H_5.SO_3.OH} = \underset{\text{[ether]}}{C_2H_5.O.C_2H_5} + H_2SO_4$$

It will be noticed that the sulphuric acid is recovered unchanged at the end of the reaction, and therefore theoretically a small amount of sulphuric acid will transform an unlimited amount of alcohol into ether. We shall find later in our study of the action of enzymes that these important agents in the chemical transformations in the body are characterised by the same property. They probably act in a manner analogous to sulphuric acid in etherification, participating in intermediate reactions, but are present unchanged in the terminal reaction.

Esters.—The formation of esters or compounds with acids is a reaction typical of all alcohols, and is analogous to the formation of salts which occurs when a metallic hydroxide reacts with an acid. Thus if sodium hydroxide and nitrous acid react together we get sodium nitrite and water, as shown in the following equation:—

$$HNO_2 + NaOH = NaNO_2 + H_2O.$$

The similar interaction of ethyl alcohol and nitrous acid, results in the formation of an ester (ethyl nitrite) and water :—

$$HNO_2 + \underset{\text{[ethyl alcohol]}}{C_2H_5.OH} = \underset{\text{[ethyl nitrite]}}{C_2H_5.NO_2} + H_2O$$

The next equation represents the reaction between alcohol and an organic acid (acetic) :—

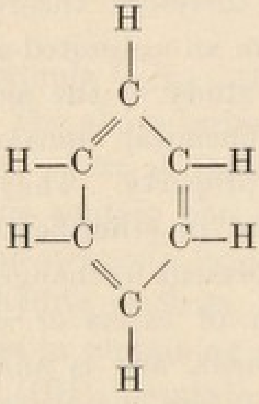
$$\underset{\text{[acetic acid]}}{CH_3.COOH} + \underset{\text{[ethyl alcohol]}}{C_2H_5.OH} = \underset{\substack{\text{[ethyl acetate or}\\ \text{acetic ester]}}}{CH_3.CO.O.C_2H_5} + H_2O$$

Amino-acids.—These are nitrogenous derivatives of the fatty acids and are the building stones from which the proteins are constructed; conversely they are the products obtained from proteins when these complex substances are broken up. We shall consider them more fully in our study of the proteins, and so we may here be content with a typical example.

Acetic acid is $CH_3.COOH$.

If one of the hydrogen atoms in the CH_3 group is replaced by the amino group (NH_2) we obtain $CH_2.NH_2.COOH$, which is *amino-acetic acid* or *glycine*.

Aromatic Compounds.—These are derivatives of the hydrocarbon called benzene, C_6H_6. The organic substances we have considered up to this point are usually spoken of as belonging to the fatty or aliphatic series; in these the carbon atoms are united together in an open chain. The aromatic compounds on the other hand are characterised by the carbon atoms being united together by alternate single and double bonds into a ring, and the formula for benzene, the simplest member of the group, may therefore be written graphically in the following way :—

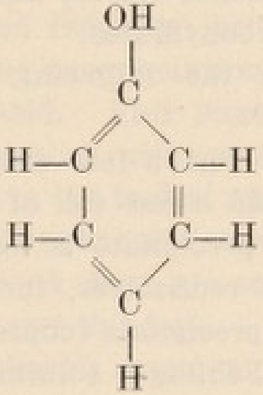


By substituting different groups (side-chains) for one or more of the hydrogen atoms, all other aromatic compounds are obtained.

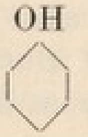
The benzene nucleus itself is extremely stable; such processes as oxidation and reduction can be applied to it without destroying it.

The production of nitro-compounds by the action of nitric acid is characteristic of derivatives of benzene, whereas such treatment will usually oxidise and decompose aliphatic substances. A few aromatic compounds are found in the animal organism, for instance, hippuric acid in the urine; some, such as tyrosine, are found among the decomposition products of proteins; hence a knowledge of these substances is necessary for the student of physiology and medicine.

Phenol or carbolic acid is included among the substances tested for in the accompanying practical lesson to remind the student of the existence of aromatic compounds; it is a hydroxyl derivative of benzene, and its formula may be written $C_6H_5.OH$, or graphically :—



that is, one of the hydrogen atoms is replaced by hydroxyl (OH). It is, however, usual to write it as shown below :—



the unchanged portion of the benzene nucleus being depicted by a simple hexagon.

We shall come across more complicated derivatives of benzene in the further study of our subject; and we shall moreover meet with other ringed compounds (heterocyclic) in which nitrogen occurs within the ring; such substances as pyridine and pyrrol and their derivatives are included in this category. They are important as the mother substances of many alkaloids.

LESSON III

THE CARBOHYDRATES

Glucose, cane sugar, dextrin, starch, and glycogen are given round as typical and important carbohydrates.

1. GLUCOSE.—Perform the following tests with a solution of glucose :—

(a) *Trommer's Test.*—Put a few drops of copper sulphate solution into a test-tube and add a few c.c. of strong caustic potash. On adding the caustic potash a precipitate is formed, which, on addition of a glucose solution, rapidly redissolves, forming a blue solution. On boiling this a yellow or red precipitate (cuprous hydrate or oxide) forms.

(b) *Fehling's Test.*—Fehling's solution is a mixture of copper sulphate, caustic soda, and potassium sodium tartrate (Rochelle salt) of a certain strength. It is used for estimating glucose quantitatively (see Lesson XII). It may be used as a qualitative test also. Boil some Fehling's solution ; if it remains clear it is in good condition ; add to it an equal volume of solution of glucose and boil again. Reduction, resulting in the formation of cuprous hydrate or oxide, takes place as in Trommer's test. This test is more certain than Trommer's, and is preferable to it.

(c) *Nylander's Test.*—Mix 5 c.c. of glucose solution with 1 of Nylander's reagent (20 gr. of bismuth subnitrate and 50 gr. of Rochelle salt dissolved in 1 litre of 8 per-cent. sodium hydroxide). Boil for three minutes and allow to cool. A black precipitate of metallic bismuth separates out.

N.B.—Sugars such as glucose, fructose, maltose, and lactose, which give the preceding tests, are called reducing sugars.

(d) *Moore's Test.*—Add to the glucose solution about half its volume of 20 per-cent. potash and heat. The solution becomes yellowish-brown. Acidify with sulphuric acid (25 per cent.) and the odour of caramel becomes apparent.

(e) *Fermentation Test.*—Add a fragment of dried yeast to the glucose solution in a test-tube ; fill the test-tube up with mercury, and invert it over mercury in a trough. Place it in an incubator at body temperature for twenty-four hours. The sugar is broken up into alcohol and carbon dioxide; the latter gas collects in the upper part of the test-tube. The alcohol may be detected by Lieben's reaction (1 e, p. 11).

(f) *Molisch's Reaction.*—Add a few drops of an alcoholic solution of thymol or α-naphthol to a solution of sugar, and allow a few drops of concentrated sulphuric acid to run to the bottom of the test-tube. A purple ring forms at the surface of contact.

This test is given by all the sugars, and in fact by all carbohydrates with more or less intensity; and it is also given by those proteins which contain a carbohydrate radical.

2. **SUCROSE** or **CANE SUGAR.**—(a) The solution of cane sugar when mixed with copper sulphate and caustic potash gives a blue solution. But on boiling no reduction occurs.

(b) Take some of the cane-sugar solution and boil it with a few drops of 25 per-cent. sulphuric acid. This converts it into equal parts of glucose and fructose. Neutralise with potash or soda. It then gives Trommer's or Fehling's test in the typical way.

(c) Boil some of the cane-sugar solution with an equal volume of concentrated hydrochloric acid. A deep red solution is formed. Glucose, lactose, and maltose do not give this test.

(d) Cane sugar gives Molisch's reaction.

3. **STARCH.**—(a) Examine starch grains with the microscope. In size and other minor particulars the starch grains differ according to their source. Potato starch is readily obtained by mounting a scraping from the surface of a freshly cut potato; these are specially large; those from rice are smaller. Note the concentric markings on the starch grains. If a drop of iodine solution is run in under the cover-slip the grains are stained blue.

(b) Starch is not soluble in cold water. Mix a little starch with cold water and pour boiling water into the paste. Continue to boil until an opalescent solution is formed; this, if strong, gelatinises on cooling.

(c) Add iodine solution. An intense blue colour is produced, which disappears on heating, and if not heated too long reappears on cooling.

N.B.—Prolonged heating drives off the iodine, and consequently no blue colour returns after cooling.

(d) Conversion into dextrin and glucose. To some starch solution in a flask add a few drops of 25 per-cent. sulphuric acid, and boil for fifteen minutes. Take some of the liquid, which is now clear, neutralise with soda, and show the presence of dextrin and glucose.

4. **DEXTRIN.**—(a) Add iodine solution to solution of dextrin; a reddish-brown colour is produced. The colour disappears on heating and reappears on cooling.

(b) Saturate a dextrin solution by grinding it in a mortar with finely powdered ammonium sulphate; filter. The erythro-dextrin is precipitated, but only *incompletely* ; therefore the filtrate gives a red-brown colour with a drop of iodine solution.

(c) Dextrin gives a slight reduction with Fehling's solution.

5. GLYCOGEN.—Solution of glycogen is given round: (a) it is opalescent like that of starch.

(b) With iodine solution it gives a brown colour very like that given by dextrin. The colour disappears on heating and reappears on cooling.

(c) By boiling with 25 per-cent. sulphuric acid it is converted into glucose. Neutralise and test with Fehling's solution.

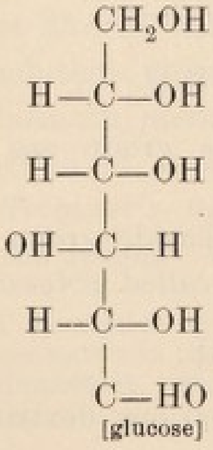
(d) Saturate the solution with ammonium sulphate as in 4 b, and filter. The glycogen is *completely* precipitated, and therefore the filtrate gives no coloration with iodine. This easily distinguishes it from dextrin.

The carbohydrates are found chiefly in vegetable tissues, and many of them form important foods. Some carbohydrates are, however, found in or formed by the animal organism. The most important of these are *glycogen*, or animal starch; *glucose*; and *lactose*, or milk sugar.

The carbohydrates may be conveniently defined as compounds of carbon, hydrogen, and oxygen, the two last-named elements being in the proportion in which they occur in water. But this definition is only a rough one, and if pushed too far would include many substances, such as acetic acid, lactic acid, and inosite, which are not carbohydrates. Research has shown that the chemical constitution of the simplest carbohydrates is that of an aldehyde, or a ketone, and that the more complex carbohydrates are condensation products of the simple ones.

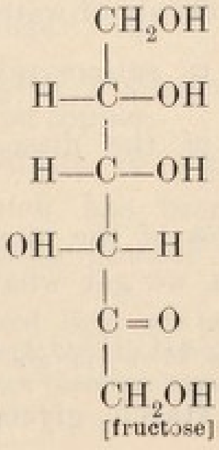
The meaning of the terms "aldehyde" and "ketone" has been explained in the preceding lesson, but there we drew most of our examples from simple aldehydes and ketones derived by oxidation from monohydric alcohols. In the case of the sugars, we have to start from more complex alcohols, namely, those which are called hexahydric, on account of their containing six OH groups. The majority of the known sugars are aldehydes (*aldoses*). Sugars which are ketones are called *ketoses*, but only one of these, namely, fructose, is of physiological interest. This constitution of the sugars explains why it is that they are reducing agents.

Three hexahydric alcohols, all with the same empirical formula $C_6H_8(OH)_6$, may be mentioned; they are isomerides, and their names are sorbitol, mannitol, and dulcitol. By careful oxidation their aldehydes and ketones can be obtained; these are the simple sugars; thus, glucose is the aldehyde of sorbitol; mannose is the aldehyde of mannitol; fructose is the ketone of mannitol; and galactose is the aldehyde of dulcitol. These sugars all have the empirical formula $C_6H_{12}O_6$.



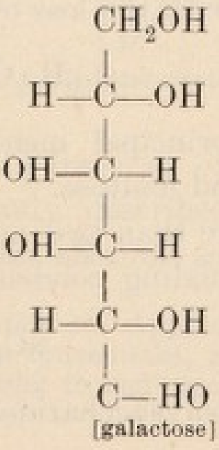
$$\begin{array}{c} CH_2OH \\ \mid \\ H-C-OH \\ \mid \\ H-C-OH \\ \mid \\ OH-C-H \\ \mid \\ H-C-OH \\ \mid \\ C-HO \end{array}$$

[glucose]



$$\begin{array}{c} CH_2OH \\ \mid \\ H-C-OH \\ \mid \\ H-C-OH \\ \mid \\ OH-C-H \\ \mid \\ C=O \\ \mid \\ CH_2OH \end{array}$$

[fructose]



$$\begin{array}{c} CH_2OH \\ \mid \\ H-C-OH \\ \mid \\ OH-C-H \\ \mid \\ OH-C-H \\ \mid \\ H-C-OH \\ \mid \\ C-HO \end{array}$$

[galactose]

They furnish an excellent example of what is called stereochemical isomerism; that is, the position of the atoms or groups of atoms in space within the sugar molecule varies. The constitutional formulæ of three important simple sugars are shown on p. 23. The six carbon atoms in each case form an open chain, but the way in which the hydrogen and hydroxyl atoms are linked to them differs.

The aldehyde constitution of glucose and of galactose is at once evident from these formulæ, the typical aldehyde group (CHO or more accurately $O=C-H$) being at the end of the chain. The ketone constitution of fructose is also shown by the typical ketone group (CO) not at the end of the chain.

By further oxidation, the sugars yield various acids. If we take these sugars as typical specimens, we see that their general formula is

$$C_nH_{2m}O_m$$

and as a general rule $n=m$; that is, the number of oxygen and carbon atoms are equal. This number in the case of the sugars already mentioned is six. Hence they are called *hexoses.*

Sugars are known to chemists, in which this number is 3, 4, 5, 7, etc., and these are called trioses, tetroses, pentoses, heptoses, etc. The majority of these have no physiological interest. It should, however, be mentioned that a pentose has been obtained from certain nucleic acids presently to be described (see p. 64), which are contained in animal organs (pancreas, liver, etc.), and in plants (for instance, yeast). If the pentoses which are found in various plants are given to an animal, they are excreted in great measure unchanged in the urine.

The hexoses are of great physiological importance. The principal ones are glucose, fructose, and galactose. These are called *monosaccharides.*

Another important group of sugars is that of the *disaccharides*; these are formed by the combination of two molecules of monosaccharides with the loss of a molecule of water, thus:—

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

The principal members of the disaccharide group are sucrose, lactose, and maltose.

If more than two molecules of the monosaccharide group undergo a corresponding condensation, we get what are called *polysaccharides.*

$$nC_6H_{12}O_6 = (C_6H_{10}O_5)_n + nH_2O.$$

The polysaccharides are starch, glycogen, various dextrins, cellulose, etc. We may therefore arrange the important carbohydrates of the hexose family in a tabular form as follows:—

1. Monosaccharides, $C_6H_{12}O_6$.	2. Disaccharides, $C_{12}H_{22}O_{11}$.	3. Polysaccharides, $(C_6H_{10}O_5)_n$.
+ Glucose. − Fructose. + Galactose.	+ Sucrose. + Lactose. + Maltose.	+ Starch. + Glycogen. + Dextrin. Cellulose.

The signs + and − in the above list indicate that the substances to which they are prefixed are dextro- and lævo-rotatory respectively as regards polarised light.[1] The formulæ given above are merely empirical: the quantity n in the starch group is variable and often large. The following are the chief facts in relation to each of the principal carbohydrates.

MONOSACCHARIDES

Glucose.—This carbohydrate (which is also known as dextrose and grape sugar) is found in fruits, honey, and in minute quantities in the blood (0·12 per cent.) and numerous tissues, organs, and fluids of the body. It is the form of sugar found in larger quantities in the blood and urine in the disease known as diabetes.

Glucose is soluble in hot and cold water and in alcohol. It is crystalline, but not so sweet as cane sugar. When heated with strong potash certain complex acids are formed which have a yellow or brown colour. This constitutes *Moore's test* for sugar. In alkaline solutions glucose reduces salts of silver, bismuth, mercury, and copper. The reduction of cupric hydrate to cuprous hydrate or oxide constitutes *Trommer's test*, which has been already described at the head of the lesson. On boiling glucose with an alkaline solution of

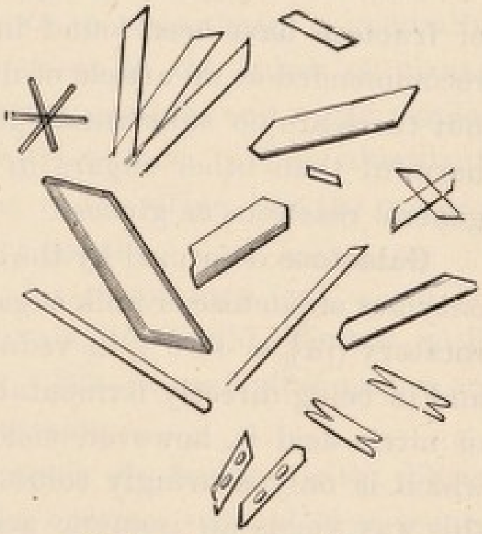


FIG. 1.—Inositol crystals.

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[1] For a description of polarised light and polarimeters see Appendix. This and the other matter in the Appendix are placed there for convenience, not because they are unimportant. Students are therefore urged to refer to and carefully study these subjects.

picric acid, a dark red opaque solution due to reduction of the picric to picramic acid is produced. Another important property of glucose is that under the influence of yeast it is converted into alcohol and carbonic acid ($C_6H_{12}O_6 = 2C_2H_6O + 2CO_2$).

Glucose may be estimated by the fermentation test, by the polarimeter (its specific rotation is $[\alpha]_D = +52{\cdot}5°$), and by the use of Fehling's solution. The last method is the most important: it rests on the same principles as Trommer's test, and we shall study it and other methods of estimating sugar in connection with diabetic urine (see Lesson XII).

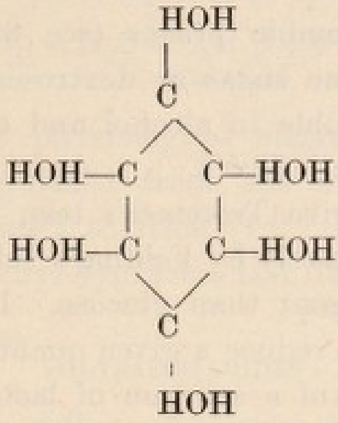
Fructose.—This sugar is also known as lævulose on account of its action on polarised light. When cane sugar is heated with dilute mineral acids it undergoes a process known as inversion—*i.e.* it takes up water and is converted into equal parts of glucose and fructose. The previously dextro-rotatory solution of cane sugar then becomes lævo-rotatory, the lævo-rotatory power of the fructose ($[\alpha]_D = -92°$) being greater than the dextro-rotatory power of the glucose formed. Hence the term *inversion.* The same hydrolytic change is produced by certain enzymes, such as the invertase of the intestinal juice, and of yeast.

Pure fructose can be crystallised with difficulty. Small quantities of fructose have been found in blood, urine, and muscle. It has been recommended as an article of diet in diabetes in place of ordinary sugar; but there are no substantial grounds for believing that fructose is less harmful than other sugars in this disease. Fructose gives the same general reactions as glucose.

Galactose is formed by the action of dilute mineral acids or inverting enzymes on lactose or milk sugar. It resembles glucose in being dextro-rotatory ($[\alpha]_D = +83°$), in reducing cupric hydrate in Trommer's test, and in being directly fermentable with yeast. When oxidised by means of nitric acid it, however, yields an acid called mucic acid ($C_6H_{10}O_8$), which is only sparingly soluble in water. Dextrose when treated in this way yields an isomeric acid—*i.e.* an acid with the same empirical formula, called saccharic acid, which is readily soluble in water.

Inositol or Inosite was discovered by Scherer in 1850 as a constituent of muscle, and for a long time was known as muscle sugar. It occurs also in small quantities in other animal organs (liver, kidney, etc.), and in plants it is a fairly constant constituent of roots and leaves, especially growing leaves.

It has the same molecular formula as the simple sugars ($C_6H_{12}O_6$), but it is only faintly sweet and gives none of the chemical reactions of these substances. Maquenne ascertained that it has the following constitutional formula :—



A mere glance at this formula will show that it is very different from those of the sugars given on p. 23. The six carbon atoms, instead of forming an open chain, are linked into a ring, as in the benzene derivatives. It is in fact a reduced hexa-hydroxy-benzene. It probably represents a transition stage between the carbohydrates and the benzene compounds. By a closing-up of the open chain of the carbohydrate molecule its formation from the latter is theoretically possible. On the other hand, the opening of the inosite ring would give rise to an open chain, and it has in fact been found that lactic acid is formed from inosite by the action of certain bacteria.

DISACCHARIDES

Sucrose.—This sugar (commonly known as cane sugar) is generally distributed throughout the vegetable kingdom in the juices of plants and fruits, especially the sugar cane, beetroot, mallow, and sugar maple. It is a substance of great importance as a food. After abundant ingestion of cane sugar traces may appear in the urine, but the greater part undergoes inversion in the alimentary canal.

Pure cane sugar is crystalline and dextro-rotatory ($[\alpha]_D = +67^\circ$). It holds cupric hydrate in solution in an alkaline liquid—that is, with Trommer's test it gives a blue solution. But no reduction occurs on boiling. After inversion it is strongly reducing.

Inversion may be brought about readily by boiling with dilute mineral acids, or by means of an inverting enzyme, such as that occurring in the succus entericus or intestinal juice. It then takes up water and is split into equal parts of glucose and fructose :—

$$\underset{\text{[sucrose]}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{[glucose]}}{C_6H_{12}O_6} + \underset{\text{[fructose]}}{C_6H_{12}O_6}$$

With yeast, cane sugar is first inverted by means of a special enzyme (*invertase*) produced by the yeast cells, and then there is an alcoholic fermentation of the monosaccharides so formed, which is accomplished by another enzyme called *zymase*.

Lactose, or **milk sugar,** occurs in milk. It sometimes also occurs in the urine of women in the early days of lactation or after weaning.

It crystallises in rhombic prisms (see fig. 2). It is much less soluble in water than cane sugar or dextrose, and has only a slightly sweet taste. It is insoluble in alcohol and ether; aqueous solutions are dextro-rotatory ($[\alpha]_D = +52{\cdot}5°$).

Solutions of lactose give Trommer's test, but when the reducing power is tested quantitatively by Fehling's solution it is found to be a less powerful reducing agent than glucose. If it required seven parts of a solution of glucose to reduce a given quantity of Fehling's solution, it would require ten parts of a solution of lactose of the same strength to reduce the same quantity of Fehling's solution.

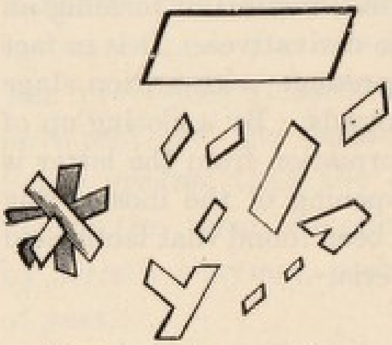


FIG. 2.—Lactose crystals.

Lactose, like cane sugar, can be hydrolysed by the same agencies as those already enumerated in connection with cane sugar. The monosaccharides formed are glucose and galactose:—

$$\underset{\text{[lactose]}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{[glucose]}}{C_6H_{12}O_6} + \underset{\text{[galactose]}}{C_6H_{12}O_6}$$

With yeast it is first inverted, and then alcohol is formed. This, however, occurs slowly.

The lactic-acid fermentation which occurs when milk turns sour is brought about by enzymes secreted by micro-organisms which are somewhat similar to yeast-cells. This may also occur as the result of the action of putrefactive bacteria in the alimentary canal. The two stages of the lactic-acid fermentation are represented by the following equations:—

$$(1) \qquad \underset{\text{[lactose]}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{[lactic acid]}}{4C_3H_6O_3}$$

$$(2) \qquad \underset{\text{[lactic acid]}}{4C_3H_6O_3} = \underset{\text{[butyric acid]}}{2C_4H_8O_2} + 4CO_2 + 4H_2$$

Maltose is the chief end product of the action of malt diastase on starch, and is also formed as an intermediate product in the action of dilute sulphuric acid on the same substance. It is also the chief sugar formed from starch by the diastatic enzymes contained in the saliva (ptyalin) and pancreatic juice (amylase). It can be obtained in form of acicular crystals; it is strongly dextro-rotatory ($[\alpha]_D = +140°$). It gives Trommer's test; but its reducing power, as measured by Fehling's solution, is one-third less than that of glucose.

By prolonged boiling with water, or, more readily, by boiling with a dilute mineral acid, or by means of an inverting enzyme, such as occurs in the intestinal juice, it is converted into glucose.

$$\underset{\text{[maltose]}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{[glucose]}}{C_6H_{12}O_6} + \underset{\text{[glucose]}}{C_6H_{12}O_6}$$

The three important physiological sugars (glucose, lactose, and maltose) may be distinguished from one another by their relative reducing action on Fehling's solution (1·0 : 0·71 : 0·63), by their rotatory power, or by the phenyl-hydrazine test described in Lesson XIII.

POLYSACCHARIDES

Starch is widely diffused through the vegetable kingdom. It occurs in nature in the form of microscopic grains, varying in size and appearance, according to their source. Each consists of a central spot (*hilum*) round which more or less concentric envelopes of starch proper or granulose alternate with layers of cellulose. Cellulose has very little digestive value, but starch is a most important food.

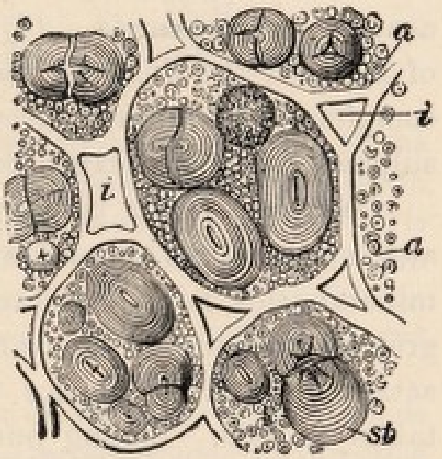


FIG. 3.—Section of pea showing starch and aleurone grains embedded in the protoplasm of the cells: *a*, aleurone grains; *st*, starch grains; *i*, intercellular spaces. (Yeo, after Sachs.)

Starch is insoluble in cold water: it forms an opalescent solution in boiling water, which if concentrated gelatinises on cooling. Its most characteristic reaction is the blue colour it gives with iodine solution.

On heating starch with dilute mineral acids glucose is formed. By the action of diastatic enzymes, maltose is the chief end product. In both cases dextrin is an intermediate stage in the process.

Before the formation of dextrin the starch solution loses its opalescence, a substance called *soluble starch* being formed. This, like native starch, gives a blue colour with iodine solution. Although the molecular weight of starch is unknown, the formula for soluble starch is probably $(C_6H_{10}O_5)_{200}$. Equations that represent the formation of sugars and dextrins from this are very complex, and are at present hypothetical.

Dextrin is the name given to the intermediate products in the hydrolysis of starch, and two chief varieties are distinguished—*erythro-dextrin*, which gives a reddish-brown colour with iodine solution; and *achroö-dextrin*, which does not.

It is readily soluble in water, but insoluble in alcohol and ether. It is an amorphous yellowish powder. It does not ferment with yeast. It is dextro-rotatory. By hydrolysis it is converted into glucose.

Glycogen, or animal starch, is found in liver, muscle, colourless blood corpuscles and other tissues.

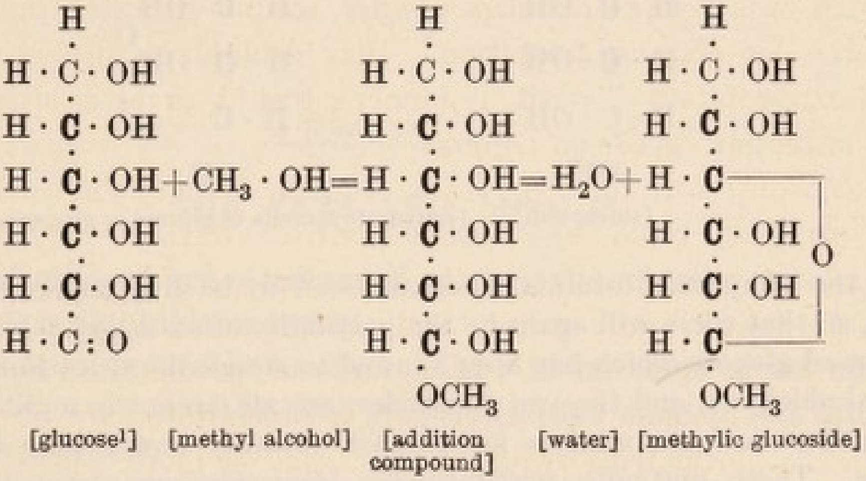
Glycogen is a white tasteless powder, soluble in water, but it forms, like starch, an opalescent solution. It is insoluble in alcohol and ether. It is dextro-rotatory. With Trommer's test it gives a blue solution, but no reduction occurs on boiling.

With iodine solution it gives a reddish or port-wine colour, very similar to that given by erythro-dextrin. Dextrin may be distinguished from glycogen by (1) the fact that it gives a clear, not an opalescent, solution with water; and (2) it is not precipitated by basic lead acetate as glycogen is. It is, however, precipitated by basic lead acetate and ammonia. (3) Glycogen is precipitated by 55 per cent. of alcohol; the dextrins require 85 per cent. or more. (4) Glycogen is completely precipitated from solution by saturation with ammonium sulphate; erythro-dextrin is only partially precipitable by this means.

Cellulose.—This is the colourless material of which the cell-walls and woody fibres of plants are composed. By treatment with strong mineral acids, it is, like starch, converted into glucose, but with much greater difficulty. The various digestive enzymes have little or no action on cellulose; hence the necessity of boiling starch before it is taken as food. Boiling bursts the cellulose envelopes of the starch grains, and so allows the digestive juices to get at the starch proper. Cellulose is found in a few animals, as in the test or outer investment of the Tunicates.

Salting out of the Colloid Carbohydrates.—By saturating solutions of the colloid carbohydrates (starch, soluble starch, glycogen, and erythro-dextrin partially) with such neutral salts as magnesium sulphate or ammonium sulphate the carbohydrate is thrown out of solution in the form of a white precipitate. The remaining carbohydrates (sugars and some of the smaller moleculed dextrins such as achroö-dextrin) are not precipitated by this means. We shall find in connection with the proteins that this method, known as "salting out," is one largely employed there for precipitating and distinguishing between classes of proteins. The student is therefore warned that a precipitate obtained under such circumstances will not necessarily indicate the presence of protein.

Glucosides.—Glucose is an aldehyde, and therefore has the power of combining with other compounds such as alcohols, organic acids, and phenols; a hydrogen atom of the compound unites with the oxygen of the $H \cdot C : O$ group of the sugar, and the rest of the molecule with the carbon of the same group ; the addition compound so formed then loses water, and a substance known as a glucoside is left. This may be illustrated by the simple glucoside which can be made synthetically by warming together (in the presence of anhydrous hydrochloric acid) methylic alcohol and glucose. The reaction is shown in the following equation :—



One notes that whereas glucose contains four asymmetric carbon atoms (*i.e.* carbon atoms united to four different atoms or atomic groups), printed in thick type, the addition compound and the glucoside each contain five. It is possible to obtain two methyl glucosides, α and β, one with the dextro-configuration of the carbon atom placed lowest in the formula, the other with the lævo-configuration. In one of these the lowest group of all is OCH_3, as shown in the above formula ; in the other it is CH_3O.

Numerous glucosides are found in nature ; thus amygdalin in bitter almonds is a compound of glucose with mandelic nitrile (=benzaldehyde+hydrocyanic acid) ; salicin is a compound of glucose and salicylic alcohol ; the indican of plants is a compound of glucose and indoxyl, and there are many others.

Mutarotation and Tautomerism.—The optical activity of glucose when freshly dissolved is about twice as great as when the solution has stood some time. If the glucose is crystallised out from this solution, and again dissolved, the fresh solution has again a high rotatory power, and this sinks once more on standing. It is evident that a change occurs in its constitution when it is left in solution, and this change is reversed on crystallisation. The explanation advanced to account for this *mutarotation* is that in solution the sugar forms an addition compound with water (a hypothetical aldehydrol), and then loses water to form a ring anhydride isomeric with the original glucose and analogous to a glucoside. The formulæ will be :—

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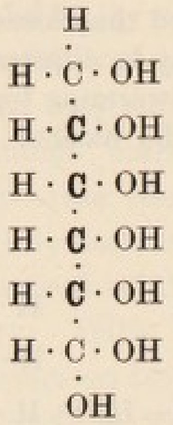
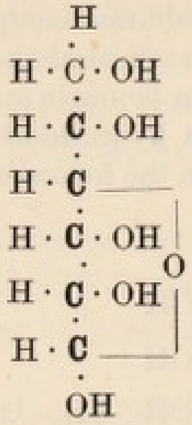
[1] This formula for glucose is a general one and does not show the typical stereochemical arrangement depicted in the formula on p. 23.



$$\begin{array}{c} H \\ H\cdot C\cdot OH \\ H\cdot C\cdot OH \\ H\cdot C\cdot OH \\ H\cdot C\cdot OH \\ H\cdot C\cdot OH \\ H\cdot C\cdot OH \\ OH \end{array}$$

[aldehydrol]

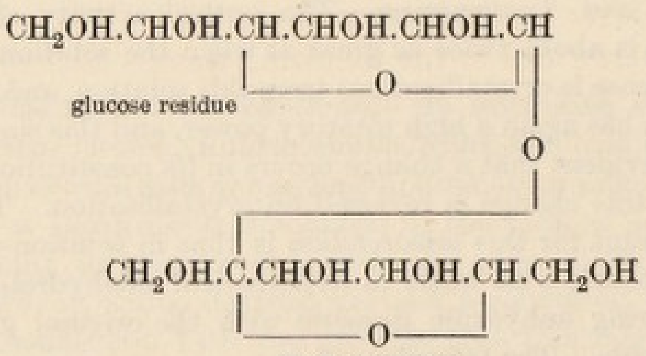


$$\begin{array}{c} H \\ H\cdot C\cdot OH \\ H\cdot C\cdot OH \\ H\cdot C\text{—} \\ H\cdot C\cdot OH \quad O \\ H\cdot C\cdot OH \\ H\cdot C\text{—} \\ OH \end{array}$$

[anhydride formula of glucose or glucoside of water]

In the anhydride formula there are seen to be five asymmetric carbon atoms, so that there will again be the possibility of an α and a β form. In a solution of glucose which has been allowed to stand, the three isomerides, the original aldehyde, and the two glucosides, are all present in equilibrium with one another, and the mixture has a lower rotatory power than the original glucose. These mutually transformable isomerides are termed *tautomeric* forms of glucose. Tautomerism of this kind is a frequent occurrence in organic chemistry.

Our knowledge of glucosides has thrown light on the constitution of the disaccharides; thus in maltose we have one glucose molecule forming a glucoside with another glucose molecule which retains its characteristic aldehyde (H . C : O) group unaltered. In lactose the place of the first glucose molecule is taken by galactose; it may thus be considered as a glucose-galactoside. In sucrose, on the other hand, the carboxyl groups of its two constituent hexoses (glucose and fructose) are involved in the union of the two molecules, which are therefore both in the glucosidic condition, as shown in the following formula :—



This indicates that there is in sucrose no real sugar group (either the aldehyde group H . C : O or the ketone group : C : O) at all, and this accounts for the fact that sucrose does not give the typical sugar reactions (reduction with Fehling's solution, and the formation of an osazone with phenyl hydrazine).

Further information regarding the carbohydrates is given in Lesson XIII.

LESSON IV

THE FATS AND LIPOIDS

Lard and olive oil are given round as examples of fats.

1. They are insoluble in water.

2. They dissolve readily in ether. On pouring some of the ethereal solution on to a piece of blotting paper, a greasy stain is left after the ether has evaporated.

3. **FAT SPLITTING BY LIPASE.**—Boil a few c.c. of fresh milk in order to destroy any lactic acid organisms which may be persent ; cool under the tap, and add a few drops of a glycerol extract of pancreas [1] containing *lipase* ; a few drops of phenolphthalein solution and dilute potash until a faint pink colour persists. Divide this into two portions labelled A and B. Boil A to destroy the enzyme, and keep both tubes at a temperature of about 36° C. The pink colour in B will gradually disappear, showing that fatty acids have been set free from the fat by the action of the fat-splitting enzyme, lipase ; A undergoes no change.

4. **SAPONIFICATION BY ALKALI.**—By boiling with potash, fat yields a solution of soap. On adding some sulphuric acid to this, the fatty acid collects in a layer on the surface of the fluid. This experiment may conveniently be performed in the following way :—Melt some lard in an evaporating basin and pour it into a solution of potash in alcohol [2] contained in a small flask and heated carefully *on a water-bath* nearly to boiling point. Continue to boil and saponification is soon completed. The completion of the process is recognised by dropping some of the solution into a test-tube containing about 10 c.c. of water ; the solution of soap will be clear, and no oil globules should separate out. If there is any separation of oil globules continue the boiling.

Then drop the soap solution into some 25 per-cent. sulphuric acid contained in a smaller beaker ; the fatty acids soon separate out and float on the surface. On cooling under the tap they solidify.

5. **REACTION OF FATTY ACIDS.**—Wash the fatty acid obtained

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[1] This is easily prepared by mixing finely minced pig's pancreas with two volumes of glycerin and straining the mixture through muslin.

[2] 30 grammes of potash are dissolved in 20 c.c. of water, and 200 c.c. of 90 per-cent. alcohol are added.

in experiment 4 repeatedly with water, until the wash water is no longer acid, and divide it into three portions. Dissolve one portion in ether; this solution reacts acid to phenolphthalein; to show this, place a few drops of phenolphthalein in 5 c.c. of alcohol containing a drop of 20 per-cent. potash. If this red solution is dropped into the solution of fatty acid, the colour is discharged. Place the second portion of fatty acid in some half-saturated solution of sodium carbonate and warm; a solution of sodium soap is obtained and carbon dioxide comes off. Note with the third portion that it produces a greasy stain on paper.

6. **REACTIONS OF SOAPS.**—A solution of soap may be prepared by heating some stearic acid with a few c.c. of water and adding caustic potash drop by drop until a clear solution results. (a) Add to this solution some sulphuric or hydrochloric acid; the fatty acid separates out as described under 4.

(b) Add to the solution powdered sodium chloride and shake; the soap is salted out and is rendered insoluble. This property of soap is used in soap manufacture.

(c) Add to the solution some calcium chloride. A precipitate of insoluble calcium soap is formed, and the solution loses its property of frothing on shaking.

7. **OSMIC ACID TEST.**—Fat, if it contains olein or oleic acid, is blackened by osmic acid. Try this with both the lard and the olive oil.

8. **TEST FOR GLYCEROL.**—The most important reaction for glycerol, the other constituent of a fat, is the acrolein test, which is performed in the following way:—Place some lard in a dry test-tube, add some crystals of acid potassium sulphate and heat. Acrolein is given off, which is recognised by its characteristic unpleasant odour, and by the fact that it blackens a piece of filter paper previously moistened with ammoniacal silver nitrate solution.

9. **EMULSIFICATION.**—(a) Take two test-tubes and label them A and B. Place water in A and soap solution in B. To each add a few drops of olive oil and shake. In B an emulsion is formed, but not in A.

(b) Shake a few drops of rancid oil (or olive oil containing a small amount of oleic acid), with a dilute solution of potash; an emulsion is formed because the potash and free fatty acid unite to form a soap. Divide this into two parts, and to one of them add a little gum solution or egg albumin; the emulsion is much more permanent in this specimen. These experiments illustrate the favourable action of soap and of a *suspending medium* such as mucilage upon the formation of an emulsion.

Fat is found in small quantities in many animal tissues. It is, however, found in large quantities in three situations, viz. bone marrow, adipose tissue, and milk. The consideration of the fat in milk is postponed to Lesson VI.

The contents of the fat cells of adipose tissue are fluid during life, the normal temperature of the body (37° C., or 99° F.) being considerably above the melting-point (25° C.) of the mixture of the fats found there. These fats are three in number, and are called palmitin, stearin, and olein. They differ from one another in chemical composition and in certain physical characters, such as melting-point and solubilities. Olein solidifies at − 5° C., palmitin at 45° C., and stearin at 53–65° C. Thus, it is olein which holds the other two dissolved at the body temperature. Fats are all soluble in hot alcohol, ether, and chloroform, but insoluble in water.

Chemical Constitution of the Fats.—The fats are compounds of fatty acids with glycerol, and may be termed glycerides or glyceric ethers.

The fatty acids, as we have already seen (p. 16), form a series of acids derived from the monohydric primary alcohols by oxidation. Formic acid is the first in the series, acetic acid comes next, and so on. The sixteenth term of the series is called **Palmitic acid**, and has the formula $C_{15}H_{31}.COOH$. The eighteenth is called **Stearic acid**, and has the formula $C_{17}H_{35}.COOH$.

Oleic acid is not a member of this series of fatty acids, but belongs to a somewhat similar series of acids known as the acrylic series, of which the general formula is $C_{n-1}H_{2n-3}COOH$. It is the eighteenth term of the series, and its formula is $C_{17}H_{33}.CO.OH$.

The first member of the group of alcohols from which this acrylic series of acids is obtained is called *allyl alcohol* ($CH_2 : CH.CH_2OH$); the aldehyde of this is *acrolein* ($CH_2 : CH.CHO$), and the formula for the acid (acrylic acid) is $CH_2 : CH.COOH$. It will be noticed that two of the carbon atoms are united by two valencies, and these substances are therefore unsaturated; they are unstable and are prone to undergo by uniting with another element a conversion into compounds in which the carbon atoms are united by one bond only. This accounts for their reducing action, and it is owing to this construction that the colour reactions with osmic acid and Sudan III. (red coloration) are due. Fat which contains any member of the acrylic series such as oleic acid blackens osmic acid, by reducing it to a lower (black) oxide. The fats palmitin and stearin do not give this reaction.

The formulæ for the fatty acids may also be written in a slightly different way, as shown below:—

Acetic acid	$(CH_3CO)OH$
Palmitic acid	$(C_{15}H_{31}CO)OH$
Stearic acid	$(C_{17}H_{35}CO)OH$
Oleic acid	$(C_{17}H_{33}CO)OH$

Each consists of a complex group placed in the above formulæ within brackets, united to hydroxyl. The group within brackets is called the fatty acid radical, and the fatty acid radicals of the four just mentioned acids have received the following names:—

Acetyl CH_3CO is the radical of acetic acid
Palmityl $C_{15}H_{31}CO$ „ „ palmitic acid
Stearyl $C_{17}H_{35}CO$ „ „ stearic acid
Oleyl $C_{17}H_{33}CO$ „ „ oleic acid

Glycerol (popularly known as glycerin) is a trihydric alcohol, $C_3H_5(OH)_3$—*i.e.* three atoms of hydroxyl united to a radical glyceryl (C_3H_5). The hydrogen in the hydroxyl atoms is replaceable by other organic radicals. As an example take the radical of acetic acid called acetyl $(CH_3.CO)$. The following formulæ represent the derivatives that can be obtained by replacing one, two, or all three hydroxyl hydrogen atoms in this way:—

$$C_3H_5\left\{\begin{array}{l}OH\\OH\\OH\end{array}\right.$$

[glycerol]

$$C_3H_5\left\{\begin{array}{l}OH\\OH\\O.CH_3.CO\end{array}\right.$$

[monoacetin]

$$C_3H_5\left\{\begin{array}{l}OH\\O.CH_3.CO\\O.CH_3.CO\end{array}\right.$$

[diacetin]

$$C_3H_5\left\{\begin{array}{l}O.CH_3.CO\\O.CH_3.CO\\O.CH_3.CO\end{array}\right.$$

[triacetin]

Triacetin is a type of a neutral fat; stearin, palmitin, and olein ought more properly to be called tristearin, tripalmitin, and triolein respectively. Each consists of glycerol in which the three atoms of hydrogen in the hydroxyls are replaced by radicals of the fatty acid. This is represented in the following formulæ:—

Palmitin $C_3H_5(OC_{15}H_{31}.CO)_3$
Stearin $C_3H_5(OC_{17}H_{35}.CO)_3$
Olein $C_3H_5(OC_{17}H_{33}.CO)_3$

If we substitute the letter R for the fatty acid radical, the general formula for a neutral fat may be written:—

$$\begin{array}{l}CH_2.OR\\|\\CH.OR\\|\\CH_2.OR\end{array}$$

Decomposition Products of the Fats.—The fats split up into the substances out of which they are built up.

Under the influence of superheated steam, mineral acids, and in the body by means of certain enzymes (for instance, the fat-splitting enzyme *lipase* of the pancreatic juice), a fat combines with water and splits into glycerol and the fatty acid. The following equation represents what occurs in a fat, taking tripalmitin as an example :—

$$\underset{\text{[palmitin—a fat]}}{C_3H_5(O.C_{15}H_{31}CO)_3} + 3H_2O = \underset{\text{[glycerol]}}{C_3H_5(OH)_3} + \underset{\text{[palmitic acid—a fatty acid]}}{3C_{15}H_{31}CO.OH}$$

In the process of saponification, much the same sort of reaction occurs, the final products being glycerol and a compound of the base with the fatty acid, which is called a soap. Suppose, for instance, that potassium hydrate is used ; we get—

$$\underset{\text{[palmitin—a fat]}}{C_3H_5(O.C_{15}H_{31}CO)_3} + 3KHO = \underset{\text{[glycerol]}}{C_3H_5(OH)_3} + \underset{\text{[potassium palmitate—a soap]}}{3C_{15}H_{31}CO.OK}$$

Emulsification.—Another change that fats undergo in the body is very different from saponification. It is a physical rather than a chemical change ; the fat is broken up into very small globules, such as are seen in the natural *emulsion*—milk. The conditions under which emulsions are formed are described in the practical exercises at the head of this lesson.

THE LIPOIDS

The name lipoid was originally applied by Overton to a heterogeneous group of substances found in the protoplasm of all cells, especially in their outer layer or cell-membrane, which, like the fats, are soluble in such reagents as ether and alcohol. These substances, though present in smaller amount than proteins, appear to be essential constituents of protoplasm, and the labile character of their molecules is a property many of them share with the proteins. The lipoids are found mixed with fat in the ether-alcohol extract of tissues and organs, and they are specially abundant in nervous tissues, where we shall again have to refer to them (Lesson XXI).

They may be classified in the following way :—

(1) Those which, like the fats, are free from both nitrogen and phosphorus. The most important member of this group is cholesterol.

(2) Those which are free from phosphorus but contain nitrogen. These yield the reducing sugar called galactose when broken up, and were termed cerebro-galactosides by Thudichum. They may be simply called galactosides.

(3) Those which contain both phosphorus and nitrogen. These are called the phosphatides, and are grouped according to the proportion of nitrogen and phosphorus in their molecules, as follows :—

(*a*) Mono-amino-mono-phosphatides, N : P=1 : 1. *e.g.* lecithin and kephalin.

(*b*) Diamino-mono-phosphatides, N : P=2 : 1. *e.g.* sphingomyelin.

(*c*) Mono-amino-diphosphatides, N : P=1 : 2. One of these, named cuorin, has been separated out from the heart by Erlandsen, and a similar substance is found in egg-yolk.

(*d*) Diamino-diphosphatides, N : P=2 : 2. One of these was separated from brain by Thudichum, but has not since been examined.

(*e*) Triamino-mono-phosphatides, N : P=3 : 1. One of these is present in egg-yolk.

This classification is obviously capable of extension as new phosphatides are discovered.

We may now take some of the most important of these substances and describe them with greater detail.

Cholesterol or cholesterin is found in small quantities in all forms of protoplasm. It is a specially abundant constituent of nervous tissues, particularly in the white substance of Schwann. It is found in small quantities in the bile, but it may occur there in excess and form the concretions known as gall stones. It can be readily extracted from the brain by the use of cold acetone. In the brain it occurs in the free state.

It is a monohydric unsaturated alcohol with the empirical formula $C_{27}H_{45}.OH$. Recent research has shown it to belong to the terpene series which had hitherto only been found as excretory products of plant life.

Windaus finds that it contains five reduced benzene rings linked together and a double linkage at the end of an open chain.

Cholesterol is now believed to be not merely a waste product of metabolism, but to exert an important protective influence on the body cells against the entrance of certain poisons called toxins. One of the poisons contained in cobra venom dissolves red blood-corpuscles; the presence of cholesterol in the envelope of the blood-corpuscles to some extent hinders this action, and it has been stated that the administration of cholesterol increases the resistance of the animal. It is certainly the case that with artificial blood-corpuscles, membranous bags containing hæmoglobin, the impregnation of the membrane with cholesterol, prevents the solvent action of toxins.

In order that cholesterol and its derivatives may act in this way, it is necessary that the double linkage and the hydroxyl group should be intact. The latter would not be the case in an ester.

From alcohol or ether containing water it crystallises in the form of rhombic tables, which contain one molecule of water of crystallisation: these are easily recognised under the microscope (fig. 4). It gives a number of colour tests which we shall study under Bile (Lesson VIII).

A substance called *iso-cholesterol* is found in the fatty secretion of the skin (sebum); it is largely contained in the preparation called *lanoline*, made from sheep's wool fat. It differs from cholesterol in being dextro-rotatory instead of lævo-rotatory in solution, and in some of its colour reactions.

Cholesterols isomeric with animal cholesterol are also found in many plants; these are termed phyto-cholesterols, or phytosterols for short.

Cholesterol compounds exhibit the physical phenomenon recently studied by Lehmann, namely, the formation of liquid crystals; this is also shown by several other lipoids. Virchow in 1855 described what he termed "myelin forms"; if brain-substance is mixed with water, where the water touches the brain material, threads are observable shooting out and twisting into fantastic shapes; these are termed "myelin forms," although the word "myelin" has no definite chemical meaning. It has now been shown that these "myelin forms" are distorted liquid crystals due to the presence of cholesterol and other lipoids. The fat globules seen in the adrenal cortex, during cell proliferation in cancer, and in the liver and other organs during fatty degeneration, are not wholly composed of fat, for the polarisation microscope shows them to be anisotropic or doubly refracting, and further investigation has shown them to be lipoids in the fluid crystalline condition. Pure cholesterol and pure cholesterol esters do not exhibit the phenomenon; but mixtures of cholesterol and fatty acids do.

The Cerebro-Galactosides.—The substance known as protagon can be separated out from the brain by means of warm alcohol; on cooling the extract, protagon is deposited as a white precipitate. This, however, also contains cholesterol, which can be dissolved out by ether. Another method of preparing protagon is to take brain and extract the cholesterol first with cold acetone; then hot acetone is employed to extract the protagon. Protagon is a substance originally described by Couerbe, under the name cérébrote, but named protagon by Liebreich, who regarded it as a definite compound, and the mother substance of all the other phosphorised and non-phosphorised constituents of the brain. It has now been definitely proved in confirmation of what Thudichum stated in 1874, that protagon is not a definite chemical unit, but a mixture of phosphorised and non-phosphorised substances in such proportions that it usually contains about 1 per cent. of phosphorus. By treatment with appropriate solvents and recrystallisation, protagon can be separated into its constituents; those which are free from phosphorus and sulphur and comprise about 70 per cent. of the original protagon are the galactosides. Although these have received many names, the known galactosides are only two in number, namely, *phrenosin* and *kerasin*. The former is a crystalline product and is dextro-rotatory, the latter is of somewhat waxy consistency and is lævo-rotatory. Phrenosin (also called cerebrin or cerebron) yields on decomposition three substances:—

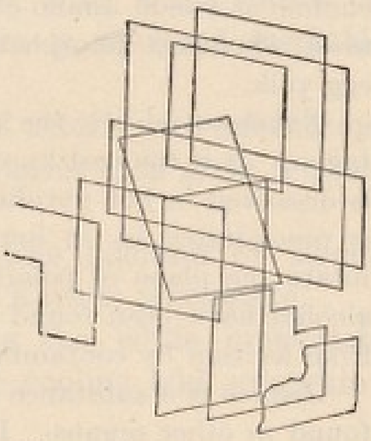


FIG. 4.—Cholesterol crystals.

(1) A reducing sugar, galactose.

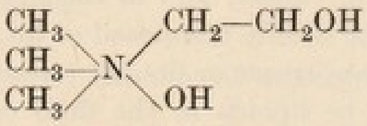
(2) A base termed sphingosine ($C_{17}H_{35}NO_2$), which represents an unsaturated mono-amino-dihydroxy alcohol.

(3) A fatty acid called cerebronic (or neuro-stearic acid), which has the constitution of α-hydroxypentacosanic acid ($C_{25}H_{50}O_3$).

Kerasin also yields galactose and sphingosine, but the fatty acid is different. It is lignoceric acid, $C_{24}H_{48}O_2$.

The Phosphatides.—The best known of these is *lecithin* ($C_{42}H_{84}NPO_9$).

This is a very labile substance, but it yields on decomposition four materials; namely, glycerol and phosphoric acid united together as glycero-phosphoric acid, two fatty acid radicals, of which one is usually oleyl, and an ammonium-like base termed choline ($C_5H_{15}NO_2$). The fatty acid radicals are united to glycerol as in an ordinary fat, the place of the third fatty acid radical being taken by the radical of phosphoric acid, which in its turn is united in an ester-like manner to the choline. The formula of choline is



Kephalin resembles lecithin in being a mono-amino-mono-phosphatide. It differs from lecithin in being insoluble in alcohol. On decomposition it yields glycero-phosphoric acid, certain fatty acids which are less saturated than oleic acid, and probably belong to the linoleic series. Instead of choline it yields amino-ethyl alcohol (hydroxyethylamine). Kephalin is the most abundant phosphatide in nerve-fibres, and has also been found in egg-yolk.

Sphingomyelin is the phosphatide obtained from the mixture called protagon. It is the best known of the diamino-mono-phosphatides. If protagon is dissolved in hot pyridine, and the solution allowed to cool, sphingomyelin is precipitated in an impure form as sphæro-crystals, which in suspension rotate the plane of polarised light to the left. Choline, fatty acids, and an alcohol have been found among its cleavage products. It, however, differs from lecithin by containing no glycerol.

Jecorin is a substance first separated from the liver by Drechsel and since found in other organs. It appears to be a mixture or possibly a compound of kephalin or a diamino-mono-phosphatide with sugar.

LESSON V

THE PROTEINS

1. TESTS FOR PROTEINS.—The following tests are to be tried with a mixture of one part of white of egg to ten of water. (Egg-white contains a mixture of albumin and globulin.)

(a) *Heat Coagulation.*—**Faintly acidulate with a few drops of 2 per-cent. acetic acid and boil. The protein is rendered insoluble (coagulated protein).**

(b) Filter some of the egg-white solution, and acidify with a few drops of 20 per-cent. acetic acid; potassium ferrocyanide then gives a white precipitate.

(c) *Precipitation with Nitric Acid.*—**The addition of strong nitric acid to the original solution also produces a white precipitate.**

(d) *Xanthoproteic Reaction.*—**On boiling the white precipitate produced by nitric acid it turns yellow; after cooling add ammonia; the yellow becomes orange.**[1]

(e) *Millon's Test.*—**Millon's reagent**[2] **gives a white precipitate, which turns brick-red on boiling.**

(f) *Rose's or Piotrowski's Test.*—**Add** *one drop* **of a 1 per-cent. solution of cupric sulphate to the original solution and then caustic potash, and a violet solution is obtained.**

Repeat experiment (f) with a solution of commercial peptone, and note that a rose-red solution is obtained. This is called the *biuret reaction.*

(g) *Rosenheim's Formaldehyde Reaction.*—**Add to the solution of commercial peptone a very dilute solution of formaldehyde (1 : 2500), and then about one-third of the volume of strong sulphuric acid containing (as most commercial specimens of the acid do) a trace of an oxidising agent such as ferric chloride or nitrous acid. A purple ring develops at the surface of contact. This reaction is the foundation of the original Adamkiewicz reaction.**

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[1] Consult p. 56 for an explanation of this and the following colour reactions.

[2] Mercury is dissolved in its own weight of nitric acid. The solution so obtained is then diluted with twice its volume of water. The decanted clear liquid is Millon's reagent; it is a mixture of the nitrates of mercury containing excess of nitric acid.

(h) *Adamkiewicz Reaction*; here glacial acetic acid was used instead of the formaldehyde. Most commercial specimens of glacial acetic acid contain hydrogen peroxide as an impurity; the oxidising action of this on the acetic acid leads to the formation of traces of glyoxylic acid and formaldehyde; the necessary factors for the occurrence of the formaldehyde reaction are thus present. Glyoxylic acid gives the reaction by virtue of its decomposition into formaldehyde, which, when present in minute traces, also reacts with pure sulphuric acid.

The same reactions (g and h) are given by the solution of egg-white, but not so markedly.

2. ACTION OF NEUTRAL SALTS.—(a) Saturate the solution of egg-white with magnesium sulphate by adding crystals of the salt and grinding it up thoroughly in a mortar. A white precipitate of egg-globulin is produced. Filter. The filtrate contains egg-albumin. The precipitate of the globulin is very small.

(b) Half saturate the solution of egg-white with ammonium sulphate. This may be done by adding to the solution an equal volume of a saturated solution of ammonium sulphate. The precipitate produced consists of the globulin; the albumin remains in solution.

(c) Completely saturate another portion with ammonium sulphate by adding crystals of the salt and grinding in a mortar—a precipitate is produced of both the globulin and albumin. Filter. The filtrate contains no protein.

(d) Repeat the last experiment (c) with a solution of commercial peptone. A precipitate is produced of the proteoses it contains. Filter. The filtrate contains the true peptone. This gives the biuret reaction (see above), but large excess of strong potash must be added on account of the presence of ammonium sulphate. *Ammonium sulphate added to saturation precipitates all proteins except peptone.*

3. ACTION OF ACIDS AND ALKALIS ON ALBUMIN.—Take three test-tubes and label them A, B, and C. In each place an equal amount of diluted egg-white.

To A add a few drops of 0·1 per-cent. solution of caustic potash.

To B add the same amount of 0·1 per-cent. solution of caustic potash.

To C add a rather larger amount of 0·1 per-cent. sulphuric acid.

Put all three into the warm bath[1] at about the temperature of the body (36–40° C.).

After five minutes remove test-tube A, and boil. The protein is no longer coagulated by heat, having been converted into *alkali-meta-protein*. After cooling, colour with litmus solution and neutralise with

---

[1] A convenient form of warm bath suitable for class purposes may be made by placing an ordinary tin pot half full of water over a bent piece of iron which acts as a warm stage. The stage is kept warm by a small gas flame.

0·1 per-cent. acid. At the neutral point a precipitate is formed which is soluble in excess of either acid or alkali.

Next remove B. This also now contains alkali-metaprotein. Add to it a few drops of sodium phosphate, colour with litmus, and neutralise as before. Note that the alkali-metaprotein now requires more acid for its precipitate than in A, the acid which is first added converting the sodium phosphate into acid sodium phosphate. This exercise shows that the presence of inorganic salts which react with acids may modify the reactions of alkali-metaprotein.

Now remove C from the bath. Boil it. Again there is no coagulation, the proteins having been converted into *acid-metaprotein.* After cooling, colour with litmus and neutralise with 0·1 per-cent. alkali. At the neutral point a precipitate is formed, soluble in excess of acid or alkali. (Acid-metaprotein is formed more slowly than alkali-metaprotein, so it is best to leave this experiment to the last.)

Other acids, such as acetic or oxalic, may be employed instead of sulphuric acid for making acid-metaprotein. The following exercise with oxalic acid gives good results:—To half a test-tubeful of diluted egg-white add 5 to 10 drops of a saturated solution of oxalic acid. Keep the mixture at a temperature of 40°–50° C. for a few minutes. Then gradually heat the solution to boiling point; no coagulum results.

4. **GELATIN.**—Take some *gelatin* and dissolve it in hot water. On cooling, the solution sets into a jelly (gelatinisation).

Take a dilute solution of the gelatin, and try all the protein tests with it enumerated on p. 41. Carefully note down your results.

5. **KERATIN.**—Suspend some horn shavings or hair in water, and try all the colour tests for protein with this. Repeat also with this the sulphur test (Lesson I, Exercise 7 b, p. 6).

6. **MUCIN.**—Add a few drops of acetic acid to some saliva. A stringy precipitate of mucin is formed.

7. **MUCOID.**—A tendon has been soaked for a few days in lime water. The fibres are not dissolved, but they are loosened from one another owing to the solution of the interstitial or ground substance by the lime water. Take some of the lime water extract and add acetic acid. A precipitate of mucoid is obtained. The fibres themselves consist of collagen, which yields gelatin on boiling. Vitreous humour or the Whartonian jelly of the umbilical cord is much richer in ground substance than tendon, and, if treated in the same way, a much larger yield of mucoid is obtained.

The **Proteins** are the most important substances that occur in animal and vegetable organisms, and *protein metabolism* is the most characteristic sign of life.

They are highly complex compounds of carbon, hydrogen, oxygen, nitrogen, and sulphur occurring in a solid viscous condition, or in solution in nearly all parts of the body. The different members of the group present great similarities, for instance, in the large size of their molecules, and in giving certain colour tests; there are, on the other hand, considerable differences between the various proteins.

The proteins in the food form the source of the proteins in the body tissues, but the latter are usually different in composition from the former. The food proteins are in the process of digestion broken up into simple substances, usually called *cleavage products*, and it is from these that the body cells reconstruct the proteins peculiar to themselves. As a result of katabolic processes in the body the proteins are finally again broken down, carbonic acid, water, sulphuric acid (combined as sulphates), urea, and creatinine being the principal final products which are discharged in the urine and other excretions. The intermediate substances between the proteins and such final katabolites as urea will be discussed under Urine.

The following figures will show how different the proteins are even in elementary composition; Hoppe-Seyler many years ago gave the variations in percentage composition as follows:—

	C	H	N	S	O
From	51·5	6·9	15·2	0·3	20·9
To	54·5	7·3	17·0	2·0	23·5

and recent research has since shown that the variations are even greater than those given by Hoppe-Seyler.

Differences are also seen when the cleavage products are separated and estimated. These differ both in kind and in amount, but nearly all of them are substances which are termed *amino-acids*. Emil Fischer, to whom we owe so much of our knowledge in this direction, considers that the proteins are linkages of a greater or lesser number of these amino-acids, and there is great hope that in the future his work will result in an actual synthesis of the protein molecule, and with that will come an accurate knowledge of its constitution.

When the protein molecule is broken down in the laboratory by processes similar to those brought about by the digestive enzymes which occur in the alimentary canal, the essential change is due to what is called *hydrolysis*: that is, the molecule unites with water and then breaks up into smaller molecules. The first cleavage products, which are called *proteoses*, retain many of the characters of the original protein; and the same is true, though to a less degree, of the *peptones*, which come next in order of formation. The peptones in their turn are decomposed into short linkages of amino-acids which are called *polypeptides*, and finally the individual amino-acids are obtained separated from each other.

What we have already learnt about the fatty acids will help us in understanding what is meant by an amino-acid.

If we take acetic acid, which is one of the simplest of the fatty acids, we see that its formula is

$$CH_3.COOH.$$

If one of the three hydrogen atoms in the CH_3 group is replaced by NH_2, we get a substance which has the formula

$$CH_2.NH_2.COOH.$$

The combination NH_2 which has stepped in is called the *amino-group*, and the new substance now formed is called *amino-acetic acid*; it is also termed **glycine** or *glycocoll*.

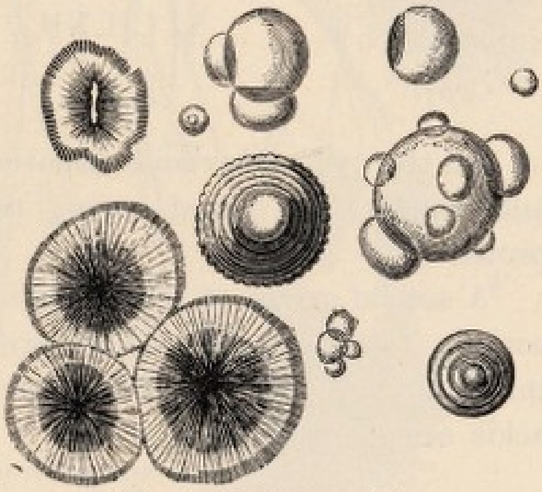


FIG. 5.—Leucine crystals.

We may take another example from another fatty acid. Propionic acid is $C_2H_5.COOH$; if we replace an atom of hydrogen by the amino group as before, we obtain $C_2H_4.NH_2.COOH$, which is amino-propionic acid or **alanine**.

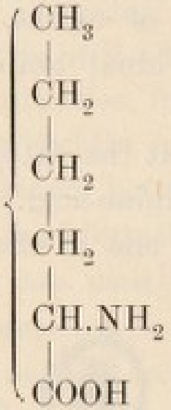
If instead of propionic we take hydroxy-propionic acid, its amino-derivative (amino-hydroxy-propionic acid) is termed **serine.**

A third amino-acid is similarly obtained by the introduction of the NH_2 into valeric acid $C_4H_9.COOH$. Amino - valeric acid $C_4H_8.NH_2.COOH$ is called **Valine.**

Going to the next fatty acid in the series, caproic acid $C_5H_{11}.COOH$, we obtain from it in an exactly similar way, $C_5H_{10}.NH_2.COOH$, which is amino-caproic acid or **leucine**. Impure leucine crystallises in spheroidal clumps of crystals, as shown in fig. 5. With pure leucine the needle-like crystals are obtained separately.

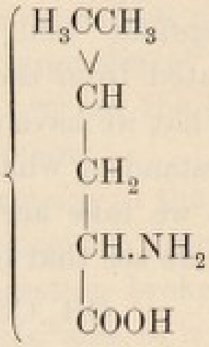
According to the way in which the amino-group is linked, a large number of isomeric amino-caproic acids, all with the same empirical formula, are theoretically possible. Many of these have been prepared synthetically, and it has been shown that the amino-caproic acid called leucine, formed by hydrolysis from proteins, is the lævo-rotatory variety, and should be more accurately named α-amino-isobutyl-acetic acid. The structure of this compound as compared with that of normal amino-caproic acid is shown in the following graphic formulæ :—

Normal α-amino-caproic acid



$$\begin{array}{l} CH_3 \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CH.NH_2 \\ | \\ COOH \end{array}$$

Iso-butyl α-amino-acetic acid



$$\begin{array}{l} H_3CCH_3 \\ \quad \vee \\ \;\; CH \\ \;\; | \\ \;\; CH_2 \\ \;\; | \\ \;\; CH.NH_2 \\ \;\; | \\ \;\; COOH \end{array}$$

All the five amino-acids mentioned (glycine, alanine, serine, valine, and leucine) are found among the final cleavage products of most proteins.

A second group of amino-acids is obtained from fatty acids, which contain two carboxyl (COOH) groups in their molecules. The most important of the amino-derivatives obtained from these *dicarboxylic* acids are :—

Amino-succinamic acid (asparagine),
Amino-succinic acid (aspartic acid),
Amino-glutaric acid (glutamic acid).

The third group of amino-acids is a very important one ; these are termed the *aromatic amino-acids* ; that is, amino-acids united to the benzene ring, and of these we will mention two, namely, phenyl-alanine and tyrosine. We shall also have to consider a nearly related substance called tryptophane.

Phenyl-alanine is alanine or amino-propionic acid in which an atom of hydrogen is replaced by phenyl (C_6H_5).

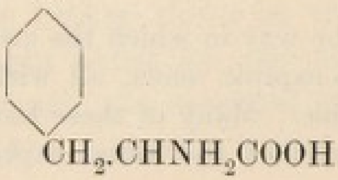
Propionic acid has the formula $C_2H_5.COOH$.

Alanine (amino-propionic acid) is $C_2H_4NH_2.COOH$.

Phenyl-alanine is $C_6H_5.C_2H_3.NH_2.COOH$.

The formula of phenyl-alanine may also be written in another way.

If an H in the benzene ring (see p. 18) is replaced by the side chain $CH_2CH.NH_2.COOH$, we obtain the formula of phenyl-alanine :—

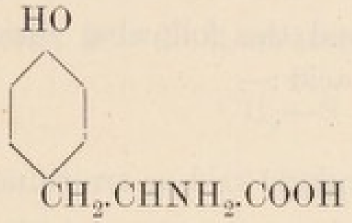


the remainder of the benzene ring, which is unaltered, being represented as usual by a simple hexagon.

Tyrosine is a little more complicated; it is *p*-oxyphenyl-alanine; that is, instead of phenyl (C_6H_5) in the formula of phenyl-alanine, we have now oxyphenyl ($C_6H_4.OH$); this gives us

$$(C_6H_4.OH)C_2H_3.NH_2.COOH$$

as the formula for tyrosine written one way, or



when written in the other way. Tyrosine crystallises in collections of very fine needles (see fig. 6).

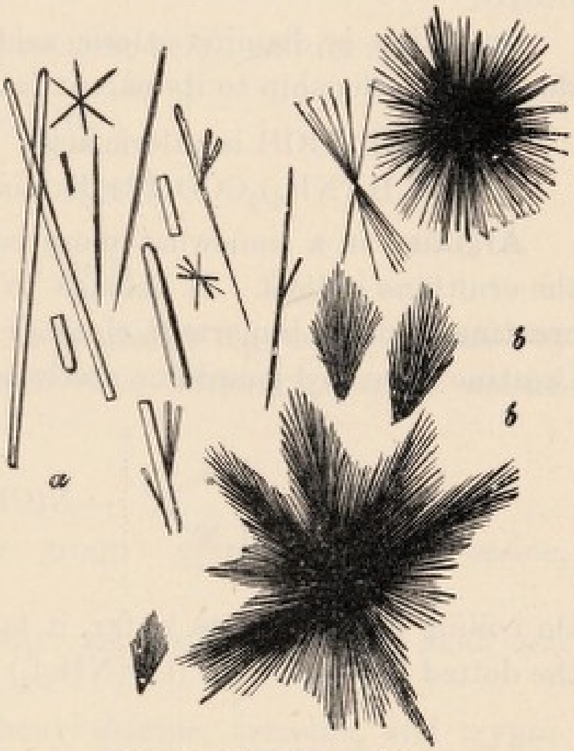
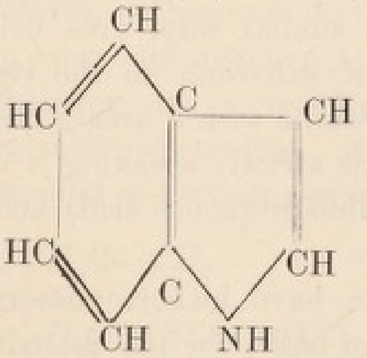


FIG. 6.—Tyrosine crystals.

Tryptophane is more complex still; it is indole aminopropionic acid: that is, aminopropionic acid united to another ringed derivative called indole. Tryptophane is the portion of the protein molecule which is the parent substance of two evil-smelling products of protein decomposition called indole and scatole or methyl indole. Indole (C_8H_7N) is a combination of the benzene and pyrrol rings, as shown below :—



Tryptophane is the radical in the protein molecule which is responsible for the colour test called the Adamkiewicz reaction.

In all the preceding cases, there is only one replacement of an atom of hydrogen by NH_2; hence they may be grouped together as *mono-amino-acids.*

Passing to the next stage in complexity, we come to another group of amino-acids which are called *diamino*-acids; that is, fatty acids in which two hydrogen atoms are replaced by NH_2 groups. Of these we may particularly mention lysine, ornithine, arginine, and histidine.

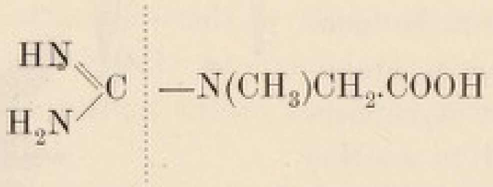
Lysine is diamino-caproic acid. Caproic acid is $C_5H_{11}.COOH$. Mono-amino-caproic acid or leucine, we have already learnt, is $C_5H_{10}.NH_2.COOH$. Lysine or diamino-caproic acid is $C_5H_9.(NH_2)_2.COOH$.

Ornithine is diamino-valeric acid, and the following formulæ will show its relationship to its parent fatty acid :—

C_4H_9COOH is valeric acid.

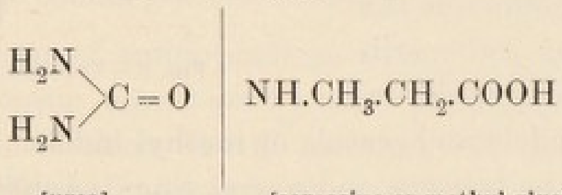
$C_4H_7(NH_2)_2COOH$ is diamino-valeric acid or ornithine.

Arginine is a somewhat more complex substance, which contains the ornithine radical. It belongs to the same group of substances as **creatine**, another important cleavage product of the protein molecule. Creatine is methyl-guanidine acetic acid, and has the formula



$$\begin{array}{l}HN\\H_2N\end{array}\!>C \;\vdots\; -N(CH_3)CH_2.COOH$$

On boiling it with baryta water, it takes up water (H_2O) and splits at the dotted line into urea ($CO(NH_2)_2$) and sarcosine, as shown below.



$$\begin{array}{l}H_2N\\H_2N\end{array}\!>C=O \;\Big|\; NH.CH_3.CH_2.COOH$$

[urea] [sarcosine or methyl-glycine]

Arginine splits in a similar way, urea being split off on the left, and ornithine instead of sarcosine on the right. Arginine is, therefore, a compound of ornithine with a urea group.

Histidine, though not strictly speaking a diamino-acid, is a diazine derivative (imidazole-amino-propionic acid) and so may be included in the same group.

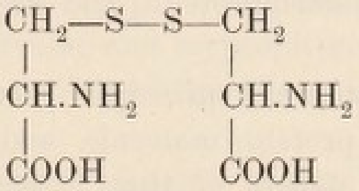
These substances we have hitherto described as acids, but they may also play the part of bases, for the introduction of a second basic group into the fatty acid molecules confers upon them basic properties. The three substances :—

Lysine	$C_6H_{14}N_2O_2$
Arginine	$C_6H_{14}N_4O_2$
Histidine	$C_6H_9N_3O_2$

are in fact often called the *hexone bases*, because each of them contains 6 atoms of carbon, as the above empirical formulæ show.

Cystine is a complex diamino-acid in which sulphur is present, and in which the greater part of the sulphur of the protein molecule is contained. The sulphur test described in Lesson I, Exercise 7 *b*, p. 6, is due to the presence of the cystine group in the protein molecule, and it is particularly well shown by such proteins as keratin, which are rich in cystine.

Chemically it is di- (thio-amino-propionic acid), and its formula is



$$\begin{array}{lll} CH_2\text{—}S\text{—} & S\text{—}CH_2 & \\ | & | & \\ CH.NH_2 & CH.NH_2 & \\ | & | & \\ COOH & COOH & \end{array}$$

We may summarise what we have learnt up to this point by enumerating the principal members of these various groups of amino-acids :—

(1) **The mono-amino-acids :**

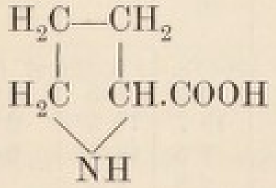
(*a*) Of the *mono-carboxylic* group: glycine, alanine, serine, valine, and leucine.

(*b*) Of the *dicarboxylic* group: asparagine, aspartic acid, and glutamic acid.

(*c*) Of the *ringed* group: phenyl-alanine, tyrosine, and tryptophane.

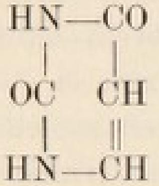
(2) **The diamino-acids :** lysine, ornithine, arginine, and cystine. We have still to mention :—

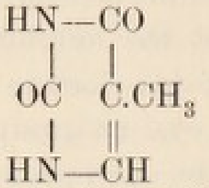
(3) **Pyrrolidine Derivatives.**—These are derivatives of a ring which reminds us of the benzene ring, but nitrogen is included in the ring formation; the most important are pyrrolidine-carboxylic acid, or *proline*, and its hydroxy derivative *oxyproline*. The formula for proline, which is a very constant product of protein cleavage, is

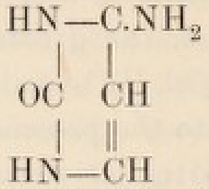


(4) **Pyrimidine Bases.**—There are derivatives of the pyrimidine ring, another *heterocyclic* nucleus.

The pyrimidine bases obtainable from the cleavage of certain proteins (nucleo-proteins) are cytosine, thymine, and uracil. Their formulæ are as follows:


[uracil or dioxypyrimidine]


[thymine or methyl uracil]


[cytosine or amino-oxypyrimidine]

(5) **Purine Bases.**—These, like the preceding, are obtained from the nucleic acid complex of nucleo-proteins, and will be described on p. 64.

(6) **Ammonia.**

Our list now represents the principal groups of chemical nuclei united together in the protein molecule, and its length makes one realise the complicated nature of that molecule and the difficulties which beset its investigation. We may put the problem another way. In the simple sugars, with six atoms of carbon, there are as many as twenty-four different ways in which the atomic groups may be linked up; the formulæ on p. 23 give only three of these which represent the structure of glucose, fructose, and galactose; but the majority of the remainder have also been prepared by the chemists. The molecule of albumin has at least 700 carbon atoms, so the possible combinations and permutations must be reckoned by thousands.

Many workers are steadily analysing the various known proteins, taking them to pieces and identifying and estimating the fragments. The following brief table gives the results obtained with some of the cleavage products of a few proteins. The numbers given are percentages:—

	Serum Albumin.	Egg Albumin.	Serum Globulin.	Caseinogen of cow's milk.	Gelatin.	Keratin, from horse hair.	Edestin, a globulin from cotton seed.	Zein, from maize.	Gliadin, from wheat.
Glycine . .	0	0	3·5	0·5	16·5	4·7	1·2	0	0·02
Leucine . .	20·0	6·1	18·7	10·5	2·1	7·1	15·5	18·6	5·6
Glutamic acid .	7·7	8·0	8·5	11·0	0·9	3·7	17·2	18·3	37·3
Tyrosine . .	2·1	1·1	2·5	4·5	0	3·2	2·1	3·5	1·2
Arginine .	...	...	...	4·8	7·6	...	11·7	1·2	3·2
Tryptophane .	+	+	+	1·5	0	...	+	0	+
Cystine	2·5	0·3	0·7	0·06	...	More than 10	0·2	...	0·4

Such numbers, of course, are not to be committed to memory, especially as the methods of analysis cannot in all cases be considered satisfactory, but they are sufficient to convey to the reader the differences between the proteins. There are several blanks left, on account of no estimations having yet been made. Where the sign + occurs, the substance in question has been proved to be present, but not yet determined quantitatively. Among the more striking points brought out are:

1. The absence of glycine from albumins.
2. The high percentage of glycine in gelatin.
3. The absence of tyrosine and tryptophane in gelatin.
4. The high percentage of the sulphur-containing substance (cystine) in keratin.
5. The high percentage of glutamic acid in vegetable proteins.

Fischer has discovered the way in which the amino-acids are linked together into groups; and the culmination of his work will be the discovery of the way in which such groups are linked together to form the protein molecule.

The groups he terms *polypeptides*; many of these have been made synthetically in his laboratory, and so the synthesis of the protein molecule is foreshadowed.

We may take as our examples of the polypeptides some of the simplest, and may write the formulæ of a few amino-acids as follows:—

$NH_2.CH_2.COOH$	Glycine
$NH_2.C_2H_4.COOH$	Alanine
$NH_2.C_5H_{10}.COOH$	Leucine

or in general terms

$$\text{HNH.R.COOH.}$$

Two amino-acids are linked together as shown in the following formula:—

$$\text{HNH.R.CO}\ \boxed{\text{OH.H}}\ \text{NH.R.COOH.}$$

What happens is that the hydroxyl (OH) of the carboxyl (COOH) group of one acid unites with one atom of the hydrogen of the next amino (HNH) group, and water is thus formed, as shown within the dotted lines: this is eliminated and the rest of the chain closes up. In this way we get a *dipeptide*. The names glycyl, alanyl, leucyl, etc., are given by Fischer to the $NH_2.R.CO$ groups which replace the hydrogen of the next NH_2 group. Thus glycyl-glycine, glycyl-leucine, leucyl-alanine, alanyl-leucine, and numerous other combinations are obtained. If the same operation is repeated we obtain tripeptides (leucyl-glycyl-alanine, alanyl-leucyl-tyrosine, etc.); then come the tetrapeptides, and so on. In the end, by coupling the chains sufficiently often and in appropriate order, Fischer has already obtained substances which give some of the reactions of peptone.

Hausmann's Method.—The ideal aim of the chemist would be to separate the complex mixture of cleavage products quantitatively in such a way as to account for the whole of the carbon, nitrogen, sulphur, etc., in the original protein. This idea has not yet been attained on account of the secondary reactions taking place during hydrolysis, such as formation of brown and black pigments, splitting off of carbonic acid, etc. Even with the best methods at his disposal, Fischer and his colleagues have succeeded so far only in separating at the utmost 50 to 70 per cent. of the amino-acids present in the cleavage products, and the chief loss appears to be in the mono-amino-acids.

Under these circumstances it is of the greatest value to be able to obtain, by a short and trustworthy procedure, at least an approximate knowledge of the nitrogen distribution in the protein molecule, even if this does not allow us to determine quantitatively the individual cleavage products. Such a method has been worked out, mainly in Hofmeister's laboratory, by Hausmann, and has been subsequently used by Osborne and others.

Hausmann's method is shortly as follows:—The total nitrogen of the protein is estimated by Kjeldahl's method. A weighed amount of the substance is then hydrolysed by means of hydrochloric acid. After complete hydrolysis the cleavage products are separated into three classes and the nitrogen estimated in each as—

1. Amide-N or ammonia nitrogen. This comprises the nitrogen of that part of the protein molecule which is easily split off as ammonia, and is determined by distilling off the ammonia after adding magnesia.

2. Diamino-N. The fluid, free from ammonia, is precipitated by phosphotungstic acid, and the nitrogen present in the precipitate determined. This represents the nitrogen of the diamino-acids (histidine, arginine, etc.).

3. Mono-amino-N is estimated in the residual fluid after removal of the amide and diamino-N.

This method has furnished most valuable information when applied to different animal and vegetable proteins, as is shown in the following table from the analyses of Osborne:—

	Total N.	Amide-N.	Diamino-N.	Mono-amino-N.
Zein (maize)	16·13	2·97	0·49	12·51
Hordein (barley) . . .	17·21	4·01	0·77	12·04
Gliadin (wheat) . . .	17·66	4·20	0·98	12·41
Glutenin (wheat) . . .	17·49	3·30	2·05	11·95
Leucosin (wheat) . . .	16·93	1·16	3·50	11·83
Edestin (hemp). . . .	18·64	1·88	5·91	10·78
Caseinogen (milk) . . .	15·62	1·61	3·49	10·31

These figures show interesting differences between otherwise similar proteins. New characteristics are given for some protein groups, *e.g.* the alcohol soluble vegetable proteins, which possess a high amide-N and low diamino-N. In Osborne's analyses (not given) of various edestins, great differences of the diamino-N were revealed. The method has also proved useful for the differentiation of proteoses, and interesting deductions as to the food value of various proteins were drawn from its results. As 80 to 90 per cent. of the carbon of proteins (according to Kossel) is present in combination with nitrogen, the method is likely to give important clues as to the constitution of different proteins.

Van Slyke's Method.—A further differentiation of the units in the protein molecule has been made possible by a method Van Slyke has introduced. The details of the method are given in Lesson XVIII; it is an application of the well-known reaction of nitrous acid on substances containing an amino-group. Since nitrous acid liberates nitrogen only from the amino-group, it is possible, by estimating the total nitrogen, to determine by difference the non-amino-nitrogen in a protein (that is, the part of the nitrogen which is in heterocyclic combination in proline, oxyproline, tryptophane, and histidine). By making use of these facts, and applying them to Hausmann's method, Van Slyke has been successful in determining from 98 to 100 per cent. of the nitrogenous products of a complete protein hydrolysis, and the whole operation can be carried out with 2 or 3 grammes of the protein material. After complete hydrolysis of the protein, the ammonia nitrogen is estimated by vacuum distillation after adding magnesia. Arginine, histidine, lysine, and cystine are precipitated, as in Hausmann's method, by phosphotungstic acid; this precipitate is dissolved, and the total nitrogen and the amino-nitrogen in it are estimated. The difference gives the non-amino-nitrogen in the histidine (which contains two-thirds non-amino-nitrogen) and arginine (which contains three-quarters non-amino nitrogen). The remaining nitrogen of this fraction is contained in the bases lysine and cystine; these contain only amino-nitrogen; cystine is determined separately by a sulphur estimation, and lysine by difference. Of the other pair of amino-acids, arginine is estimated by boiling with potash, which splits off half its nitrogen as ammonia, and histidine by difference. The amino-acids contained in the phosphotungstic filtrate are separated into two fractions: (1) the acids which contain only amino-nitrogen, and (2) those which also contain secondary nitrogen in the pyrrolidine ring (proline and oxyproline), or in the indole ring (tryptophane).

During the distillation of the ammonia (amide nitrogen), a black colouring matter is formed; the nitrogen in this is estimated and is designated humin nitrogen. The following table gives the amounts in percentages of the total nitrogen, of the nitrogen in the various fractions of certain proteins:—

	Amide-N.	Humin-N.	Cystine-N.	Arginine-N.	Histidine-N.	Lysine-N.	Mono-amino-N.	Non-amino-N.	Total N.
Wheat (gliadin) .	25·52	0·86	1·25	5·71	5·20	0·75	51·98	8·50	99·77
Edestin . .	9·99	1·98	1·49	27·05	5·75	3·86	47·55	1·70	99·37
Keratin (from hair) . .	10·05	7·42	6·60	15·33	3·48	5·37	47·50	3·10	98·85
Gelatin . .	2·25	0·07	0·00	14·70	4·48	6·32	56·30	14·90	99·02
Fibrin . .	8·32	3·17	0·99	13·86	4·83	11·51	54·30	2·70	99·58
Hæmoglobin .	5·24	3·60	0·00	7·70	12·70	10·90	57·00	2·90	100·04

It will be seen that nearly 100 per cent. of the total nitrogen is accounted for. A further striking result is the high non-amino-nitrogen percentage in gelatin, indicating that larger quantities of proline are contained in it than older analyses had revealed. The large amount of lysine in hæmoglobin is also surprising, as histidine was previously thought to be its principal di-amino-acid. The method has a great future before it.

TESTS FOR PROTEINS

Solubilities.—The proteins are insoluble in alcohol and ether. Some are soluble in water,[1] others insoluble. Many of the latter are soluble in weak saline solutions. Some are insoluble, others soluble in concentrated saline solutions.

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[1] The proteins are not truly soluble in water; they are in a state of *colloidal solution*, a condition intermediate between true solution and suspension. Many of their properties are due to this fact. (See Appendix.)

All proteins are soluble with the aid of heat in concentrated mineral acids and alkalis. Such treatment, however, decomposes as well as dissolves the protein. Proteins are also soluble in gastric and pancreatic juices; but here, again, they undergo a change, as we have already seen.

Heat Coagulation.—Most native proteins, such as white of egg, are rendered insoluble when their solutions are heated. The temperature of heat coagulation differs in different proteins; thus myosinogen and fibrinogen coagulate at 56° C., serum albumin and serum globulin at about 75° C.

The proteins which are coagulated by heat come mainly under two classes: the *albumins* and the *globulins*. These differ in solubility; the albumins are soluble in distilled water, the true globulins require salts to hold them in solution.

Indiffusibility.—The proteins (peptones excepted) belong to the class of substances called *colloids* by Thomas Graham; that is, they pass with difficulty, or not at all, through animal membranes. In the construction of dialysers, vegetable parchment is largely used.

Proteins may thus be separated from diffusible (*crystalloid*) substances such as salts, but the process is a tedious one. If some serum or white of egg is placed in a dialyser (fig. 7) and distilled water outside, the greater amount of the salts passes into the water through the membrane and is replaced by water; the two proteins albumin and globulin remain inside; the globulin is, however, precipitated, as the salts which previously kept it in solution are removed.

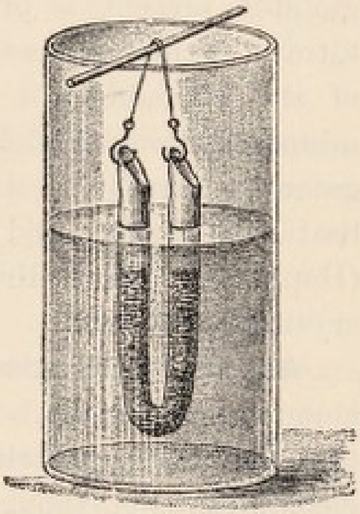

FIG. 7.—In this form of dialyser the substance to be dialysed is placed within the piece of tubing suspended in the larger vessel of water. The tubing is made of parchment paper.

The terms "diffusion" and "dialysis" should be distinguished from each other.

If water is carefully poured on the surface of a solution of any substance, this substance gradually spreads through the water, and the composition of the mixture becomes uniform in time. The time occupied is short for substances like sodium chloride, and long for substances like albumin. The phenomenon is called *diffusion*. If the solutions are separated by a membrane the term *dialysis* is employed. The word *osmosis* is properly restricted to the passage of water through membranes, and can be best studied when semi-permeable membranes are employed. See more fully article OSMOSIS in Appendix.

Crystallisation.—Hæmoglobin, the red pigment of the blood, is a protein substance and is crystallisable (for further details, see The Blood, Lesson IX). Like other proteins it has an enormously large molecule; though crystalline, it is not, however, crystalloid in Graham's sense of that term. Blood pigment, however, is not the only crystallisable protein. Long ago crystals of protein (globulin or vitellin) were observed in the aleurone grains of many seeds, and in the somewhat similar granules occurring in the egg-yolk of some fishes and amphibians. By appropriate methods these have been separated and recrystallised. Further, egg albumin itself has been crystallised. If a solution of white of egg is diluted with an equal volume of saturated solution of ammonium sulphate, the globulin present is precipitated and is removed by filtration. The filtrate is now allowed to remain some days at the temperature of the air, and as it becomes more concentrated from evaporation, minute spheroidal globules, and finally minute needles, either aggregated or separate, make their appearance (Hofmeister). Crystallisation is more rapid if a little acetic or sulphuric acid is added (Hopkins). Serum albumin (from some animals) has also been similarly crystallised (Gürber).

Action on Polarised Light.—All proteins are lævo-rotatory, the amount of rotation varying with individual proteins. Several of the compound proteins, *e.g.* hæmoglobin, and nucleo-proteins are dextro-rotatory, though their protein components are lævo-rotatory (Gamgee).

Colour Reactions.—The principal colour reactions have been already described in the heading of this lesson.

(1) The xantho-proteic reaction depends on the conversion of the aromatic group of the protein molecule into nitro-derivatives.

(2) Millon's reaction is due to the presence of the tyrosine group, and is given by all benzene derivatives which contain a hydroxyl group (OH) replacing hydrogen.

(3) The formaldehyde reaction (and the Adamkiewicz reaction) is due to the presence of the tryptophane radical (indole amino-propionic acid).

The presence, absence, or intensity of these colour tests in various proteins depends respectively on the presence, absence, or amount of the groups to which they are due.

(4) In the copper sulphate test the proteoses and peptones behave differently from the native proteins; the latter give a violet and the former a rose-red colour, which is called the *biuret reaction*, because the

same tint is also given by the substance called biuret.[1] The name does not imply that biuret is present in protein, but both biuret and protein give the reaction because they possess the same atomic groups, namely, two $CONH_2$ groups linked either to a carbon atom, or to a nitrogen atom, or directly to one another (Schiff). The native proteins give a violet colour because the red tint of the copper compound with the biuret group is mixed with another copper compound which has a blue colour.

Precipitants of Proteins.—Proteins are precipitated by a large number of reagents; the peptones and proteoses are exceptions in many cases, and will be considered separately afterwards (see Lesson VII).

Solutions of the proteins are precipitated by—

1. Strong acids, such as nitric acid.
2. Picric acid.
3. Acetic acid and potassium ferrocyanide.
4. Acetic acid and excess of neutral salts such as sodium sulphate.
5. Salts of the heavy metals, such as copper sulphate, mercuric chloride, lead acetate, silver nitrate, &c.
6. Tannin.
7. Alcohol.
8. Saturation with certain neutral salts, such as ammonium sulphate.

It is necessary that the words *coagulation* and *precipitation* should, in connection with the proteins, be carefully distinguished. The term *coagulation* is used when an insoluble protein (coagulated protein) is formed from a soluble one. This may occur—

1. When the protein is heated—*heat coagulation.*
2. Under the influence of an enzyme; for instance, when a curd is formed in milk by rennet or a clot in shed blood by the fibrin ferment —*enzyme coagulation.*

There are, however, certain precipitants of proteins in which the precipitate formed is readily soluble in suitable reagents, such as saline solutions, and the protein continues to show its typical reactions. This precipitation is not coagulation. Such a precipitate is produced by saturation with ammonium sulphate. Certain proteins, called globulins, are more readily precipitated by such means than others. Thus, serum globulin is precipitated by half-saturation with ammonium sulphate. Full saturation with ammonium sulphate precipitates all proteins but peptone. The globulins are precipitated by certain salts

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[1] Biuret is obtained by heating solid urea; ammonia is given off and leaves biuret thus:—

$$\underset{\text{[urea]}}{2CON_2H_4} = \underset{\text{[biuret]}}{C_2O_2N_3H_5} + \underset{\text{[ammonia]}}{NH_3}$$

such as sodium chloride and magnesium sulphate, which do not precipitate the albumins. The precipitation of proteins by salts in this way is conveniently termed "salting out."

The precipitate produced by alcohol is peculiar in that after a time it becomes a coagulum. Protein freshly precipitated by alcohol is readily soluble in water or saline media; but after it has been allowed to stand some time under alcohol it becomes more and more insoluble. Albumins and globulins are most readily rendered insoluble by this method; proteoses and peptones are never rendered insoluble by the action of alcohol. This fact is of value in the separation of these proteins from others.

CLASSIFICATION OF PROTEINS

The knowledge of the chemistry of the proteins which is slowly progressing under Emil Fischer's leadership will no doubt in time enable us to give a classification of these substances on a strictly chemical basis. The following classification must be regarded as a provisional one, which, while it retains the old familiar names as far as possible, yet attempts also to incorporate some of the new ideas. The classes of animal proteins, then, beginning with the simplest, are as follow:—

1. Protamines.
2. Histones.
3. Albumins.
4. Globulins.
5. Sclero-proteins.
6. Phospho-proteins.
7. Conjugated proteins.
    - (*a*) Gluco-proteins.
    - (*b*) Nucleo-proteins.
    - (*c*) Chromo-proteins.

We shall take these classes one by one.

1. The Protamines

These substances are obtainable from the heads of the spermatozoa of certain fishes, where they occur in combination with nuclein. Kossel's view that they are the simplest proteins in nature has met with general acceptance, and they give such typical protein reactions as the copper sulphate test (Rose's or Piotrowski's reaction). On hydrolytic decomposition they first yield substances of smaller molecular weight analogous to the peptones which are called *protones*, and then they split up into amino-acids. The number of resulting amino-acids is small as compared with other proteins; hence the hypothesis that they are simple proteins is confirmed. Notable among their decomposition products are the diamino-acids or hexone bases, especially arginine. The protamines differ in their composition according to their source, and yield these products in different proportions.

Salmine (from the salmon roe) and *clupeine* (from the herring roe) appear to be identical, and have the empirical formula $C_{30}H_{57}N_{17}O_6$: its principal decomposition product is arginine, but small amounts of valine, serine, and proline are also found. *Sturine* (from the sturgeon) yields the same products with lysine and histidine in addition. With one exception, the protamines yield no aromatic amino-acids; the exception is *cyclopterine* (from *Cyclopterus lumpus*); this substance is thus an important chemical link between the other protamines and the more complex members of the protein family.

2. The Histones

These are substances which have been separated from blood corpuscles; *globin*, the protein constituent of hæmoglobin, is a well-marked instance. They yield a larger number of amino-compounds than do the protamines, but diamino-acids are still relatively abundant. They are coagulable by heat, soluble in dilute acids, and precipitable from such solutions by ammonia. The precipitability by ammonia is a property possessed by no other protein group.

3. The Albumins

These are typical proteins, and yield the majority of the cleavage products enumerated on pp. 45 to 50.

They enter into colloidal solution in water, in dilute saline solutions, and in saturated solutions of sodium chloride and magnesium sulphate. They are, however, precipitated by saturating their solutions with ammonium sulphate. Their solutions are coagulated by heat usually at 70°–73° C. Serum albumin, egg-albumin, and lact-albumin are instances.

4. The Globulins

The globulins give the same general tests as the albumins: they are coagulated by heat, but differ from the albumins mainly in their solubilities. This difference in solubility may be stated in tabular form as follows:—

Reagent.	Albumin.	Globulin.
Water	soluble	insoluble
Dilute saline solution	soluble	soluble
Saturated solution of magnesium sulphate or sodium chloride	soluble	insoluble
Half-saturated solution of ammonium sulphate	soluble	insoluble
Saturated solution of ammonium sulphate	insoluble	insoluble

In general terms globulins are more readily salted out than albumins; they may therefore be precipitated and thus separated from the albumins by saturation with such salts as sodium chloride, or, better, magnesium sulphate, or by half saturation with ammonium sulphate.

The typical globulins are also insoluble in water, and so may be precipitated by removing the salt which keeps them in solution. This may be accomplished by dialysis (see p. 55).

Their temperature of heat coagulation varies considerably. The following are the commoner globulins:—fibrinogen and serum globulin in blood; egg-globulin in white of egg, para-myosinogen in muscle, and crystallin in the crystalline lens. We must also include under the same heading certain proteins which are the result of enzyme coagulation on globulins, such as fibrin (see Blood) and myosin (see Muscle).

The most striking and real distinction between globulins and albumins is that the latter on hydrolysis yield no glycine, whereas the globulins do.

5. The Sclero-proteins

These substances form a heterogeneous group of substances, which are frequently termed *albuminoids*. The prefix *sclero-* indicates the skeletal origin and often insoluble nature of the members of the group. The principal proteins under this heading are the following:—

1. **Collagen**, the substance of which the white fibres of connective tissue are composed. Some observers regard it as the anhydride of gelatin.

2. **Ossein**.—This is the same substance derived from bone.

In round numbers the solid matter in *bone* contains two-thirds inorganic or earthy matter, and one-third organic or animal matter. The inorganic constituents are calcium phosphate (84 per cent. of the ash), calcium carbonate (13 per cent.), and smaller quantities of calcium chloride, calcium fluoride, and magnesium phosphate. The organic constituents are ossein (this is the most abundant), elastin from the membranes lining the Haversian canals, lacunæ, and canaliculi, and other proteins and nuclein from the bone corpuscles. There is also a small quantity of fat even after removal of all the marrow. *Dentine* is like bone chemically, but the proportion of earthy matter is rather greater. *Enamel* is the hardest tissue in the body; the mineral matter is like that found in bone and dentine; but the organic matter is so small in quantity as to be practically non-existent (Tomes). Enamel is epiblastic, not mesoblastic, like bone and dentine.

3. **Gelatin**.—This substance is produced by boiling collagen with water. It possesses the peculiar property of setting into a jelly when a solution made with hot water cools. On digestion it is like ordinary

proteins converted into peptone-like substances and is readily absorbed. Though it will replace in diet a certain quantity of such proteins and thus acts as a "protein-sparing" food, it cannot altogether take their place as a food. Animals whose sole nitrogenous food is gelatin waste rapidly. The reason for this is that gelatin contains neither the tyrosine nor the tryptophane radicals, and so it gives neither Millon's nor the Adamkiewicz reaction. Animals which receive in their food gelatin to which tyrosine and tryptophane are added thrive better.

4. **Chondrin.**—This is the name given to the mixture of gelatin and mucoid which is obtained by boiling cartilage.

5. **Elastin.**—This is the substance of which the yellow or elastic fibres of connective tissue are composed. It is a very insoluble material. The sarcolemma of muscular fibres and certain basement membranes are composed of a similar substance.

6. **Keratin,** or horny material, is the substance found in the surface layers of the epidermis, in hairs, nails, hoofs, and horns. It is very insoluble, and chiefly differs from most other proteins in its high percentage of the sulphur-containing amino-acid called cystine. A similar substance, called *neurokeratin*, is found in neuroglia and nerve fibres. In this connection it is interesting to note that the epidermis and the nervous system are both formed from the same layer of the embryo—the epiblast.

6. The Phospho-proteins

Vitellin (from egg-yolk), *caseinogen*, the principal protein of milk, and *casein*, the result of the action of rennet on caseinogen (see Milk), are the principal members of this group. Among their decomposition products is a considerable quantity of phosphoric acid. They have been frequently confused with the nucleo-proteins which we shall be studying immediately; but they do not yield the products (purine and other bases) which are characteristic of nucleo-compounds. The phosphorus is contained within the protein molecules, and not in another molecular group united to the protein, as in the case of the nucleo-proteins. The phospho-proteins are of special value in the nutrition of young and embryonic animals. Many other proteins, such as the globulin of blood serum, contain traces of phosphorus.

7. The Conjugated Proteins

These very complex substances are compounds in which the protein molecule is united to other organic materials, which are, as a rule, also of complex nature. This second constituent of the compound is usually termed a *prosthetic group*. They may be divided into the following sub-classes.

i. **Chromo-proteins.**—These are compounds of proteins with a pigment, which usually contains iron. They are exemplified by hæmoglobin and its allies, which will be fully considered under Blood.

ii. **Gluco-proteins.**—These are compounds of protein with a carbohydrate group. This class includes the mucins and the mucoids.

The *mucins* are widely distributed and may occur in epithelial cells, or be shed out by these cells (mucus, mucous glands, goblet cells). The mucins obtained from different sources vary in composition and reactions, but they all agree in the following points:—

(*a*) Physical character. Viscid and tenacious.

(*b*) They are soluble in dilute alkalis, such as lime water, and are precipitable from solution by acetic acid.

The mucoids generally resemble the mucins, but differ from them in minor details. The term is applied to the mucin-like substances which form the chief constituent of the ground substance of connective tissues (tendo-mucoid, chondro-mucoid, etc.). Another, ovo-mucoid, is found in white of egg, and others (pseudo-mucin and para-mucin) are occasionally found in dropsical effusions, and in the fluid of ovarian cysts.

The differences between the mucins and the mucoids are probably due to the nature of the protein to which the carbohydrate group is united. The carbohydrate substance is not sugar, but a nitrogenous substance which has a similar reducing power to sugar, and which is called *glucosamine* ($C_6H_{11}O_5NH_2$): that is, glucose in which HO is replaced by NH_2.

Pavy and others have shown that a *small* quantity of the same carbohydrate derivative can be split off from various other proteins which we have already placed among the albumins and globulins. It is, however, probable that this must not be considered a prosthetic group, but is more intimately united within the protein molecule.

iii. **Nucleo-proteins.**—These are compounds of protein with a complex organic acid called nucleic acid which contains phosphorus. They are found both in the nuclei and cell-protoplasm of cells. In physical characters they often simulate mucin.

Nuclein is the name given to the chief constituent of cell-nuclei. It is identical with the chromatin of histologists (see fig. 8).

On decomposition it yields an organic acid called nucleic acid, together with a variable but usually small amount of protein. It contains a high percentage (10–11) of phosphorus. The nuclein obtained from the nuclei or heads of the spermatozoa consists of nucleic acid without any protein admixture. In fishes' spermatozoa, however, there is an exception to this rule, for there it is, as we have already seen, united to protamine.

The nucleo-proteins of cell protoplasm are compounds of nucleic acid with a much larger quantity of protein, so that they usually contain only 1 per cent. or less of phosphorus. Some also contain iron, and it is probable that the normal supply of iron to the body is contained in the nucleo-proteins or *hæmatogens* (Bunge) of plant or animal cells.

Nucleo-proteins may be prepared from cellular structures such as thymus, testis, kidney, etc., by two principal methods :—

1. *Wooldridge's Method.*—The organ is minced and soaked in water for twenty-four hours. Dilute acetic acid added to the aqueous extract precipitates the nucleo-protein.

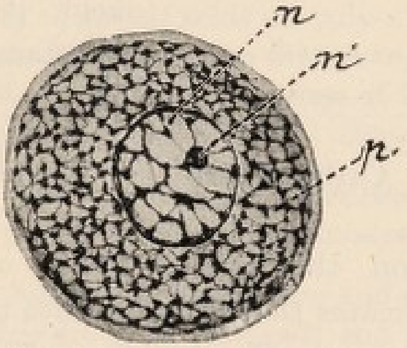

FIG. 8.—Diagram of a cell : *p*, protoplasm composed of spongioplasm and hyaloplasm ; *n*, nucleus with intranuclear network of chromatin or nuclein ; and *n'*, nucleolus (Schäfer).

2. *Sodium chloride Method.*—The minced organ is ground up in a mortar with solid sodium chloride ; the resulting viscous mass is poured into excess of water, and the nucleo-protein rises in strings to the top of the water.

The solvent usually employed for a nucleo-protein, by whichever method it is prepared, is a 1 per-cent. solution of sodium carbonate. The relationship of nucleo-proteins to the coagulation of the blood is described under that heading.

Nucleic acid yields, among its decomposition products, phosphoric acid, a carbohydrate, various bases of the purine group, and bases also of the pyrimidine group. The following diagrammatic way of representing the decomposition of nucleo-protein will assist the student in remembering the relationships of these substances :—

NUCLEO-PROTEIN
subjected to gastric digestion yields

Protein—converted into peptone, which goes into solution.

Nuclein, which remains as an insoluble residue. If this is dissolved in alkali, and hydrochloric acid added, it yields

Protein—converted into acid meta-protein in solution.

A precipitate consisting of nucleic acid. If this is heated in a sealed tube with hydrochloric acid, it yields the following substances.

Phosphoric acid. Carbohydrate. Purine bases. Pyrimidine bases.

Recent research on the nucleic acids obtained from various mammalian organs indicates that they fall into two main classes :—

(1) *Nucleic Acid Proper.*—This yields on decomposition—

(*a*) Phosphoric acid.

(*b*) A carbohydrate of the hexose group which has not yet been identified. In nucleic acid from vegetable cells (*e.g.* from yeast) the sugar which takes its place is a pentose (*d*-ribose) (Levene).

(*c*) Two members of the purine group in the same proportion, namely, adenine and guanine.

(*d*) Two pyrimidine bases, namely, cytosine and thymine (see p. 50).

The *purine bases* are specially interesting because of their close relationship to uric acid, and we shall have to deal with them again in our description of that substance. They are all derivatives of a ringed complex, named purine by Fischer, and their relationship to each other is best seen by their formulæ :—

Purine $C_5H_4N_4$

Purine bases
- Hypoxanthine (monoxy-purine) $C_5H_4N_4O$
- Xanthine (dioxy-purine) $C_5H_4N_4O_2$
- Adenine (amino-purine) $C_5H_3N_4.NH_2$
- Guanine (amino-oxy-purine) $C_5H_3N_4O.NH_2$

Uric acid (trioxy-purine) $C_5H_4N_4O_3$

The two bases obtained from nucleic acid are the two which contain the NH_2 group. If xanthine and hypoxanthine are obtained, they are the secondary effects of oxidative and de-amidising enzymes.

(2) *Guanylic Acid.*—This is a simpler form of nucleic acid found in certain organs (pancreas, liver, etc.), mixed with the nucleic acid proper. It yields on decomposition only three substances, namely :—

(*a*) Phosphoric acid.
(*b*) A carbohydrate of the pentose group.
(*c*) Guanine, but no adenine.

From his work on the nucleic acid of yeast, Levene finds that it is composed of complexes consisting of phosphoric acid, carbohydrate (ribose), and a base. These are termed *nucleotides*. Guanylic acid, described above, is a mono-nucleotide, but the majority of nucleic acids are poly-nucleotides. When these are broken down by chemical reagents, the first change is the removal of the phosphoric acid, leaving intact the combinations of base and carbohydrate ; these latter combinations are called *nucleosides* ; thus—

Adenine+ribose=adenosine.
Guanine+ribose=guanosine.

These nucleosides may be further split into base and ribose ; or they may be de-amidised (*i.e.* the amino-group is removed) and nucleosides obtained in which hypoxanthine and xanthine are united with the ribose, and these in their turn may be split into base and ribose.

The same cleavages are accomplished in the body by the action of tissue-enzymes contained in varying degrees in the different organs and tissues. As these enzymes are specific, the number which may come into successive play in the decompositions which occur in the body is very numerous. These enzymes are spoken of under the general term *nucleases*.

Protein-hydrolysis

When protein material is subjected to hydrolysis, as it is when heated with mineral acid, or superheated steam, or to the action of such enzymes as trypsin in the alimentary canal, it is finally resolved into the numerous amino-acids of which it is built. But before this ultimate stage is reached, it is split into substances of progressively diminishing molecular size, which still retain many of the protein characters. These may be classified in order of formation as follows :—

1. Meta-proteins.
2. Proteoses.
3. Peptones.
4. Polypeptides.
5. Amino-acids.

The polypeptides are linkages of two or more amino-acids as already explained. Although most of the polypeptides at present known are products of laboratory synthesis, some have been definitely separated from the digestion of proteins, and so they must appear in our classification. The proteoses, peptones, and some of the longer polypeptides give the biuret reaction; the peptones, which are probably complex polypeptides, cannot be salted out of solution like the proteoses; their molecules are smaller than those of the proteoses. We shall study them more fully under Digestion.

It is, however, convenient to add here a brief description of the meta-proteins, since some of the practical exercises at the head of this lesson deal with them.

They are obtained as the first stage of hydrolysis by enzymes, and also from the action of dilute acids or alkalis on either albumins or globulins. The general properties of the *acid* and *alkali-meta-proteins* which are thereby formed are as follows:—they are insoluble in pure water, but are soluble in either acid or alkali, and are precipitated by neutralisation unless certain disturbing influences such as sodium phosphate are present. They are precipitated, as globulins are, by saturation with such neutral salts as sodium chloride or magnesium sulphate. They are not coagulated by heat if in solution. In alkali-meta-protein some of the sulphur in the original protein is removed.

A variety of meta-protein (probably a compound containing a large quantity of alkali) may be formed by adding strong potash to undiluted white of egg. The resulting jelly is called *Lieberkühn's jelly*. A similar jelly is obtainable by adding strong acetic acid to undiluted egg-white.

The word "albuminate" is used for compounds of protein with mineral substances. Thus if a solution of copper sulphate is added to a solution of albumin a precipitate of copper albuminate is formed. Similarly, by the addition of other salts of the heavy metals, other metallic albuminates are obtainable. The halogens (chlorine, bromine, iodine) also form albuminates in this sense, and may be used for the precipitation of proteins.

It should be noted, in conclusion, that the foregoing classification of proteins is mainly applicable to those of animal origin. The vegetable proteins may roughly be arranged under the same main headings, although it is doubtful if a real and complete analogy exists in all cases. The cleavage products of the vegetable proteins are in the main the same as those of the animal proteins, but the quantity of each yielded is usually different. Many vegetable proteins, for instance, give a very much higher yield of glutamic acid than do those of animal origin.

There are also certain vegetable proteins, such as gliadin from the gluten of wheat, hordein from barley, and zein from maize, which stand apart from all other members of the group in being soluble in alcohol.

The vegetable proteins which have been mainly studied are those contained in the seeds of plants. They may provisionally be grouped into four main classes :—

1. Albumins, such as leucosin in wheat.

2. Globulins, such as edestin of hemp and other seeds ; most of these are readily crystallisable.

3. Glutelins. These are insoluble in water and saline solutions, and are soluble only in dilute alkali. They are probably not very strongly marked off from the globulins, since it has been shown that the solubility of globulins in dilute saline solutions is also due to a trace of alkali. The best example of this third class is the glutenin of wheat gluten.

4. Gliadins ; the proteins soluble in alcohol just alluded to. They are characterised also by the absence of lysine among their cleavage products, and usually yield a very high percentage of glutamic acid on decomposition. The gluten of wheat flour, which is formed when water is added to it, has been shown to consist of two proteins—one (gliadin) soluble in alcohol, the other (glutenin) soluble in alkali. It is to the former that the gluten of dough owns its cohesiveness ; and grains such as rice, which contain no gliadin, cannot in consequence be employed for making bread.

LESSON VI

FOODS

A. MILK.—**1.** Examine a drop of milk with the microscope.

2. Note the specific gravity of fresh milk with the lactometer; compare this with the specific gravity of milk from which the cream has been removed (skimmed milk). The specific gravity of skimmed milk is higher owing to the removal of the lightest constituent—the cream.

3. The reaction of fresh milk is neutral or slightly alkaline to litmus.

4. Warm some milk in a test-tube to the temperature of the body, and add a few drops of rennet. After standing, a *curd* is formed from the conversion of *caseinogen*, the chief protein in milk, into *casein*. The casein entangles the fat globules. The liquid residue is termed *whey*. No curdling is produced if the rennet solution is previously boiled, because heat destroys rennet as it does all enzymes.

5. Take some milk to which 0·2 per cent. of potassium oxalate has been added; warm to 40° C. and add rennet. No curdling takes place because the oxalate has precipitated the calcium salts which are necessary in the coagulation process.

Take a second specimen of oxalated milk and add a few drops of 2 per-cent. solution of calcium chloride, and then rennet; curdling or coagulation takes place if the mixture is kept warm in the usual way.

6. To another portion of warm milk diluted with water add a few drops of 20 per-cent. acetic acid. A lumpy precipitate of caseinogen entangling the fat is formed.

7. Filter off this precipitate, and in the filtrate test for *lactose* or *milk-sugar* by Fehling's solution (see Lesson III); for *lact-albumin* by boiling, or by Millon's reagent (see Lesson V); and for earthy (that is, calcium and magnesium) *phosphates* by ammonia, which precipitates these phosphates. Phosphates may also be detected in the following way: add nitric acid, boil and filter; warm the filtrate with ammonium molybdate; a yellow crystalline precipitate of ammonium phospho-molybdate is formed.

8. Fat (*butter*) may be extracted from the precipitate obtained in 6 by shaking it with ether; on evaporation of the ethereal extract the fat is left behind, forming a greasy stain on paper. The presence of fat may also be demonstrated by the black colour produced by the addition of osmic acid to the milk.

9. Shake up a little milk with twice its volume of ether ; the opacity of the milk remains nearly as great as before. Repeat this, but first add to the milk a few drops of caustic potash before adding the ether and then shake. The milk which lies beneath the ethereal solution of fat becomes translucent. As a matter of fact ether dissolves the fat without the addition of alkali, and the opacity of milk is therefore not due to the fat globules alone, but largely to their protein envelopes. The clearing which takes place when ether and alkali are added is due to an action of the reagents on the caseinogen.

10. *Caseinogen*, like globulin, is precipitated by saturating milk with sodium chloride or magnesium sulphate, and by half saturation with ammonium sulphate, but differs from the globulins in not being coagulated by heat. The precipitate produced by saturation with salt floats to the surface with the entangled fat, and the clear salted whey is seen below after an hour or two.

B. FLOUR.—Mix some wheat flour with a little water into a stiff dough. Wrap this up in a piece of muslin and knead it under a tap or in a capsule of water. The starch grains come through the holes in the muslin (identify by iodine test), and an elastic sticky mass remains behind. This is a protein called *gluten*. Suspend a fragment of gluten in water ; add nitric acid and boil ; it turns yellow ; cool and add ammonia ; it turns orange (xanthoproteic reaction). Boil another fragment with Millon's reagent ; it turns a brick-red colour.

C. BREAD contains the same constituents as flour, except that some of the starch has been converted into dextrin and dextrose during baking (most flours, however, contain a small quantity of sugar). Extract breadcrust with cold water, and test the extract for dextrin (iodine test) and for dextrose (Trommer's or Fehling's test). If hot water is used, starch also passes into solution.

D. MEAT.—This is our main source of protein food. Cut up some lean meat into fine shreds and grind these up with salt solution. Filter and test for proteins.

THE PRINCIPAL FOOD-STUFFS

We can now proceed to apply the knowledge we have obtained of the proteins, carbohydrates, and fats to the investigation of some important foods. The chief chemical substances in food are:—

$$\left.\begin{array}{l}\text{1. Proteins}\\ \text{2. Carbohydrates}\\ \text{3. Fats}\end{array}\right\}\text{organic.}$$

$$\left.\begin{array}{l}\text{4. Water}\\ \text{5. Salts}\end{array}\right\}\text{inorganic.}$$

In milk and in eggs, which form the exclusive foods of young animals, all varieties of these principles are present in suitable proportions. Hence they are spoken of as perfect foods. Eggs, though a perfect food for the developing bird, contain too little carbohydrate for a mammal. In most vegetable foods carbohydrates are in excess, while in animal foods, such as meat, the proteins are predominant. In a suitable diet these should be mixed in proper proportions, which vary for herbivorous and carnivorous animals. We must, however, limit ourselves to the omnivorous animal, man.

A healthy and suitable diet must possess the following characters:—

1. It must contain the proper amount and proportion of the various proximate principles.

2. It must be adapted to the climate, to the age of the individual, and to the amount of work done by him.

3. The food must contain, not only the necessary amount of proximate principles, but these must be present in a digestible form. As an instance of this, many vegetables (peas, beans, lentils) contain even more protein than beef and mutton, but are not so nutritious, as they are less digestible, much passing off in the fæces unused.

The nutritive value of a diet depends mainly on the amount of carbon and nitrogen it contains in a readily digestible form. A man doing a moderate amount of work, and taking an ordinary diet, will eliminate, chiefly from the lungs in the form of carbonic acid, from 250 to 280 grammes of carbon *per diem*. During the same time he will eliminate, chiefly in the form of urea in the urine, about 15 to 18 grammes of nitrogen. These substances are derived from the food, and from the metabolism of the tissues; various forms of energy, work and heat being the chief, are simultaneously liberated. During muscular exercise the output of carbon greatly increases; the increased excretion of nitrogen is insignificant. Taking, then, the state of moderate exercise, it is necessary that the waste of the tissues should be replaced by fresh material in the form of food; and the proportion of carbon to nitrogen should be the same as in the excretions: 250 to 15, or 16·6 to 1. The proportion of carbon to nitrogen in protein is, however, 53 to 15, or 3·5 to 1. The extra supply of carbon comes from non-nitrogenous foods—viz. fat and carbohydrate.

Voit gives the following daily diet:—

Protein	120 grammes.
Fat	100 ,,
Carbohydrate	333 ,,

Ranke's diet closely resembles Voit's; it is—

Protein	100 grammes.
Fat	100 ,,
Carbohydrate	250 ,,

In preparing diet tables, such adequate diets as those just given should be borne in mind. The following dietary (from G. N. Stewart) will be seen to be rather more liberal, but may be taken as fairly typical of what is usually consumed by an adult man in the twenty-four hours, doing an ordinary amount of work.

Food-stuff.	Quantity.		Grammes of					
			Nitrogen.	Carbon.	Proteins.	Fats.	Carbohydrates.	Salts.
	Metric system.	English weights.						
Lean meat .	250 grammes	9 oz.	8	33	55	8·5	0	4
Bread . .	500 ,,	18 ,,	6	112	40	7·5	245	6·5
Milk . .	500 ,,	¾ pint	3	35	20	20	25	3·5
Butter . .	30 ,,	1 oz.	0	20	0	27	0	0·5
Fat with meat	30 ,,	1 ,,	0	22	0	30	0	0
Potatoes .	450 ,,	16 ,,	1·5	47	10	0	95	4·5
Oatmeal .	75 ,,	3 ,,	1·7	30	10	4	48	2
			20·2	299	135	97	413	21

For a certain time at any rate, a man will maintain his weight and health on diets even scantier than those of Voit and Ranke. The most convincing of these experiments have been performed by Chittenden upon himself and others. Chittenden urges that the normal diet should contain only about half the customary quantity of protein. The body certainly does not assimilate the larger amount usually taken, for the greater part of the nitrogenous constituents is converted into amino-acids, which are rapidly transformed by the liver into urea and cast out of the body, leaving the non-nitrogenous remainder to be utilised in the same way as fats and carbohydrates are, for the production of heat and energy. Although Chittenden's views have not met with general acceptance, his work will bring home to many people that temperance is necessary in food as well as in drink. The majority of well-to-do people certainly eat an excess of meat, and so throw an unnecessary strain upon their digestive and excretory organs. One should hesitate, however, in accepting Chittenden's conclusions to the full, for it is doubtful if the *minimum* is also the *optimum* diet. It may be that there is a real need for an excess of protein beyond the apparent minimum. In diamond mining a large quantity of earth must be crushed to obtain the precious stones. It may be that among the many cleavage products of protein the majority may be compared to this waste earth, and we get rid of them as quickly as possible in the excretions, but some few may be unusually precious for protein synthesis in the body, and that, in order to get an adequate supply of these, a comparatively large amount of protein must be ingested.

Recent research has shown that there is something else in an adequate diet which is necessary, especially during the time of growth. These unknown constituents (vitamines) will be discussed in the concluding section of this chapter.

MILK

Milk is often spoken of as a "perfect food," and it is so for infants. For those who are older it is so voluminous that unpleasantly large quantities of it would have to be taken in the course of the day to ensure the proper supply of nitrogen and carbon. Moreover, for adults it is relatively too rich in protein and fat. It also contains too little iron (Bunge); hence children weaned late become anæmic.

The microscope reveals that it consists of two parts: a clear fluid and a number of minute particles that float in it. These consist of minute fat globules, varying in diameter from 0·0015 to 0·005 millimetre.

The milk secreted during the first few days of lactation is called *colostrum*. It contains very little caseinogen, but large quantities of globulin instead. Microscopically, cells from the acini of the mammary gland are seen, which contain fat globules in their interior: they are called colostrum corpuscles.

Reaction and Specific Gravity.—The reaction of fresh cow's milk and of human milk is amphoteric towards litmus. This is due to the presence of both acid and alkaline salts ; the latter are usually in excess. Milk readily turns acid or sour as the result of fermentative change, part of its lactose being transformed into lactic acid (see p. 28). The specific gravity of milk is usually ascertained with the hydrometer. That of normal cow's milk varies from 1028 to 1034. When the milk is skimmed the specific gravity rises, owing to the removal of the light constituent, the fat, to 1033 to 1037. In all cases the specific gravity of water, with which other substances are compared, is taken as 1000.

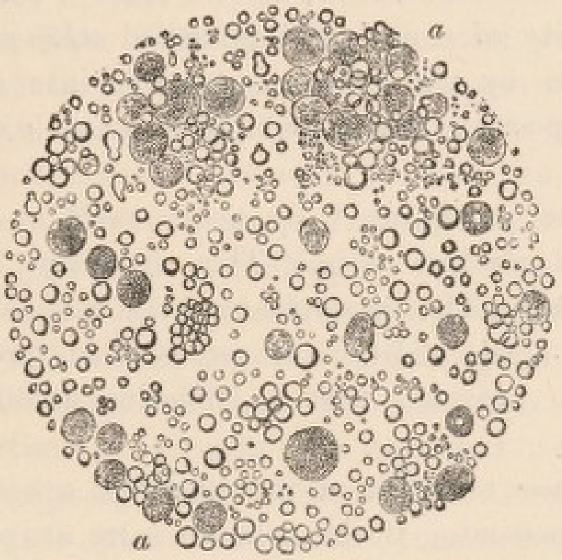

FIG. 9.—Microscopic appearance of milk in the early stage of lactation, showing colostrum corpuscles (*a*) in addition to fat globules. (Yeo.)

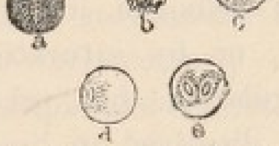

FIG. 10.—*a*, *b*, colostrum corpuscles with fine and coarse fat globules respectively ; *c*, *d*, *e*, pale cells devoid of fat. (Heidenhain.)

Composition.—Bunge gives the following table, contrasting the milk of woman and cow :—

	Woman.	Cow.
	Per cent.	Per cent.
Proteins (chiefly caseinogen) . .	1·7	3·5
Butter (fat)	3·4	3·7
Lactose	6·2	4·9
Salts	0·2	0·7

Hence, in feeding infants on cow's milk, it will be necessary to dilute it, and add sugar and a little cream to make it approximately equal to natural human milk.

The Proteins of Milk.—The principal protein in milk is called *caseinogen* : this is the one which is coagulated by rennet to form *casein*. Cheese consists of casein with the entangled fat. The other protein in milk is an albumin. It is present in small quantities only; it differs in some of its properties (specific rotation, coagulation temperature, and solubilities) from serum-albumin; it is called *lact-albumin.*

The Coagulation of Milk.—Rennet is the agent usually employed for this purpose; it is an enzyme secreted by the stomach, especially by sucking animals, and is generally obtained from the calf.

The *curd* consists of the casein and entangled fat: the liquid residue called *whey* contains the sugar, salts, and albumin of the milk. There is also a small quantity of a new protein called *whey-protein,* which differs from caseinogen by not being convertible into casein. It is produced by the decomposition of the caseinogen molecule during the process of curdling.

Caseinogen itself may be precipitated by acids such as acetic acid, or by saturation with neutral salts. This, however, is not coagulation, but precipitation. The precipitate may be collected and dissolved in lime water; the addition of rennet then produces coagulation in this solution, provided that a sufficient amount of calcium salts is present.

In milk also, rennet produces coagulation, provided that a sufficient amount of calcium salts is present. If the calcium salts are precipitated by the addition of potassium oxalate, rennet causes no formation of casein. The process of curdling in milk is a double one; the first action due to rennet is to produce a change in caseinogen; the second action is that of the calcium salt, which precipitates the altered caseinogen as casein. In blood also calcium salts are necessary for coagulation; but there they act in a different way, namely, in the production of fibrin-ferment (see COAGULATION OF BLOOD).

Caseinogen is not coagulable by heat. We have already classed it with vitellin as a phospho-protein (see p. 61).

Caseinogen, as was originally pointed out by Hammarsten, is a protein with acid properties: it is quite insoluble in water, but it forms soluble salts with such metallic bases as potassium, sodium, and calcium. The caseinogen as it exists in milk is combined with calcium as calcium caseinogenate, When acetic acid is added to milk, we therefore get calcium acetate, and a precipitate of free caseinogen. On " dissolving " this caseinogen in an alkali such as soda or potash, we have the formation of sodium caseinogenate or potassium caseinogenate, as the case may be. The precipitate obtained in milk by the addition of alcohol, or by " salting out," is not free caseinogen, but calcium caseinogenate. When we add potassium oxalate to milk, we get the reaction represented in the following equation:—Calcium caseinogenate + potassium oxalate = calcium oxalate + potassium caseinogenate. When we add calcium chloride to oxalated milk, the following equation represents what occurs :—Potassium caseinogenate + calcium chloride = calcium caseinogenate + potassium chloride.

Calcium caseinogenate forms an opalescent or colloidal solution in water, and reacts with the rennet enzyme. The caseinogenates of magnesium, barium, and strontium have similar characters. The caseinogenates of potassium, sodium, and ammonium differ from the above by forming a nearly clear solution in water, and they do not react with the rennet enzyme (W. A. Osborne).

The Fats of Milk.—The chemical composition of the fat of milk (butter) is very like that of adipose tissue. It consists chiefly of palmitin, stearin, and olein. There are, however, smaller quantities of fats derived from fatty acids lower in the series, especially butyrin and caproin. The old statement that each fat globule is surrounded by a film of protein is, according to Ramsden's recent work, correct. Milk also contains small quantities of lipoids, namely, lecithin and cholesterol; and a yellow fatty pigment or lipochrome.

Milk Sugar or Lactose.—This is a disaccharide ($C_{12}H_{22}O_{11}$). Its properties have already been described in Lesson III, p. 28.

Souring of Milk.—When milk is allowed to stand, the chief change which it is apt to undergo is a conversion of a part of its lactose into lactic acid. This is due to the action of micro-organisms, and would not occur if the milk were contained in cold sterilised vessels. Equations showing the change produced are given on p. 28. When souring occurs, the acid which is formed precipitates a portion of the caseinogen. This must not be confounded with the formation of casein from caseinogen which is produced by rennet. There are, however, some bacterial growths which produce true coagulation like rennet.

Alcoholic Fermentation in Milk.—When yeast is added to milk, the sugar does not readily undergo the alcoholic fermentation. Other somewhat similar fungoid growths are, however, able to produce the change, as in the preparation of koumiss; the milk sugar is first inverted, that is, glucose and galactose are formed from it (see p. 28), and it is from these sugars that alcohol and carbonic acid originate.

The Salts of Milk.—The chief salt present is calcium phosphate; a small quantity of magnesium phosphate is also present. The other salts are chiefly chlorides of sodium and potassium.

Differences between different Milks.—It is an undoubted fact that the milk provided by nature for the growing offspring is different in the various classes of the animal kingdom. The quantitative variations are often enormous, and it has been shown that the milk best adapted for the nutrition of the young animal is that which comes from its mother, or, at least, from an animal of the same species. The practical application of this rule comes home most to us when dealing with the feeding of children, and it is universally acknowledged that, after all, cows' milk is but a poor substitute for human milk. Cows' milk is, of course, diluted, and sugar and cream added, so as to make it quantitatively like mothers' milk, but even then the question arises whether the essential difference between the two kinds of milk is not deeper than one of mere quantity; and, in particular, the pendulum of scientific opinion has swung backwards and forwards in relation to the question whether the principal protein, called caseinogen in both, is really identical in the two cases. The caseinogen of human milk curdles in small flocculi in the stomach, so contrasting with the heavy curd which cows' milk forms; and even although the curdling of cows' milk be made to occur in smaller fragments by mixing the milk with barley water or lime water, its digestion proceeds with comparative slowness in the child's alimentary canal. These are practical points well known to every clinical observer, and in the past they have been attributed, not so much to fundamental differences in the caseinogen itself, as to accidental concomitant factors; the excess of citric acid in human milk, for instance, or its paucity in calcium salts, having been held responsible for the differences observed in the physical condition of the curd and in its digestibility.

This question is far from settled even to-day, but there are some data now available that point to a qualitative difference between caseinogens. Some of these depend on the application of the "biological test" carried out on the line of immunity experiments, which has been so signally successful in the distinction between the blood-proteins of different species of animals (see Lesson IX). The differences, however, which lead to the formation of specific precipitins are so slight, that ordinary chemical methods of analysis are, at present, unable to reveal them. But, in the case of milk, there are differences which the chemist can detect. One cannot lay much stress on mere percentage composition, although differences have been noted in that, because we have no guarantee that the proteins investigated were separated from all impurities; there are also small differences in the percentage of mono-amino-acids obtained after hydrolysis, but the present methods of estimating these with accuracy leave much to be desired. A deeper chemical distinction noted is contained in the recent work of Bienenfeld, who finds that human caseinogen contains a carbohydrate complex which is absent from that of the cow.

A few years ago it was stated that human caseinogen will not curdle with rennet; but this has been shown to be a mistake. The conditions of rennet curdling are somewhat different in the two kinds of milk we are considering, but provided the reaction in the stomach is acid, human milk is curdled by rennet when acted on by gastric juice.

EGGS

In this country the eggs of hens and ducks are those particularly selected as foods. The *shell* is made of calcareous matter, especially calcium carbonate. The *white* is composed of a richly albuminous fluid enclosed in a network of firmer and more fibrous material. The amount of solids is 13·3 per cent.: of this 12·2 is protein in nature. The proteins are albumin, with smaller quantities of egg-globulin and ovo-mucoid (p. 62). The remainder is made up of sugar (0·5 per cent.), traces of fats, lecithin and cholesterol, and 0·6 per cent. of inorganic salts. The *yolk* is rich in food materials for the development of the future embryo. In it there are two varieties of yolk-spherules, one kind yellow and opaque (due to admixture with fat and a yellow lipochrome), and the other smaller, transparent and almost colourless: these are protein in nature, consisting of the phospho-protein called *vitellin* (p. 61). Lecithin, cholesterol, small quantities of sugar, and inorganic salts are also present.

The nutritive value of eggs is high, as they are so readily digestible; but the more an egg is cooked the more insoluble do its protein constituents become.

MEAT

This is composed of the muscular and connective (including adipose) tissues of certain animals. The flesh of some animals is not eaten; in some cases this is a matter of fashion; some flesh, like that of the carnivora, is stated to have an unpleasant taste; and in other cases (*e.g.* the horse) it is more lucrative to use the animal as a beast of burden.

Meat is the most concentrated and most easily assimilable of nitrogenous foods. It is our chief source of nitrogen. Its chief solid constituent is protein, and the principal protein is myosin. In addition to the extractives and salts contained in muscle, there is always a certain percentage of fat, even though all visible adipose tissue is dissected off. The fat-cells are placed between the muscular fibres, and the amount of fat so situated varies in different animals. It is particularly abundant in pork; hence the indigestibility of this form of flesh; the fat prevents the gastric juice from obtaining ready access to the muscular fibres.

The following table gives the chief substances in some of the principal meats used as food :—

Constituents.	Ox.	Calf.	Pig.	Horse.	Fowl.	Pike.
Water	76·7	75·6	72·6	74·3	70·8	79·3
Solids	23·3	24·4	27·4	25·7	29·2	20·7
Proteins, including gelatin	20·0	19·4	19·9	21·6	22·7	18·3
Fat	1·5	2·9	6·2	2·5	4·1	0·7
Carbohydrate	0·6	0·8	0·6	0·6	1·3	0·9
Salts	1·2	1·3	1·1	1·0	1·1	0·8

The large percentage of water in meat should be particularly noted ; if a man wished to take his daily quantity of 100 grammes of protein entirely in the form of meat, it would be necessary for him to consume about 500 grammes (*i.e.* a little more than 1 lb.) of meat *per diem.*

FLOUR

The best wheat flour is made from the interior of wheat grains, and contains the greater proportion of the starch of the grain and most of the protein. Whole flour is made from the whole grain *minus* the husk, and thus contains, not only the white interior, but also the "germ" or embyro plant, and the harder and browner outer portion of the grain. This outer region contains a somewhat larger proportion of the proteins of the grain. Whole flour contains 1 to 2 per cent. more protein than the best white flour, but it has the disadvantage of being less readily digested. Brown flour contains a certain amount of bran in addition ; it is still less digestible, but is useful as a mild laxative, the insoluble cellulose mechanically irritating the intestinal canal as it passes along.

The best flour contains very little sugar. The presence of sugar indicates that germination has commenced in the grains. In the manufacture of malt from barley this is purposely allowed to go on.

When mixed with water, wheat flour forms a sticky adhesive mass called dough. This is due to the formation of gluten, and the forms of grain poor in gluten cannot be made into dough (oats, rice, etc.). Gluten does not exist in the flour as such, but is formed on the addition of water from the pre-existing soluble proteins in the flour. It is a mixture of the two proteins gliadin and glutenin (see p. 67).

The following table contrasts the composition of some of the more important vegetable foods :—

Constituents.	Wheat.	Barley.	Oats.	Rice.	Lentils.	Peas.	Potatoes.
Water	13·6	13·8	12·4	13·1	12·5	14·8	76·0
Protein	12·4	11·1	10·4	7·9	24·8	23·7	2·0
Fat	1·4	2·2	5·2	0·9	1·9	1·6	0·2
Starch	67·9	64·9	57·8	76·5	54·8	49·3	20·6
Cellulose	2·5	5·3	11·2	0·6	3·6	7·5	0·7
Mineral salts	1·8	2·7	3·0	1·0	2·4	3·1	1·0

We see from this table—

1. The great quantity of starch always present.

2. The small quantity of fat; that bread is generally eaten with butter is a popular recognition of this fact.

Protein, except in potatoes, is pretty abundant, and especially so in the pulses (lentils, peas, etc.). The protein in the pulses is not gluten, but consists mainly of globulins.

In the mineral matters in vegetables, salts of potassium and magnesium are, as a rule, more abundant than those of sodium and calcium.

BREAD

Bread is made by cooking the dough of wheat flour mixed with yeast, salt, and flavouring materials. An enzyme in the flour acts at the commencement of the process when the temperature is kept a little over that of the body, and forms dextrin and sugar from the starch, and then the alcoholic fermentation, due to the action of the yeast, begins. The bubbles of carbonic acid, burrowing passages through the bread, make it light and spongy. This enables the digestive juices subsequently to soak into it readily and affect all parts of it. During baking the gas and alcohol are expelled from the bread, the yeast is killed, and a crust forms from the drying of the outer portions of the loaf.

White bread contains, in 100 parts, 7 to 10 of protein, 55 of carbohydrate, 1 of fat, 2 of salts, and the rest water.

COOKING OF FOOD

The cooking of foods is a development of civilisation, and much relating to this subject is a matter of education and taste rather than of physiological necessity. Cooking, however, serves many useful ends :—

1. It destroys all parasites and danger of infection. This relates, not only to bacterial growths, but also to larger parasites, such as tapeworms and trichinæ.

2. In the case of vegetable foods it breaks up the starch grains, bursting the cellulose and allowing the digestive juices to come into contact with granulose.

3. In the case of animal foods it converts the insoluble collagen of the universally distributed connective tissues into the soluble gelatin. The loosening of the fibres is assisted by the formation of steam between them. By thus loosening the binding material, the more important elements of the food, such as muscular fibres, are rendered accessible to the gastric and other juices. Meat before it is cooked is generally kept a certain length of time to allow *rigor mortis* to pass off.

Of the two chief methods of cooking, roasting and boiling, the former is the more economical, as by its means the meat is first surrounded with a coat of coagulated protein on its exterior, which keeps in the juices to a great extent, letting little else escape but the dripping (fat). Whereas in boiling, unless bouillon and bouilli are used, there is considerable waste. Cooking, especially boiling, renders the proteins more insoluble than they are in the raw state, but this is counterbalanced by the other advantages that cooking possesses.

Beef Tea.—In making beef tea and similar extracts of meat it is necessary that the meat should be placed in cold water, and this is gradually and carefully warmed. In cooking a joint it is usual to put the meat into boiling water at once, so that the outer part is coagulated, and the loss of material minimised.

An extremely important point in this connection is that beef tea and similar meat extracts should not be regarded as foods. They are valuable as pleasant stimulating drinks for invalids, but they contain very little of the nutritive material of the meat, their chief constituents, next to water, being the salts and extractives (creatine, hypoxanthine, lactic acid, etc.), of flesh.

Many invalids restricted to a liquid diet get tired of milk, and imagine that they get sufficient nutriment by taking beef tea instead. It is very important that this erroneous idea should be corrected. One of the greatest difficulties that a physician has to deal with in these cases is the distaste which many adults evince for milk. It is essential that this should be obviated as far as possible by preparing the milk in different ways to avoid monotony. Some can take koumiss; but a less expensive variation may be introduced in the shape of junkets, which, although well known in the West of England, are comparatively unknown in other parts. The preparation of a junket consists of adding to warm milk in a bowl or dish a small quantity of rennet (Clark's essence is very good for this purpose) and flavouring material according to taste. The mixture is then put aside, and in a short time the milk sets into a jelly (coagulation of casein), which may then be served with or without cream.

Soup contains the extractives of meat, a small proportion of the proteins, and the principal part of the gelatin. The gelatin is usually increased by adding bones and fibrous tissue to the stock. It is the presence of this substance which causes the soup when cold to gelatinise.

ACCESSORIES TO FOOD

Among these must be placed *alcohol*, the value of which within moderate limits is not as a food, but as a stimulant; *condiments* (mustard, pepper, ginger, curry powder, etc.), which are stomachic stimulants, the abuse of which is followed by dyspeptic troubles; and *tea*, *coffee*, *cocoa*, and similar drinks. These are stimulants chiefly to the nervous system: tea, coffee, maté (Paraguay), guarana (Brazil), cola nut (Central Africa), bush tea (South Africa), and a few other plants used in various countries all owe their chief property to an alkaloid called *theine* or *caffeine* ($C_8H_{10}N_4O_2$); cocoa to the closely related alkaloid, *theobromine* ($C_7H_8N_4O_2$); coca to *cocaine*. These alkaloids are all poisonous, and used in excess, even in the form of infusions of tea and coffee, produce over-excitement, loss of digestive power, and other disorders well known to physicians. Coffee differs from tea in being rich in aromatic matters; tea contains a bitter principle, tannin. To avoid the injurious solution of too much tannin, tea should only be allowed to infuse (draw) for a few minutes. Cocoa is not only a stimulant, but a food as well: it contains about 50 per cent. of fat and 12 per cent. of protein. In cocoa, as manufactured for the market, the amount of fat is reduced to 30 per cent., and the amount of protein rises proportionately to about 20 per cent.

Green vegetables are taken as a palatable adjunct to other foods rather than for their nutritive properties. Their potassium salts are, however, abundant. Cabbage, turnips, and asparagus contain 80 to 92 water, 1 to 2 protein, 2 to 4 carbohydrates, and 1 to 1·5 cellulose per cent. The small amount of nutriment in most green foods accounts for the large meals made by and the vast capacity of the alimentary canal of herbivorous animals.

VITAMINES

If an animal is fed upon a mixture of pure protein, fat, and carbohydrate, with a due admixture of salts and water, it does not thrive, but shows evidence of malnutrition, although the quantities given may be theoretically correct. If a growing animal is fed on such a diet it ceases to grow. But if, as Hopkins showed recently, a small amount of a natural food, such as milk, is mixed with the artificial diet just referred to, the animals thrive and grow normally. There is something extra, something which is at present unknown, which is absolutely essential, and quite small amounts of it are usually sufficient.

If this unknown constituent is absent from a man's diet, he undergoes just the same sort of malady, and illnesses so produced, such as scurvy and Beri-beri, are termed "Deficiency diseases."

A good deal of work has recently been done in relation to one of these diseases, namely, Beri-beri (the Kak-ke of Japan). This is prevalent among the natives whose staple article of diet is polished rice, that is, rice grains deprived of their external layer. This disease is characterised by malnutrition of the nerves, and neuritis or inflammation of the nerves is followed by nerve-degeneration and paralysis. It can also be produced in birds by feeding them on polished rice; and in both man and bird can be rapidly cured by adding the polishings of the rice grains. The outer layer of the grain contains the extra something, and Funk and others have recently separated out from the polishings a base which they have termed *vitamine*, although at present there is no certainty of its chemical composition. Funk's hypothesis that the vitamines are derivatives of pyrimidine should be taken with caution. At any rate this base is the curative principle, and very small doses will cure the condition.

Vitamine is not confined to rice grains, but is found in many other vegetable and animal foods. The value of whole meal bread, for example, does not depend on the small extra amount of protein it contains, but probably here also upon a vitamine. The amount of vitamine varies considerably. Thus, in pigeons fed upon polished rice, as much as 20 grammes of meat daily must be added to prevent the occurrence of Beri-beri; whereas 3 grammes of egg-yolk are sufficient, and half a gramme of yeast is enough.

LESSON VII

THE DIGESTIVE JUICES

SALIVA

1. The reaction of saliva is alkaline to litmus paper.

2. To a little saliva in a test-tube add acetic acid. Mucin is precipitated in stringy flakes.

3. Filter off the mucin thus precipitated; the filter will also catch any cells which may be present. Apply the xanthoproteic or Millon's test to the filtrate; the presence of a small amount of protein is shown.

4. The presence of potassium thiocyanate (KCNS) in saliva may be shown by the red colour given by a drop of ferric chloride solution; this colour is discharged by the addition of a drop of mercuric chloride solution. The presence and amount of potassium thiocyanate in saliva are, however, very inconstant.

5. Put some 0·5 per-cent. solution of starch into three test-tubes, A, B, and C. Add some saliva to B and C; faintly acidulate C with 0·2 per-cent. hydrochloric acid; then place the three tubes in the water-bath at 40° C. After five or six minutes remove the test-tubes and examine their contents. The starch to which no saliva was added (tube A) will be unaltered, and will give a deep blue colour on the addition of a drop of iodine solution. The same is true for the acidulated specimen (tube C). The contents of test-tube B will give a red-brown colour with iodine owing to the presence of erythro-dextrin, and will also contain a reducing sugar (maltose), as can be shown by boiling with Fehling's solution.

6. THE ACHROMIC POINT.—By the action of ptyalin, the starch-splitting enzyme of saliva, starch is converted into (1) soluble starch, (2) erythro-dextrin, which gives a red-brown colour with iodine; (3) achroö-dextrin, which gives no colour with iodine; and (4), finally, maltose. In accurate work with amylolytic enzymes, it is usual to determine the rate of their action by determining the exact point when iodine ceases to give a coloration; this corresponds to the moment when all the erythro-dextrin has disappeared, having been converted into achroö-dextrin and maltose; this is known as the *achromic point*. This may be determined with saliva in the following way: Rinse out the mouth

thoroughly with warm distilled water; then collect some saliva, dilute it with five times its volume of distilled water and filter.

Place a number of drops of iodine solution on a white testing slab.

Then mix 5 c.c. of a 0·5 per-cent. solution of starch with an equal quantity of the diluted filtered saliva, and place the mixture in the water-bath at 40° C. Every half minute or so transfer a drop of the digesting mixture with a glass rod to a drop of iodine solution. At first, as long as starch is present, the colour struck will be blue; then as erythro-dextrin appears the colour will be violet, owing to the mixture of the blue (due to starch) and of the red (due to erythro-dextrin); a little later the colour struck will be the red-brown due to the presence of erythro-dextrin; with successive drops after this the colour will get fainter and fainter, until finally the achromic point is reached. Note the time this has taken from the moment when the starch and saliva mixture was placed in the warm bath. Compare this time with that obtained by other members of the class, and a relative measure of the activity of the saliva of different people will in this way be obtained.

GASTRIC DIGESTION

1. Half fill four test-tubes—

A with water; B with 0·2 per-cent. hydrochloric acid [1]; C with 0·2-per-cent. hydrochloric acid; D with solution of white of egg (1 to 10 of water).

2. To A add a few drops of glycerol extract of stomach [2] (this contains pepsin) and a piece of a solid protein like fibrin.

To B also add pepsin solution and a piece of fibrin.

To C add only a piece of fibrin.

To D add a few drops of pepsin solution and fill up the tube with 0·2 per-cent. hydrochloric acid.

3. Put the tubes into the water-bath at 40° C, and observe them carefully.

In A the fibrin remains unaltered.

In B it becomes swollen, and gradually dissolves.

In C it becomes swollen, but does not dissolve.

These experiments show that neither pepsin nor hydrochloric acid alone digest protein, but that both must be present for this purpose.

After half an hour examine the solution in test-tube B.

(a) Colour some of the liquid with litmus and neutralise with dilute alkali. Acid-meta-protein is precipitated.

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[1] Made by adding 994 c.c. of water to 6 c.c. of commercial concentrated hydrochloric acid.

[2] Benger's liquor pepticus may be used instead of the glycerol extract of stomach.

(b) Take another test-tube, and put into it a drop of 1 per-cent. solution of copper sulphate ; empty it out so that the merest trace of copper sulphate remains adherent to the wall of the tube ; then add the solution from test-tube B and a few drops of strong caustic potash. A pink colour (biuret reaction) is produced. This should be carefully compared with the violet tint given by unaltered albumin.

(c) To a third portion of the fluid in test-tube B add a drop of nitric acid ; proteoses or propeptones are precipitated. This precipitate dissolves on heating and reappears on cooling.

Repeat these three tests with the digested white of egg in test-tube D.

4. Examine an artificial gastric digestion which has been kept a week. Note the absence of putrefactive odour ; in this it contrasts very forcibly with an artificial pancreatic digestion under similar conditions.

ENZYMES

The word **fermentation** was first applied to the change of sugar into alcohol and carbonic acid by means of yeast. The evolution of carbonic acid causes frothing and bubbling; hence the term "fermentation." The agent yeast which produces this used to be called the ferment. Microscopic investigation shows that yeast is composed of minute rapidly growing unicellular organisms belonging to the fungus group of plants.

The souring of milk, the transformation of urea into ammonium carbonate in decomposing urine, and the formation of vinegar (acetic acid) from alcohol are produced by the growth of very similar organisms. The complex series of changes known as putrefaction, which are accompanied by the formation of malodorous gases, and which are produced by the growth of various forms of rapidly multiplying bacteria, also come into the same category.

That the change or fermentation is produced by these organisms is shown by the fact that it occurs only when the organisms are present, and stops when they are removed or killed by a high temperature or by certain substances (carbolic acid, mercuric chloride, etc.) called antiseptics.

The "germ theory" of disease explains the infectious diseases by considering that the change in the system is of the nature of fermentation, and, like the others we have mentioned, produced by microbes; the transference of the bacteria or their spores from one person to another constitutes infection. The poisons produced by the growing bacteria appear to be either alkaloidal (ptomaines) or protein in nature. The existence of poisonous proteins is a very remarkable thing, as no profound chemical differences have yet been shown to exist between them and those which are not poisonous, but which are useful as foods. Snake venom is an instance of a very virulent poison of protein nature.

The microscopic appearances of some of the micro-organisms concerned are shown in the accompanying illustration (fig. 11).

All these micro-organisms require moisture in which to act. They act best at a temperature of about 40° C. Their activity is stopped, but the organisms are not destroyed by cold; even after being subjected to the intense cold of liquid air, they resume activity when once more raised to a suitable temperature. The organisms are, however, like other living cells, killed by too great heat. Some micro-organisms act without free oxygen; these are called *anaërobic*, in contradistinction to those that require oxygen and which are therefore called *aërobic*.

Another well-known fact concerning micro-organisms is that the substances they produce in time put a stop to their activity; thus in the case of yeast, the alcohol produced, and in the case of putrefactive bacteria acting on proteins, the phenol, cresol, etc., produced, first stop the growth of and ultimately kill the organisms in question.

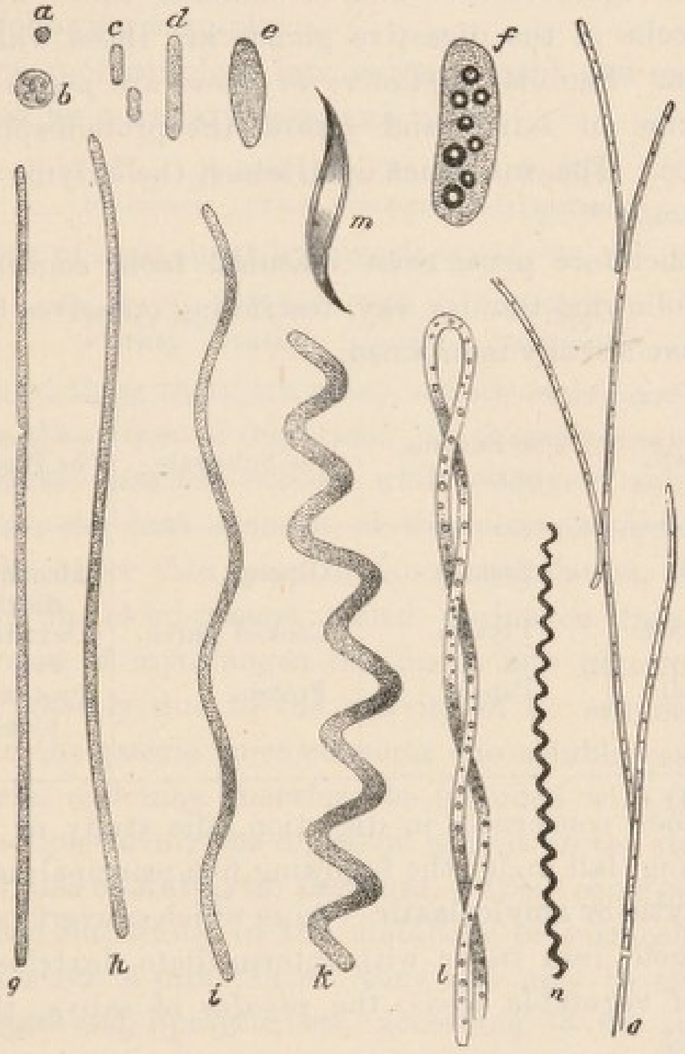

FIG. 11,—Typical forms of Schizomycetes (after Zopf): *a*, micrococcus; *b*, macrococcus; *c*, bacterium; *d*, bacillus; *e*, clostridium; *f*, monas Okenii; *g*, leptothrix; *h*, *i*, vibrio; *k*, spirillum; *l*, spirulina; *m*, spiromonas; *n*, spirochæte; *o*, cladothrix.

For a long time it was uncertain how micro-organisms were able to effect these chemical transformations. It is now, however, definitely proved that they do so by producing agents of a chemical nature which were formerly called soluble ferments, but are now usually spoken of as *enzymes*. This was first demonstrated in connection with the invertase of yeast cells, and with the enzyme secreted by the micrococcus ureæ which converts urea into ammonium carbonate in putrefying urine. For a long time, however, efforts to obtain from yeast cells an enzyme capable of bringing about the alcoholic fermentation were unsuccessful.

This is because the enzyme does not leave the yeast cells, but acts intracellularly. Buchner finally, by crushing the yeast cells, succeeded in obtaining from them the long-sought enzyme, and he termed it *zymase*; and since then other enzymes have been obtained from other micro-organisms by similar means.

Enzymes are also formed by the cells of the higher organisms, both in animal and vegetable life; and in animals those which are produced by the cells of the digestive glands are those which have been longest known. Familiar instances of these are *ptyalin*, the starch-splitting enzyme of saliva, and *pepsin*, the protein-splitting enzyme of gastric juice. The substance upon which the enzyme acts is spoken of as the *substrate*.

We may therefore place these essential facts concerning enzyme action in the following tabular way, restricting ourselves for the present to those we have already mentioned.

The Living Cell.	The Enzyme produced.	The Substrate.	The Products of Action.
The yeast cell.	Zymase.	Glucose.	Alcohol and carbon dioxide.
The salivary cell.	Ptyalin.	Cooked starch.	Dextrins and maltose.
The gastric cell.	Pepsin.	Protein.	Proteoses and peptones.

The enzymes concerned in digestion, the study of which we are now commencing, fall under the following five principal headings:—

1. **Amylolytic or amyloclastic**—those which convert polysaccharides (starch, glycogen) into sugar with intermediate dextrins. Examples: the *diastase* of vegetable seeds, the *ptyalin* of saliva, the *amylase* of pancreatic juice.

2. **Inverting** — those which convert disaccharides into monosaccharides. Examples:—

   (*a*) *Invertase* of yeast cells; *invertase* of intestinal juice; these convert sucrose into equal parts of glucose and fructose.
   (*b*) *Maltase* of intestinal juice; this converts maltose into glucose.
   (*c*) *Lactase* of intestinal juice; this converts lactose into equal parts of glucose and galactose.

3. **Lipolytic or lipoclastic**—those which split fats into fatty acids and glycerol. An example, *lipase*, is found in pancreatic juice.

4. **Proteolytic or proteoclastic**—those which split proteins into

proteoses, peptones, polypeptides, and finally amino-acids. Examples: the *pepsin* of gastric, and the *trypsin* of pancreatic juice.

5. **Peptolytic or peptoclastic** — those which split proteoses and peptones into polypeptides and amino-acids. The *erepsin* of intestinal juice is an example of these.

The enzymes in the foregoing list are hydrolytic; that is, water is added to the substrate, which then splits into simpler molecules. This is seen in the following examples:—

(*a*) Conversion of cellulose into carbonic acid and methane by the enzyme secreted by putrefactive organisms:—

$$\underset{\text{[cellulose]}}{(C_6H_{10}O_5)_n} + \underset{\text{[water]}}{nH_2O} = \underset{\text{[carbonic acid]}}{3nCO_2} + \underset{\text{[methane]}}{3nCH_4}$$

(*b*) Inversion of cane sugar by invertase:—

$$\underset{\text{[sucrose]}}{C_{12}H_{22}O_{11}} + \underset{\text{[water]}}{H_2O} = \underset{\text{[glucose]}}{C_6H_{12}O_6} + \underset{\text{[fructose]}}{C_6H_{12}O_6}$$

In addition to these there are many others which are concerned in other processes than those of digestion. Of these we may mention:—

6. **Coagulative enzymes**—those which convert soluble into insoluble proteins; the best example of this class is *thrombin* or *fibrin-ferment*, which comes into play in blood-coagulation, converting the soluble protein in blood-plasma called fibrinogen into fibrin. The similar conversion of myosinogen in muscle into myosin during *rigor mortis* is also possibly due to the activity of an enzyme. *Rennet* or *rennin* found in the gastric juice converts the soluble caseinogenate of milk into casein, and may therefore be included with these enzymes; it comes into action during the digestion of milk in the stomach.

7. **Intracellular or Autolytic Enzymes.**—These come into play during cell life, and are important in the metabolic or intracellular chemical changes which occur in protoplasm; they also may be subdivided into proteolytic, peptolytic, lipolytic, etc., according to the substrate upon which they act. After death their activity continues, and so they produce self-digestion or *autolysis* of the cells in which they are situated, if the tissue or organ is kept at an appropriate temperature and under aseptic conditions.

8. **Oxidases,** which are oxygen carriers and produce oxidation: they are mainly found as intracellular enzymes, and are important in tissue respiration.

9. **Reductases.**—These are the counterpart of the oxidases and produce reduction in the tissues.

10. **Deamidases.**—These remove the amino-group from amino-compounds.

These ten groups do not by any means exhaust the list; there is in the living organism no group of agents as important as enzymes in bringing about the chemical changes necessary for its continued metabolism and well-being. We shall come across many examples of enzymic action in our subsequent studies, for instance in the formation of urea, of uric acid, etc. etc.

There are, however, certain considerations of a more general nature which we may enumerate here. The first of these is:—

The Chemical Nature of Enzymes.—This is a subject which is very difficult to investigate; the enzymes are substances that to a great extent elude the grasp of the chemist. No one has ever yet been successful in obtaining any enzyme in a state of absolute purity; but we are perhaps safe in saying that if they are not protein in character, they are substances closely allied to the proteins.

Zymogens.—These are the parent substances or precursors of the enzymes. The granules seen in many secreting cells consist very largely of zymogen, which in the act of secretion is converted into the active enzyme. Thus pepsin is formed from pepsinogen, trypsin from trypsinogen, thrombin from thrombogen or prothrombin, and so forth.

Activation of Enzymes. Co-enzymes.—Many enzymes contained in secretions are in a condition ready for action. In other cases this is not so, and their action only occurs after they have been rendered energetic by the presence or action of other substances termed activating agents or co-enzymes. The *modus operandi* of the co-enzyme appears to vary in different cases; in some cases the co-enzyme is itself an enzyme; in other cases it is a more or less simple organic substance, or even an inorganic material. Pepsin, for instance, will only act in an acid medium, and its most favourable ally is hydrochloric acid; a compound of the two substances, pepsin-hydrochloric acid, appears to be the effective agent in the proteolysis which occurs in the stomach. Trypsin is not present as such in the fresh pancreatic juice; what is present is trypsinogen; this is converted into the active enzyme trypsin when it meets the entero-kinase of the succus entericus; and entero-kinase is itself an enzyme, an enzyme of enzymes, as Pawlow terms it. Thrombo-kinase is regarded by some as the activating agent for thrombin or fibrin ferment, though here, as also in rennet action, the presence of calcium is essential too. Bile salts act as coadjutors in the action of pancreatic lipase, phosphates and other phosphorus-containing substances in the action of zymase, and so forth.

The Specificity of Enzyme Action.—An enzyme which acts upon starch will not act upon protein; and one which acts upon protein will not act upon fat. In some cases the action of an enzyme is extraordinarily limited; thus there are three separate enzymes to hydrolyse the three principal disaccharides, sucrose, lactose, and maltose, neither of which will act upon either of the other two sugars in the list. Arginase splits arginine into ornithine and urea, but will act upon no other substance. Some of the peptolytic enzymes obtained in extracts of tissues and organs will act upon certain polypeptides, resolving them into their constituent amino-acids, whereas others act in a similar way on other groups of polypeptides. The "lock and key" simile first introduced by Emil Fischer will aid us in understanding this specificity of action. Each lock must have its special key: so the configuration of an enzyme must be related in some way to the configuration of the substrate to enable it to enter and unlock its parts from one another.

The Inexhaustibility of Enzymes.—A small amount of enzyme will act on an unlimited amount of substrate, provided sufficient time is given, and provided also the products of action are removed. "A little leaven leaveneth the whole lump." This is perhaps analogous to the part played by sulphuric acid in the etherification of alcohol (see p. 17). The enzyme appears to take a share in intermediate reactions, and there is some evidence that in certain stages it combines with the substrate; but subsequently when the substrate breaks up into simpler materials, the enzyme is liberated unchanged, and so ready to similarly act on a fresh amount of substrate in which the same series of events is repeated.

Catalytic Action of Enzymes.—The analogy of enzymic action is, in fact, so close to that of inorganic catalysts, that the view at present current regarding it is that the action is a catalytic one. This is to say, the presence of the enzyme induces a chemical reaction to occur rapidly which in its absence also occurs, but so slowly that any action at all is difficult to discover. To use the technical phrase, its action is to increase the *velocity* of chemical reactions. It is, for instance, quite conceivable that, if starch and water were mixed together, the starch would in time take up the water and split into its constituent molecules of sugar. But an action of this kind would be so slow, occupying perchance many years, that for practical purposes it does not take place at all. If an inorganic catalyst is added, such as sulphuric acid, and the temperature raised to boiling point, the action takes place in a few minutes; if an organic catalyst, such as the enzyme ptyalin, is added, the velocity of the change is equally great or even greater, but what is of more importance for the well-being of the animal, a moderate temperature, namely that of the body, amply suffices. The organic catalysts or enzymes are, however, colloidal in nature (possibly protein), and this explains the points in which they differ from the inorganic catalysts, for instance in their destrucibility by high temperatures.

Reaction Velocity.—Most of the reactions in inorganic chemistry take place between *electrolytes,* that is substances which are conductors of the electric current. They conduct electricity when they are in solution because they are largely broken up into their constituent ions; thus sodium chloride is broken up into ions of sodium and ions of chlorine; the sodium ions are called *kations* because they become charged with positive electricity and move towards the kathode or negative pole; the chlorine ions are called *anions* because they are charged with negative electricity and move towards the anode or positive pole. Substances which are not ionised in solution are called *non-electrolytes* and do not conduct electricity.

The reactions of inorganic salts, bases, and acids are really reactions between ions, and ionic reactions occur at such enormous velocity as to be practically instantaneous. Ionic reactions take place between the inorganic constituents of living cells, but such reactions occurring as they do in a colloidal medium are somewhat slowed down, but even so are completed in an immeasurably short time. The most important substances (fats, carbohydrates, proteins) in living tissues are, however, not electrolytes, and reactions between them are spoken of as *molecular reactions,* and occur so slowly that it is possible to ascertain the rate at which they take place. *Reaction velocity* is defined as the quantity of the substance transformed, measured in gramme-molecules per litre, which disappears in the unit of time (one minute). When starch is transformed into sugar, or protein into amino-acids, there is only one substance transformed, and such reactions, which compose the majority of the reactions in living cells, are called *unimolecular reactions,* or reactions of the first order. When, for instance, starch is changed into sugar by the action of an acid, it is the starch alone which is altered; the acidity undergoes no diminution. Similarly when the change is brought about by an enzyme, the starch only is changed; the enzyme is still present in its original quantity. Reaction velocity is thus of special importance in a study of the changes produced by enzymes, and these are the most frequent of all changes in living structures.

Since the quantity of the substance acted upon is continually diminishing, the velocity of the reaction cannot remain the same throughout, but must diminish in a certain ratio. Suppose 20 parts out of 100 are transformed in the first minute, there will be only 80 parts remaining at the commencement of the second minute:—

$$100 - \frac{100}{5} = 80.$$

Similarly at the commencement of the third minute we have only 64 left, 16 having disappeared:—

$$80 - \frac{80}{5} = 64.$$

In the fourth minute, 12·8 disappears and 51·2 is left:—

$$64 - \frac{64}{5} = 51{\cdot}2\,;$$

and so on.

In order to express this in general terms, we may label the original concentration 100 by the symbol C_0, and for 80, 64, 51·2, etc., use the terms C_1, C_2, C_3, etc. . . . C_t. The constant figure in the above example is $\frac{1}{5}$ or 0·2. This may be represented by k. The equations then run:—

$$C_0 - C_0k = C_1, \text{ or } C_0(1-k) = C_1.$$

$$\begin{array}{ll} \text{Further} & C_0(1-k) - C_0(1-k) \times k = C_2\,; \\ \text{or} & C_0(1-k)^2 = C_2. \\ \text{Further} & C_0(1-k)^3 = C_3. \\ \text{Finally} & C_0(1-k)^t = C_t. \end{array}$$

If this is plotted out in the form of a curve, we obtain the curve known as a logarithmic curve.

In other cases the law is a different one, and we find that the reaction velocity is not directly proportional to the quantity of reacting substance, but to the square of this quantity. In all such cases, two substances are simultaneously changed in their concentration. Such a process takes place in the decomposition of esters (compounds of organic acids with alcohols), under the influence of an alkali; here not only is the amount of ester becoming less, but the alkali is also used up in the formation of salts of the organic acid. Such reactions are called *bimolecular reactions*, or reactions of the second order. Certain reactions in living cells are of this order, but reactions of higher orders still are not as yet known in living cells.

The Optimum Temperature of Enzyme Action.—As the temperature rises the velocity of the action increases until a temperature is reached at which the activity of the enzyme is greatest. Most enzymes act best at 40° C., but there are exceptions to this rule; malt diastase, for instance, acts best at 60° C. Beyond the optimum temperature a further rise inhibits activity, until a point is reached when the enzyme is destroyed. The susceptibility of enzymes to the influence of a high temperature is a striking piece of evidence in favour of the view that these agents are protein-like in nature. The fatal temperature is as a rule in the neighbourhood of 50° C.

The effect of a rise of temperature is thus complicated by being of a twofold nature. In the first place, and between certain limits, a rule known as the law of Arrhenius is followed : that is a rise of 10° C. doubles or more than doubles the velocity of the action of the enzyme, as it does other chemical reactions. But as the temperature rises, the velocity of the disintegration of the enzyme increases also. The optimum temperature will therefore be one at which the accelerating effect is strong enough to finish the reaction quickly, and the retarding effect due to destruction of the enzyme is not so great as to paralyse the accelerating effect.

Reversibility of Enzyme Action. — We have just seen that the majority of enzymic actions are unimolecular, and that the law followed is the simple logarithmic law.

But in these reactions we usually meet with the peculiarity that it is not completed when the reaction ceases. A certain quantity of the substrate never disappears. Thus a small amount of sucrose remains unchanged whether the hydrolysis is brought about by the action of an acid or of an enzyme. This phenomenon is due to the fact that two reactions are always taking place in opposite directions. Simultaneously with the splitting up, the synthetical reaction begins, and synthesis or building up increases in proportion as the splitting of the compound advances. The velocity of the splitting process decreases at the same rate as the velocity of the synthetic process increases. At a certain point, both have the same velocity, and therefore no further change occurs in the mixture when this condition of equilibrium is reached. This rule is expressed by writing the chemical equation connected by a double arrow instead of the sign of equation. Two examples follow :—

$$\underset{\text{[Ethyl alcohol]}}{C_2H_5 . OH} + \underset{\text{[Acetic acid]}}{CH_3 . COOH} \rightleftarrows \underset{\text{[Ethyl acetate]}}{C_2H_5 . COO . CH_3} + \underset{\text{[Water]}}{H_2O}$$

$$\underset{\text{[Glucose]}}{C_6H_{12}O_6} + \underset{\text{[Fructose]}}{C_6H_{12}O_6} \rightleftarrows \underset{\text{[Sucrose]}}{C_{12}H_{22}O_{11}} + \underset{\text{[Water]}}{H_2O}$$

This phenomenon is termed "*reversibility*," and was first demonstrated by Croft Hill in his experiments with sucrose and invertase.

In intracellular action this is a factor of importance, for the same enzyme can in the presence of different proportions of the substrate and its cleavage products both tie (in anabolism) and untie (in katabolism) the knot.

It should further be noted that hydrolytic actions are *isothermic*; that is, the total energy of the products is equal to that of the substance broken up.

The simple logarithmic law of enzyme action has been demonstrated for the majority of enzymes (invertase, trypsin, erepsin, lipase, etc.). The effect in a given time is directly proportional to the quantity of enzyme present. But there is an exception to this rule in the case of pepsin, as was first pointed out by Schütz in 1885. He found that peptic activity is proportional to the square root of the amount of pepsin present. Thus if a certain quantity of pepsin produced an amount of digestive action which we will call a, in order to produce a digestive action equal to $2a$ in the same time, it would be necessary to employ four times the amount of pepsin; and in order to produce a digestive action equal to $3a$, it would be necessary to use nine times the amount of pepsin. This rule (Schütz's law) has been often confirmed, and a few years ago Arrhenius explained it on mathematical lines.

Anti-enzymes.—Many chemical substances, such as strong acids and alkalis, alcohol, formaldehyde, iodine, potassium cyanide, and salts of the heavy metals, hinder enzyme activity. But the term anti-enzyme is generally limited to substances produced in the metabolism of living organisms. Excess of these organic anti-enzymes can be readily produced by injecting an enzyme into the blood-stream of an animal. This stimulates the production of an anti-enzyme, so that when the blood-serum is mixed with the original enzyme, its power is inhibited. Anti-enzymes are specific, that is, they inhibit the enzyme which was injected into the blood, and no other.

THE SALIVA

The secretion of **saliva** is a reflex action; the taste or smell of food excites the nerve-endings of the afferent nerves (glossopharyngeal and olfactory); the efferent or secretory nerves are contained in the chorda tympani (a branch of the seventh cranial nerve), which supplies the submaxillary and sublingual glands, and in a branch of the glossopharyngeal, which supplies the parotid. The sympathetic branches which supply the blood-vessels with constrictor nerves contain in some animals secretory fibres also.

The parotid gland is called a serous or albuminous gland; before

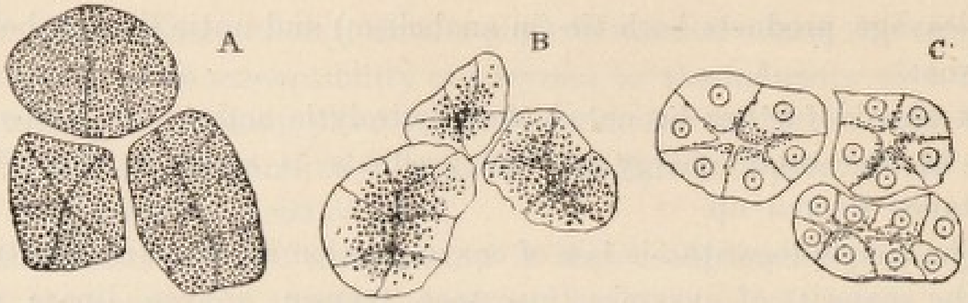

FIG. 12.—Alveoli of serous gland; A, loaded before secretion; B, after a short period of active secretion; C, after a prolonged period. (Langley.)

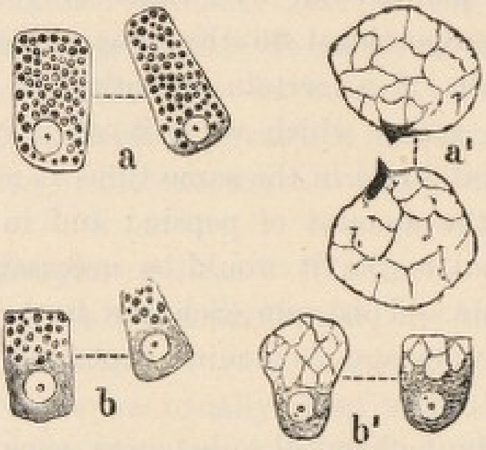

FIG. 13.—Mucous cells from a fresh submaxillary gland of dog: *a*, loaded with mucinogen granules before secretion; *b*, after secretion: the granules are fewer, especially at the attached border of the cell; *a'* and *b'* represent cells in a loaded and discharged condition respectively which have been irrigated with water or dilute acid. The mucous granules are swollen into a transparent mass of mucin traversed by a network of protoplasmic cell-substance. (Foster, after Langley.)

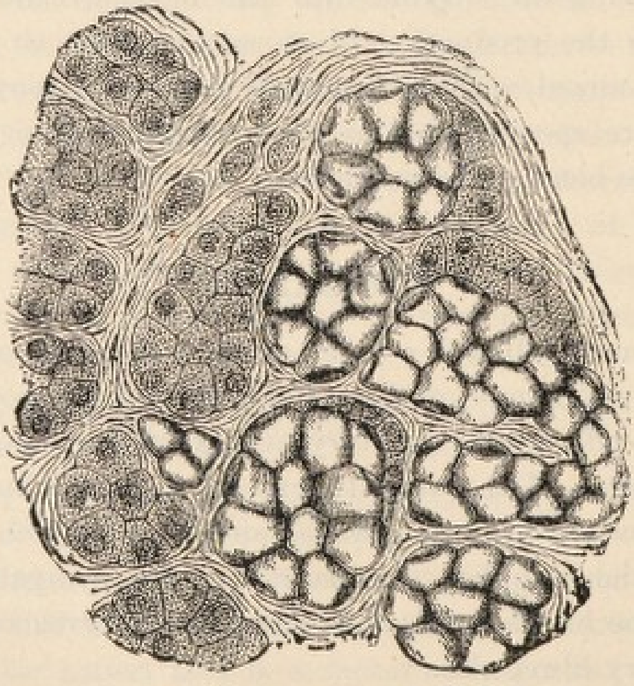

FIG. 14.—Section of part of the human submaxillary gland. (Heidenhain.) To the right is a group of mucous alveoli, to the left a group of serous alveoli.

secretion the cells of the acini are swollen out with granules; after secretion has occurred the cells shrink, owing to the granules having been shed out to contribute to the secretion (see fig. 12).

The submaxillary and sublingual glands are called mucous glands: their secretion contains mucin. Mucin is absent from parotid saliva. The granules in the cells are larger than those of the parotid gland: they are composed of mucinogen, the precursor of mucin (see fig. 13).

In a section of a mucous gland prepared in the ordinary way the mucinogen granules are swollen out, and give a highly refracting appearance to the mucous acini (see fig. 14).

COMPOSITION OF SALIVA

On microscopic examination of mixed saliva a few epithelial scales from the mouth and salivary corpuscles from the tonsils are seen. The liquid is transparent, slightly opalescent, of slimy consistency, and may contain lumps of nearly pure mucin. On standing it becomes cloudy owing to the precipitation of calcium carbonate, the carbonic acid which held it in solution as bicarbonate escaping.

Of the three forms of saliva which contribute to the mixture found in the mouth, the sublingual is richest in solids (2·75 per cent.). The submaxillary saliva comes next (2·1 to 2·5 per cent.). When artificially obtained by stimulation of nerves in the dog the saliva obtained by stimulation of the sympathetic is richer in solids than that obtained by stimulation of the chorda tympani. The parotid saliva is poorest in total solids (0·3 to 0·5 per cent.), and contains no mucin. Mixed saliva contains in man an average of about 0·5 per cent. of solids: it is alkaline in reaction, due to the salts in it; and has a specific gravity of 1002 to 1006.

The solid constituents dissolved in saliva may be classified thus :—

Organic
- *a.* Mucin: this may be precipitated by acetic acid.
- *b.* Ptyalin: an amylolytic enzyme.
- *c.* Protein: of the nature of a globulin.
- *d.* Potassium thiocyanate.

Inorganic
- *e.* Sodium chloride: the most abundant salt.
- *f.* Other salts: sodium carbonate; calcium phosphate and carbonate; magnesium phosphate; potassium chloride.

THE ACTION OF SALIVA

The action of saliva is twofold, physical and chemical.

The physical use of saliva consists in moistening the mucous membrane of the mouth, assisting the solution of soluble substances in the food, and in virtue of its mucin lubricating the bolus of food to facilitate swallowing.

The chemical action of saliva is due to its enzyme ptyalin.

The starch is first split into erythro-dextrin, which gives a red colour with iodine, achroö-dextrin, which gives no colour with iodine, and maltose. Brown and Morris give the following equation :—

$$10\underset{\text{[starch]}}{(C_6H_{10}O_5)_n} + \underset{\text{[water]}}{4nH_2O}$$

$$= \underset{\text{[maltose]}}{4nC_{12}H_{22}O_{11}} + \underset{\text{[achro\"o-dextrin]}}{(C_6H_{10}O_5)_n} + \underset{\text{[erythro-dextrin]}}{(C_6H_{10}O_5)_n}$$

The erythro-dextrin is then converted into achroö-dextrin, and finally into maltose.

Ptyalin acts in a similar way, but more slowly on glycogen : it has no action on cellulose ; hence it is inoperative on uncooked starch grains, for in these the cellulose layers are intact.

Ptyalin acts best at about the temperature of the body (35–40°), and in a neutral medium ; a small amount of alkali makes but little difference ; a very small amount of acid stops its activity. The conversion of starch into sugar by saliva in the stomach continues for a variable time, for the swallowed masses which fall into the fundus of the stomach are not subjected to peristalsis and admixture with gastric juice until a later stage in the digestion ; but the admixture of the contents of the fundus with the rest of the gastric contents will occur more quickly if the person moves about. The hydrochloric acid which is poured out by the gastric glands first neutralises the saliva and combines with the proteins in the food ; but immediately free hydrochloric acid appears the ptyalin is destroyed, so that it does not resume work even when the semi-digested food once more becomes alkaline in the duodenum.

THE SECRETION OF GASTRIC JUICE

The juice secreted by the glands in the mucous membrane of the stomach varies in composition in the different regions, but the mixed juice is a solution of a proteolytic enzyme pepsin in a saline solution, which also contains a little free hydrochloric acid.

The gastric juice can be obtained during the life of an animal by

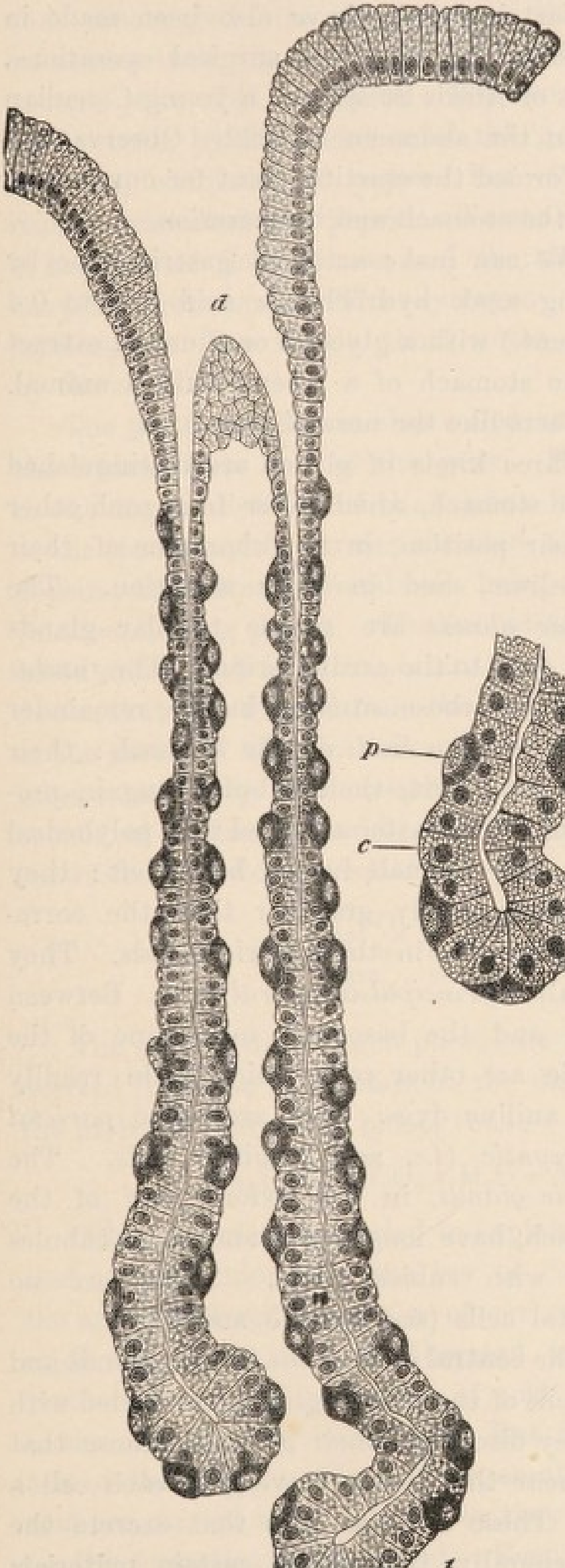

FIG. 15.—A fundus gland from the dog's stomach (Klein): *d*, duct or mouth of the gland; *b*, base of one of its tubules; on the right the base of a tubule is more highly magnified; *c*, central cell; *p*, parietal cell.

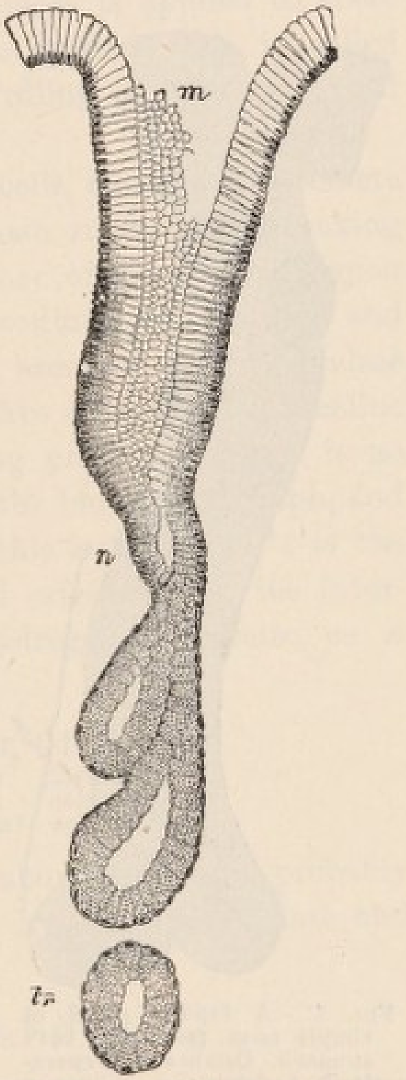

FIG. 16.—A pyloric gland from a section of the dog's stomach (Ebstein): *m*, mouth; *n*, neck; *tr*, a deep portion of tubule cut transversely.

means of a gastric fistula. Gastric fistulæ have also been made in human beings, either by accidental injury or by surgical operations. The most celebrated case is that of Alexis St Martin, a young Canadian who received a musket wound in the abdomen in 1822. Observations made on him by Dr Beaumont formed the starting point for our correct knowledge of the physiology of the stomach and its secretion.

We can make artificial gastric juice by mixing weak hydrochloric acid (0·2 to 0·4 per cent.) with a glycerol or aqueous extract of the stomach of a recently killed animal. This acts like the normal juice.

Three kinds of glands are distinguished in the stomach, which differ from each other in their position, in the character of their epithelium, and in their secretion. The *cardiac glands* are simple tubular glands quite close to the cardiac orifice. The *fundus glands* are those situated in the remainder of the cardiac half of the stomach: their ducts are short, their tubules long in proportion. The latter are filled with polyhedral cells, only a small lumen being left: they are more closely granular than the corresponding cells in the pyloric glands. They are called *principal* or *central* cells. Between them and the basement membrane of the tubule are other cells which stain readily with aniline dyes. They are called *parietal* or *oxyntic* (*i.e.* acid-forming) cells. The *pyloric glands*, in the pyloric half of the stomach, have long ducts and short tubules lined with cubical cells. There are no parietal cells (see figs. 15 and 16).

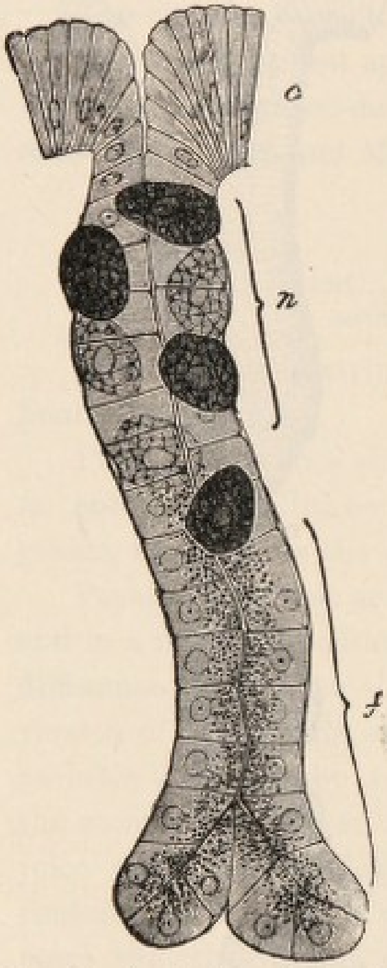

FIG. 17.—A fundus gland of simple form from the bat's stomach. Osmic acid preparation (Langley); *c*, columnar epithelium of the surface; *n*, neck of the gland, with central and parietal cells; *f*, base occupied only by principal or central cells, which exhibit the granules accumulated towards the lumen of the gland.

The **central cells** of the fundus glands and the cells of the pyloric glands are loaded with granules. During secretion they discharge their granules, those that remain being chiefly situated near the lumen, leaving in each cell a clear outer zone (see fig. 17). These are the cells that secrete the pepsin. Like secreting cells generally, they select certain materials from the lymph that bathes them: these materials are worked up by the protoplasmic activity of the cells into the secretion, which is then discharged into the lumen of the gland. The most important substance in a digestive secretion is the enzyme. In the case of a gastric juice this is pepsin. We can trace an intermediate step in this process by the presence of the granules. The granules are not, however, composed of pepsin, but of a mother-substance, which is readily converted into pepsin. We shall find a similar enzyme precursor in the cells of the pancreas, and the term *zymogen* is applied to these enzyme precursors. The zymogen in the gastric cells is called *pepsinogen*. The rennin that causes the curdling of milk is formed by the same cells.

The **parietal cells** are also called oxyntic cells, because they secrete the hydrochloric acid of the juice. Heidenhain succeeded in making in one dog a *cul-de-sac* of the fundus, in another of the pyloric region of the stomach; the former secreted a juice containing both acid and pepsin; the latter, parietal cells being absent, secreted a viscid alkaline juice containing pepsin. The formation of a free acid from the alkaline blood and lymph is an important but puzzling problem. There is no doubt that it is formed from the chlorides of the blood and lymph, and of the chemical theories advanced as to how this is done, Maly's is the most satisfactory. He considers that the acid originates by the interaction of sodium chloride and sodium dihydrogen phosphate, as is shown in the following equation:—

$$\underset{\text{[sodium dihydrogen phosphate]}}{NaH_2PO_4} + \underset{\text{[sodium chloride]}}{NaCl} = \underset{\text{[disodium hydrogen phosphate]}}{Na_2HPO_4} + \underset{\text{[hydrochloric acid]}}{HCl}$$

The sodium dihydrogen phosphate in the above equation is probably derived from the interaction of the disodium hydrogen phosphate and the carbonic acid of the blood, thus:—

$$Na_2HPO_4 + CO_2 + H_2O = NaHCO_3 + NaH_2PO_4.$$

Other theories have tried to explain the formation of such a strong acid as hydrochloric by the law of "mass action." We know that by the action of large quantities of carbonic acid on salts of the mineral acids the latter may be liberated in small quantities. We know, further, that small quantities of acid ions may be continually formed in the organism by ionisation. But in every case we can only make use of these explanations if we assume that the small quantities of acid are carried away as soon as they are formed, and thus give room for the formation of fresh acid. Even then it is impossible to explain the whole process. A specific action of the cells is no doubt exerted, for these reactions can hardly be considered to occur in the blood generally, but rather in the oxyntic cells, which possess the necessary selective powers in reference to the constituents of the blood, and the hydrochloric acid, as soon as it is formed, passes into the secretion of the gland in consequence of its high power of diffusion.

COMPOSITION OF GASTRIC JUICE

The following table gives the percentage composition of the gastric juice of man and dog :—

Constituents.	Human.	Dog.
Water	99·44	97·30
Organic substances (chiefly pepsin) .	0·32	1·71
HCl	0·20	0·50
$CaCl_2$	0·006	0·06
NaCl	0·14	0·25
KCl	0·05	0·11
NH_4Cl	...	0·05
$Ca_3(PO_4)_2$	0·01	0·17
$Mg_3(PO_4)_2$		0·02
$FePO_4$		0·008

In the foregoing table one also sees the great preponderance of chlorides over other salts : apportioning the total chlorine to the various metals present, that which remains over must be combined with hydrogen to form the free hydrochloric acid of the juice. The freshly secreted juice contains about 0·5 per cent. of the acid (as shown in the analysis of dog's gastric juice in the table). When the juice remains in the stomach this is in part neutralised by the food and saliva, and also by pancreatic juice which enters the stomach from the duodenum, so that the ultimate percentage is only 0·2.

Pepsin stands apart from nearly all other enzymes by requiring an acid medium in order that it may act. A compound of the two substances called *pepsin-hydrochloric acid* is the really active agent. Other acids may take the place of hydrochloric acid, but none act so well. Lactic acid is often found in gastric juice ; this, however, is derived by fermentative processes from the food.

Pawlow has shown that in dogs the secretory fibres for the gastric glands are contained in the vagus nerves.

By an ingenious surgical operation he succeeded in separating off from the stomach a diverticulum which pours its secretion through an opening in the abdominal wall. This small stomach was found to act in every way like the main stomach of the animal. The pure juice so obtained is clear and colourless: it has a specific gravity of 1003 to 1006. It is feebly dextro-rotatory, and gives some of the protein reactions. It contains from 0·4 to 0·6 per cent. of hydrochloric acid. It is strongly proteolytic, and inverts cane sugar. When cooled to 0° C. it deposits a fine precipitate of pepsin: this settles in layers, and the layers first deposited contain most of the acid, which is loosely combined with and carried down by the pepsin. Pepsin is also precipitable by saturation with ammonium sulphate (Kühne).

The juice is most abundant in the early periods of digestion, but it continues to be secreted in declining quantity as long as any food remains to be dealt with. When there is no food given there is no juice. But sham feeding with meat will cause it to flow.

The larger the proportion of protein in the diet, the more abundant and active is the juice secreted, provided the animal is hungry; the psychical element is important.

THE ACTIONS OF GASTRIC JUICE

Gastric juice has the following five actions:—

1. It is **antiseptic,** owing to the hydrochloric acid present; putrefactive processes do not normally occur in the stomach, and the micro-organisms which produce such processes, many of which are swallowed with the food, are in great measure destroyed, and thus the body is protected from them.

2. It **inverts** sucrose into glucose and fructose. This also is due to the acid of the juice, and is frequently assisted by inverting ferments contained in the vegetable food swallowed. The juice has no action on starch.

3. It contains **lipase,** or a fat-splitting ferment. The protein envelopes of the fat cells are first dissolved by the pepsin-hydrochloric acid, and the solid fats are melted. They are then split in small measure into their constituents, glycerol and fatty acids. This action is mainly produced by a regurgitation of the contents of the duodenum mixed with pancreatic juice; but even after the pylorus has been ligatured and regurgitation prevented, the gastric juice itself produces a *small* amount of fat-splitting, and therefore contains lipase. It is a remarkable fact that the administration of fat in the food increases the regurgitation from the duodenum.

4. It **curdles milk.** This is due to the action of the rennet enzyme or rennin. The conditions of this action we have already discussed under Milk (see p. 74); but it may here be added that Pawlow has

advanced the view that rennin is not a distinct and separate enzyme, but that milk-curdling is only one of the activities of pepsin. This hypothesis has been accepted by numerous physiologists; but, on the other hand, there is a number of equally eminent observers who still maintain that pepsin and rennin are two separate enzymes. Whichever view is correct, the curd of casein formed from the caseinogen is subsequently digested as other proteins are.

5. It is **proteolytic**; this is the most important action of all. The proteins of the food are converted by the pepsin-hydrochloric acid into peptones. It has been stated that the prolonged action of the juice leads to the further splitting of the peptones into amino-acids, but accurate work has shown that pepsin-hydrochloric acid does not split any of the known polypeptides into their ultimate cleavage products.

This action is a process of hydrolysis; and peptones may be formed by other hydrolysing agencies, such as superheated steam or heating with dilute mineral acids. The first stage in the process of hydrolysis is that of acid meta-protein; the next step is the formation of proteoses. The word "proteose" includes the albumoses (from albumin), globuloses (from globulin), vitelloses (from vitellin), etc. Similar substances are also formed from gelatin (gelatoses) and elastin (elastoses). Then peptone (a mixture of polypeptides) is produced. The products of digestion of protein may be arranged according to the order in which they are formed, as follows:—

1. Acid meta-protein.
2. Propeptone or proteoses
   - (*a*) Proto-proteose
   - (*b*) Hetero-proteose
     The primary proteoses, *i.e.* those which are formed first.
   - (*c*) Deutero- or secondary proteose.
3. Peptone (Polypeptides).

1. **Acid Meta-protein.**—The general properties of the meta-proteins, the first degradation products in the cleavage of the proteins which occurs during digestion, are described on p. 66. We shall find later that, in pancreatic digestion, an alkali-meta-protein is formed instead of the acid modification. The theory has been put forward that a protein is capable of playing the part of a base in virtue of its NH_2 groups, and also of an acid in virtue of its COOH groups.

2. **Proteoses.**—They are not coagulated by heat; they are precipitated but not coagulated by alcohol: like peptone they give the

pink biuret reaction. They are precipitated by nitric acid, *the precipitate being soluble on heating, and reappearing when the liquid cools.* This last is a distinctive property of proteoses. They are slightly diffusible.

The primary proteoses are precipitated by saturation with magnesium sulphate or sodium chloride; deutero-proteose is not; it is, however, precipitated by saturation with ammonium sulphate. Proto- and deutero-proteose are soluble in water; hetero-proteose is not; it requires salt to hold it in solution.

3. **Peptones.**—They are soluble in water, are not coagulated by heat, and are not precipitated by nitric acid, copper sulphate, ammonium sulphate, and a number of other precipitants of proteins. They are precipitated but not coagulated by alcohol. They are also precipitated by tannin, picric acid, potassio-mercuric iodide, phospho-molybdic acid, and phospho-tungstic acid.

They give the biuret reaction (rose-red solution with a trace of copper sulphate and caustic potash or soda).

Peptone is readily diffusible through animal membranes.

The annexed table will give us at a glance the chief characters of peptones and proteoses in contrast with those of the native proteins, albumins, and globulins.

Variety of Protein.	Action of Heat.	Action of Alcohol.	Action of Nitric Acid.	Action of Ammonium Sulphate.	Action of Copper Sulphate and Caustic Potash.	Diffusibility.
Albumin.	Coagulated.	Precipitated, then coagulated.	Precipitated in the cold; not readily soluble on heating.	Precipitated by complete saturation.	Violet colour.	Nil.
Globulin.	Ditto.	Ditto.	Ditto.	Precipitated by half saturation; also precipitated by $MgSO_4$.	Ditto.	Ditto.
Proteoses.	Not coagulated.	Precipitated, but not coagulated.	Precipitated in the cold; readily soluble on heating; the precipitate reappears on cooling.[1]	Precipitated by saturation.	Rose-red colour (biuret reaction).	Slight.
Peptones.	Not coagulated.	Precipitated, but not coagulated.	Not precipitated.	Not precipitated.	Rose-red colour (biuret reaction).	Great.

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[1] In the case of deutero-albumose this reaction only occurs in the presence of excess of salt.

The question has been often raised why the stomach does not digest itself during life. The mere fact that the tissues are alkaline and pepsin requires an acid medium in which to act is not an explanation, but only opens up a fresh difficulty as to why the pancreatic juice which is alkaline does not digest the intestinal wall. To say that it is the vital properties of the tissues that enable them to resist digestion only shelves the difficulty and gives no real explanation of the mechanism of defence. Recent studies on the important question of immunity (see Lesson IX.) have furnished us with the key to the problem ; just as poisons introduced from without stimulate the cells to produce antitoxins, so harmful substances produced within the body are provided with anti-substances capable of neutralising their effects ; for this reason the blood does not normally clot within the blood-vessels, and Weinland has shown that the gastric epithelium forms an antipepsin, the intestinal epithelium an antitrypsin, and so on. The bodies of parasitic worms that live in the intestine are particularly rich in these anti-bodies.

LESSON VIII

THE DIGESTIVE JUICES (*continued*)

PANCREATIC DIGESTION

1. A 1 per-cent. solution of sodium carbonate, to which a little glycerol extract of pancreas [1] has been added, forms a good artificial pancreatic fluid.

2. Half fill three test-tubes with this solution.

A. To this add half its bulk of diluted egg-white (1 in 10).

B. To this add a piece of fibrin.

C. Boil this ; cool ; then add fibrin.

3. Put all into the water-bath at 40° C. After half an hour, test A and B for alkali-metaprotein by neutralisation, for proteoses by nitric acid, and for proteoses and peptone by the biuret reaction.

4. Note in B that the fibrin does not swell up and dissolve, as in gastric digestion, but that it is eaten away from the edges to the interior.

5. In C no digestion occurs, as enzymes are destroyed by boiling.

6. Take a solution of starch, equal quantities in three test-tubes.

D. To this add a few drops of extract of pancreas (without the sodium carbonate).

E. To this add a few drops of bile.

F. To this add both bile and pancreatic extract.

7. Put these into the water-bath, and test small portions of each every half-minute by the iodine reaction. It disappears first in F ; then in D ; while E undergoes no change. Test D and F for maltose by Fehling's solution.

This shows the favourable influence bile exerts on pancreatic digestion. It is, however, more marked still in the case of fats. (See 9, below.)

8. Shake up a few drops of olive oil with artificial pancreatic juice (extract of pancreas and sodium carbonate). A milky fluid (emulsion) is formed, from which the oil does not readily separate on standing.

9. Boil 10 c.c. of fresh milk ; cool ; colour with an indicator (for instance, a few drops of an alcoholic solution of phenolphthalein), and

---

[1] Benger's liquor pancreaticus diluted with two or three times its volume of 1 per-cent. sodium carbonate may be used instead.

divide it into two parts of 5 c.c. each. To one add a few drops of glycerol extract of pancreas; to the other, the same amount of pancreatic extract and a few drops of bile. Put each into the warm bath. In each the pink colour disappears as fatty acids are liberated by lipase; this occurs more rapidly in the specimen containing bile.

The foregoing experiments illustrate the action that pancreatic juice has on all three classes of organic food.

BILE

1. Ox bile is given round. Observe its colour, taste, smell, and reaction to litmus paper.

2. Acidulate a little bile with 20 per-cent. acetic acid. A stringy precipitate of a mucinoid substance is obtained. Filter this off and boil the filtrate; no protein coagulable by heat is present.

3. Add a few drops of bile to (a) acid-metaprotein prepared as described in Lesson V, and to (b) solution of proteoses to which half its volume of 0·2 per-cent. hydrochloric acid has been added. A precipitate occurs in each case. Bile salts precipitate the unpeptonised protein which leaves the stomach.

4. **PETTENKOFER'S TEST FOR BILE SALTS.**—To a thin film of bile in a capsule add a drop of solution of cane sugar and a drop of concentrated sulphuric acid. A purple colour is produced. This occurs more quickly on the application of heat. The test may also be performed as follows:—Shake up some bile and cane sugar solution in a test-tube until a froth is formed. Pour concentrated sulphuric acid gently down the side of the tube: it produces a purple colour in the froth.

5. **GMELIN'S TEST FOR BILE PIGMENTS.**—On to a little fuming nitric acid (*i.e.* nitric acid containing nitrous acid in solution) in a test-tube pour gently a little bile. Notice the succession of colours—green, blue, red, and yellow—at the junction of the two liquids. This test may also be performed in a capsule. Place a drop of fuming nitric acid in the middle of a thin film of bile: it becomes surrounded by rings of the above-mentioned colours. Huppert's test for bile pigments is specially applicable for their detection in urine, and so is postponed to Lesson XII.

6. **HAY'S TEST FOR BILE SALTS.**—Take two beakers full of water; to one add a few drops of bile, or solution of bile salts. Sprinkle a little flowers of sulphur on the surface of each. It remains floating on the pure water; but where bile is present the surface tension of the water is reduced, and the sulphur consequently rapidly sinks. This test is very sensitive, and may be employed for the detection of bile salts in urine.

7. **CHOLESTEROL.**—(a) Examine crystals of this substance with the microscope. Heat these on a slide with a drop of sulphuric acid and water (5 : 1); the edges of the crystals turn red.

(So [illegible])

(b) *Salkowski's Reaction.*—Dissolve some cholesterol in chloroform in a *dry* test-tube, and gently shake with an equal amount of concentrated sulphuric acid ; the solution turns red, and the subjacent acid acquires a green fluorescence. The chloroformic solution of cholesterol is rendered colourless by pouring it into a wet test-tube, and the colour is restored by the addition of sulphuric acid.

(c) *Liebermann-Burchard Reaction.*—Two or three drops of acetic anhydride are added to a chloroformic solution of cholesterol and then sulphuric acid drop by drop. A rose-red colour first develops ; this becomes blue and finally bluish-green.

(d) *Preparation of Cholesterol from Brain.*—Ox or sheep's brain is minced, and in order to remove the water is mixed with three times its weight of plaster of Paris. After some hours the mixture sets into a hard mass, which can easily be broken up. Some of this powdered material is given round. Add to the quantity supplied sufficient acetone to cover it. Allow it to stand for ten minutes, shaking frequently. Filter the acetone solution through a dry filter into a beaker, and allow the acetone to evaporate spontaneously. Cholesterol crystallises out ; dissolve this in hot alcohol ; place a drop on a glass slide and examine the typical crystals with the microscope.

THE PANCREAS

The Pancreas is a compound tubulo-racemose gland; between the secreting acini are situated little masses of epithelial cells without ducts called "islets of Langerhans." Examination of the secreting cells in different stages of activity reveals changes comparable to those already described in the case of salivary and gastric cells. Granules indicating the presence of zymogens crowd the cells before secretion: these are discharged during secretion, so that in an animal whose pancreas has been powerfully stimulated to secrete, the granules are seen only at the free border of the cells (see fig. 18).

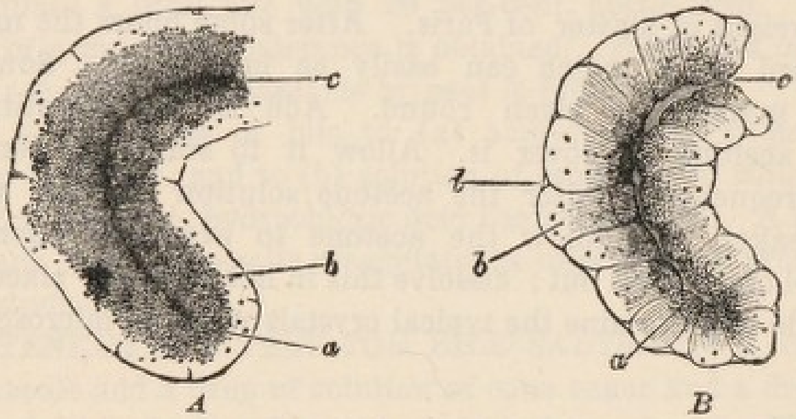


FIG. 18.—Part of an alveolus of the rabbit's pancreas: A, before discharge; B, after. (From Foster, after Kühne and Lea.)

As in the case of gastric juice, experiments on the pancreatic secretion are usually performed with an artificial juice, made by mixing a weak alkaline solution (1 per-cent. sodium carbonate) with an extract of pancreas. The pancreas should be kept some time before the extract is made, so as to ensure that the transformation of trypsinogen into trypsin has taken place.

Quantitative analysis of human pancreatic juice gives the following results:—

Water	97·6 per cent.
Organic solids	1·8 ,,
Inorganic salts	0·6 ,,

Dog's pancreatic juice is considerably richer in solids.

The organic substances in pancreatic juice are:—

(*a*) Enzymes. These are the most important both quantitatively and functionally. They are four in number:—

i. Trypsin, a proteolytic enzyme. In the fresh juice, however, this is present in the form of trypsinogen.

ii. Amylase, an amylolytic enzyme.

iii. Lipase, a fat-splitting enzyme.

iv. A milk-curdling enzyme.

(*b*) A small amount of protein matter, coagulable by heat.

(*c*) Traces of leucine, tyrosine, xanthine, and soaps.

The inorganic substances in pancreatic juice are :—

Sodium chloride, which is the most abundant, and smaller quantities of potassium chloride, and phosphates of sodium, calcium, and magnesium. The alkalinity of the juice is due to phosphates and carbonates, especially of sodium.

THE SECRETION OF PANCREATIC JUICE

One of the most effective ways of producing a flow of the juice is to introduce acid into the duodenum, and no doubt the acid of the gastric juice is the normal stimulus for the pancreatic flow. This flow still occurs when all the nerves supplying the duodenum and pancreas are cut, and it was held by Popielski and by Wertheimer and Le Page that it must be due to a local reflex, the centres being situated in the scattered ganglia of the pancreas and of the solar plexus. Starling and Bayliss, however, pointed out that it cannot be a nervous reflex, since it occurs after extirpation of the solar plexus, and destruction of all nerves passing to an isolated loop of intestine. Moreover, atropine does not paralyse the secretory action. It must therefore be due to direct excitation of the pancreatic cells by a substance or substances conveyed to the gland from the bowel by the blood-stream.

The exciting substance is not acid; injection of 0·4 per cent. of hydrochloric acid into the blood-stream has no influence on the pancreas. The substance in question must be produced in the intestinal mucous membrane under the influence of the acid. This conclusion was confirmed by experiment. If the mucous membrane of the duodenum or jejunum is exposed to the action of 0·4 per cent. hydrochloric acid, a substance is produced which, when injected into the blood-stream in minimal doses, produces a copious secretion of pancreatic juice. This substance is termed *secretin*. It is associated with another substance which lowers arterial blood-pressure. The two substances are not identical, since acid extracts of the lower end of the ileum produce a lowering of blood-pressure, but have no excitatory influence on the pancreas.

Secretin is split off from a precursor, *pro-secretin*, which is present in relatively large amounts in the duodenal mucous membrane, and gradually diminishes in amount throughout the intestine until it entirely disappears in the ileum. Pro-secretin can be dissolved out of the mucous membrane by normal saline solution. It has no influence on the pancreatic secretion. Secretin can be split off from it by boiling or by treatment with acid.

What secretin is chemically, we do not yet know. It is soluble in alcohol and ether. It is not a protein, but probably is an organic substance of low molecular weight. It is, moreover, the same substance in all animals, and not specific to different kinds of animals.

Whether there are any secretory nerves for the pancreas is at present doubtful. Pawlow thought he had discovered them in the vagus; but as he did not exclude in his experiments the passage of the acid chyme from the stomach into the duodenum, it is probable that the pancreatic secretion he obtained was due to that circumstance and the consequent formation of secretin.

Injection of secretin also stimulates the flow of bile and of succus entericus.

Secretin is an instance of the chemical messengers or *hormones* (Starling) of the body. Evidence is accumulating to show that hormones are extremely important. It has already, for instance, been shown that one called *gastrin* is formed as the result of salivary digestion, and stimulates the flow of gastric juice. Another is formed in the corpus luteum of the ovary, which, passing into the mother's circulation, stimulates the mammæ to enlarge and secrete milk.

The pancreatic juice does not act alone on the food in the intestines. There are, in addition, the bile (which see), the succus entericus and bacterial action to be considered.

The **succus entericus** or intestinal juice has no action on native proteins. It is stated to possess a slight lipolytic action, and it appears to have to some extent the power of converting starch into sugar; its best known action on carbohydrates, however, is due to an enzyme it contains called *invertase*, which converts sucrose into glucose and fructose. The original use of the term "inversion" has been explained on p. 26. It may be extended to include the similar hydrolysis of other disaccharides, although there may be no formation of lævo-rotatory substances. There are two other inverting enzymes in the succus entericus, one of which acts on maltose, and the other on milk sugar.

A few years ago, however, Pawlow showed that succus entericus has a still more important action, which is to activate the proteolytic power of the pancreatic juice. Fresh pancreatic juice has very little power on proteins, for what it contains is not trypsin, but its precursor, trypsinogen.

If fresh pancreatic and intestinal juices are mixed together, the result is a very powerful proteolytic mixture, though neither juice by itself has any proteolytic activity. The substance in the intestinal juice that activates trypsinogen or, in other words, liberates trypsin from trypsinogen, has been called by Pawlow an enzyme of enzymes, or *entero-kinase.*

Dixon and Hamill's recent work has made still clearer the mechanism of pancreatic secretion. There are in the pancreas three precursors of enzymes, namely, protrypsinogen, proamylase, and prolipase. Secretin combines chemically, or at any rate acts chemically, on all three; it liberates amylase and lipase from their precursors, and these two active enzymes pass into the pancreatic juice. It liberates trypsinogen from protrypsinogen, and trypsinogen passes into the juice; finally trypsinogen is converted into the active enzyme trypsin by the entero-kinase of the succus entericus. Trypsinogen appears to be a complex consisting of trypsin united to a protein moiety, and as long as the enzyme is combined in this way, it is inactive; entero-kinase is a proteolytic enzyme which digests the protein moiety, and thus liberates the trypsin (J. Mellanby and Woolley). The view has been advanced by Herzen that the spleen sends a hormone to the pancreas which assists in the elaboration of trypsinogen, but the evidence in favour of this hypothesis is not generally regarded as convincing.

Intestinal juice contains another enzyme called *erepsin* by its discoverer, Otto Cohnheim; this is a peptolytic enzyme, and breaks up proteoses and peptones into their final cleavage products, the amino-acids, and so assists the action of trypsin. The only native protein which it digests is caseinogen.

ACTION OF PANCREATIC JUICE

The action of pancreatic juice, which when activated is the most powerful and important of all the digestive juices, may be described under the headings of its four enzymes.

1. **Action of Trypsin.**—Trypsin acts like pepsin, but with certain differences, which are as follows:—

(*a*) It acts in an alkaline, pepsin in an acid medium.

(*b*) It acts more rapidly than pepsin; deutero-proteoses can be detected as intermediate products in the formation of peptone; the primary proteoses have not been detected.

(*c*) Alkali-metaprotein is formed in place of the acid-metaprotein of gastric digestion.

(*d*) It acts more powerfully on certain proteins (such as elastin) which are difficult of digestion in gastric juice. It does not, however, digest collagen.

(*e*) Acting on solid proteins such as fibrin, it eats them away from the surface to the interior; there is no preliminary swelling as in gastric digestion.

(*f*) Trypsin acts further than pepsin, and rapidly splits up the proteose and peptone which have left the stomach into simpler substances, the polypeptides. The polypeptides in their turn are resolved into their constituent amino-acids, such as leucine, tyrosine, alanine, aspartic acid, glutamic acid, arginine, tryptophane, and many others. The constitution and properties of these cleavage products are described on pp. 45 to 50. In addition to these there is a certain amount of ammonia. The red colour which a tryptic digest strikes with chlorine or bromine water is due to the presence of tryptophane (indole-amino-propionic acid).

When once the peptone stage is passed, the products of further cleavage no longer give the biuret reaction; hence they are frequently termed *abiuretic*.

A variable fraction of the protein molecule is broken off with comparative ease, so that certain free amino-acids appear in the mixture, at a time when the remainder are still linked together as polypeptides. But ultimately the whole molecule is resolved into amino-acids, either entirely separated, or in very short polypeptide linkages.

It will thus be seen that there are two important differences between pepsin and trypsin; one is a difference of degree, trypsin being by far the more powerful and rapid catalyst; the second is a difference of kind, pepsin not being able to cleave polypeptides into amino-acids in the way trypsin can. The preliminary action of pepsin, however, is beneficial, for trypsin cleavage occurs more readily after pepsin has acted on a protein.

2. **Action of Amylase.**—The conversion of starch into maltose is the most rapid of all the actions of the pancreatic juice. Its power in this direction is much greater than that of saliva, and it will act even on unboiled starch. The absence of this enzyme in the pancreatic juice of infants is an indication that milk, and not starch, is their natural diet.

3. **Action on Fats.**—These are split by pancreatic lipase into glycerin and fatty acids. The fatty acids unite with the alkaline bases present to form soaps (*saponification*, see p. 37). If a glycerol

extract of pancreas is filtered, the filtrate has no lipoclastic action; the material deposited on the filter is also inactive, but on mixing it with the inactive filtrate once more, a strongly lipoclastic material is obtained. In this way lipase is separable into two fractions: the material on the filter is inactive lipase; the material in the filtrate is its co-enzyme; the latter is not destroyed by boiling. Bile salts also activate the inactive lipase, and this explains the fact that bile favours fat-splitting.

Pancreatic juice also assists in the emulsification of fats; this it is able to do because it is alkaline, and it is capable of liberating fatty acids which form soaps with the alkali present; the soap forms a film on the outer surface of each fat globule, and this prevents them running together. Emulsions are much more permanent in the presence of colloids, such as gum or protein. The presence of protein in the pancreatic juice renders it therefore specially suitable for the purpose of emulsification.

4. **Milk-curdling Enzyme.**—The addition of pancreatic extracts or pancreatic juice to milk causes clotting; but this action (which differs in some particulars from the clotting caused by rennet) can hardly ever be called into play, as the milk upon which the juice has to act has been already curdled by the rennin of the stomach. This action is, as in the case of pepsin, possibly a function of trypsin.

BACTERIAL ACTION

The gastric juice is an antiseptic; the pancreatic juice is not. An alkaline fluid like pancreatic juice is just the most suitable medium for bacteria to flourish in. Even in an artificial digestion the fluid is very soon putrid, unless special precautions to exclude or kill bacteria are taken. It is often difficult to say where pancreatic action ends and bacterial action begins, as many of the bacteria that grow in the intestinal contents (having reached that situation in spite of the gastric juice) produce enzymes which act in the same way as the pancreatic juice. Some form sugar from starch, others peptone, and amino-acids from proteins, while others, again, break up fats. There are, however, certain actions that are entirely due to these putrefactive organisms.

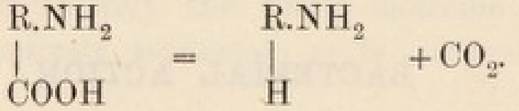
i. On carbohydrates. The most frequent fermentation they set up is the lactic acid fermentation; this may go further and result in the formation of carbonic acid, hydrogen, and butyric acid (see p. 28). Cellulose is broken up into carbonic acid and methane. This is the chief cause of the gases in the intestine, the amount of which is increased by vegetable food.

ii. On fats. In addition to acting like lipase, they produce lower acids (valeric, butyric, etc.). The formation of acid products from fats and carbohydrates gives to the intestinal contents an acid reaction. Recent researches show that the contents of the intestine become acid much higher up than was formerly supposed. These organic acids do not, however, hinder pancreatic digestion.

iii. On proteins. Fatty acids and amino-acids are produced; but the enzymes of these putrefactive organisms have a specially powerful action in liberating substances having an evil odour, such as indole (C_8H_7N), skatole (C_9H_9N), and phenol (C_6H_6O). The indole and skatole originate from the tryptophane radical of proteins. There are also gaseous products in some cases.

Ammonia-producing organisms flourish best in the lower regions of the small intestine; the ammonia neutralises the organic acid produced higher up, and in the large intestine the contents are consequently alkaline.

Bases from Amino-acids.—A very characteristic action of putrefactive bacteria is exerted on the amino-acids; this change consists in the splitting off of carbonic acid from their carboxyl (COOH) group, and the production of amines according to the following equation :—



$$\begin{array}{c} R.NH_2 \\ | \\ COOH \end{array} = \begin{array}{c} R.NH_2 \\ | \\ H \end{array} + CO_2.$$

R in the above equation represents the radical which varies in the different members of the amino-acid group (CH_2 in glycine, C_2H_4 in alanine, C_5H_{10} in leucine, etc.).

It has been known for some time that the well-known putrefactive bases *putrescine* and *cadaverine* are formed in this way from ornithine and lysine respectively :—

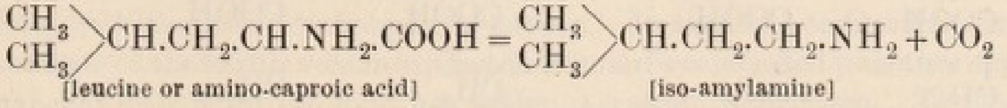
$$NH_2.CH_2.CH_2.CH_2.CH.NH_2.COOH = NH_2.CH_2.CH_2.CH_2.CH_2.NH_2 + CO_2$$

[ornithine or diamino-valeric acid] = [putrescine or tetramethylene-diamine]

$$NH_2.CH_2.CH_2.CH_2.CH_2.CH.NH_2.COOH$$
$$= NH_2.CH_2.CH_2.CH_2.CH_2.CH_2.NH_2. + CO_2.$$

[lysine or diamino-caproic acid] = [cadaverine or pentamethylene-diamine]

Recently, however, it has been recognised that this "decarboxylation" of amino-acids is a general reaction of certain putrefactive organisms, and that a whole series of bases is obtainable, which are of considerable physiological interest. For example it was found that during the putrefaction of meat and placenta leucine and tyrosine are converted into the bases iso-amylamine and oxyphenylethylamine respectively :—



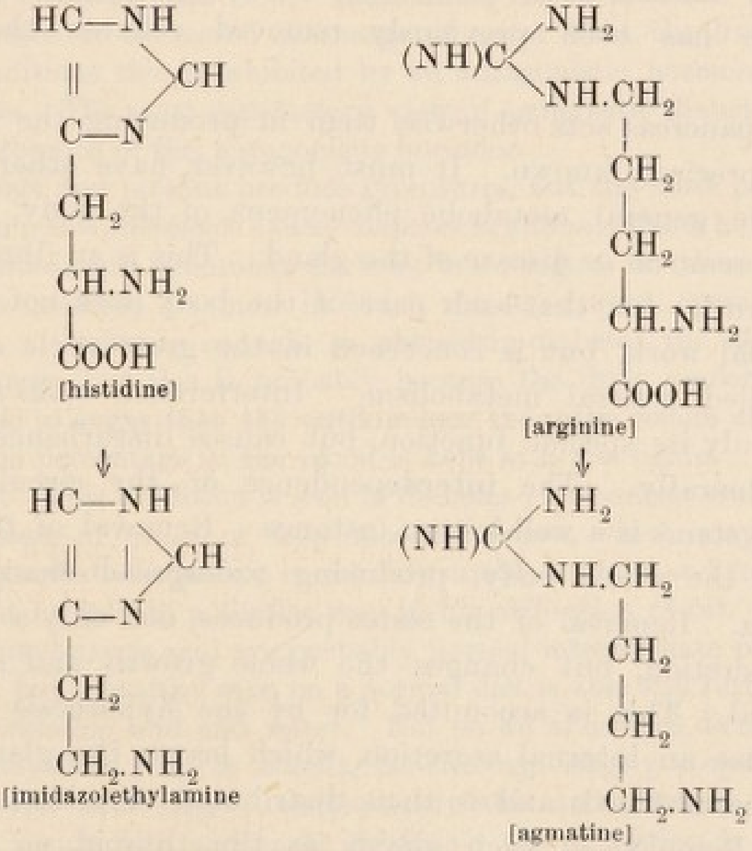
$$\left.\begin{matrix}CH_3\\CH_3\end{matrix}\right>CH.CH_2.CH.NH_2.COOH = \left.\begin{matrix}CH_3\\CH_3\end{matrix}\right>CH.CH_2.CH_2.NH_2 + CO_2$$

[leucine or amino-caproic acid] [iso-amylamine]

$$OH.C_6H_4.CH_2.CH.NH_2COOH = OH.C_6H_4.CH_2.CH_2.NH_2 + CO_2.$$

[tyrosine or oxyphenylalanine] [oxyphenylethylamine]

These two bases exert a pressor action on arterial blood-pressure, similar to that of adrenaline, and it is most probable that the pressor bases contained in normal urine during a liberal meat diet owe their origin to bacterial action in the intestine. The bases oxyphenylethylamine has also been found in extracts of ergot—the fungus which develops in the ovaries of certain grasses (*e.g.* rye) ; the enzymes of this fungus are able, like bacterial enzymes, to "decarboxylise" not only tyrosine, but also histidine and arginine, the bases formed being imidazolethylamine and agmatine respectively, as shown below :—

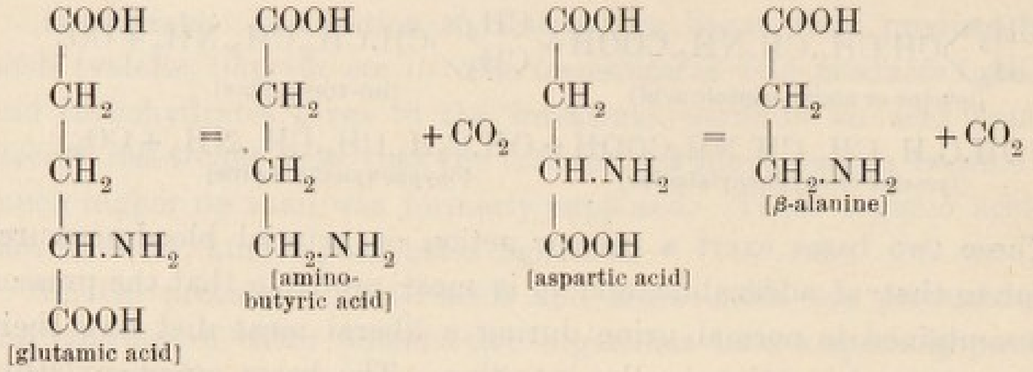


[histidine] [arginine]

[imidazolethylamine [agmatine]

Imidazolethylamine has also been obtained from histidine owing to the action of putrefactive bacteria in the intestine. Very small doses lower blood-pressure enormously. The base agmatine was first discovered in herring roe. There is no doubt that the pharmacological action of ergot is in part due to these bases.

Quite analogous to them is the formation of amino-butyric acid from glutamic acid, and of β-alanine from aspartic acid during putrefaction :—



$$\begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\CH.NH_2\\|\\COOH\\\text{[glutamic acid]}\end{array} = \begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\CH_2.NH_2\\\text{[amino-}\\\text{butyric acid]}\end{array} + CO_2 \qquad \begin{array}{c}COOH\\|\\CH_2\\|\\CH.NH_2\\|\\COOH\\\text{[aspartic acid]}\end{array} = \begin{array}{c}COOH\\|\\CH_2\\|\\CH_2.NH_2\\\text{[β-alanine]}\end{array} + CO_2$$

EXTIRPATION OF THE PANCREAS

Complete removal of the pancreas in animals and diseases of the pancreas in man produce a condition of diabetes, in addition to the loss of pancreatic action in the intestines. Grafting the pancreas from another animal into the abdomen of the animal from which the pancreas has been previously removed relieves the diabetic condition.

How the pancreas acts otherwise than in producing the pancreatic juice is not precisely known. It must, however, have other functions related to the general metabolic phenomena of the body, which are disturbed by removal or disease of the gland. This is an illustration of a universal truth—viz. that each part of the body does not merely do its own special work, but is concerned in the great cycle of changes which is called general metabolism. Interference with any organ upsets, not only its specific function, but causes disturbances through the body generally. The interdependence of the circulatory and respiratory systems is a well-known instance. Removal of the thyroid gland upsets the whole body, producing widespread changes known as myxœdema. Removal of the testes produces, not only a loss of the spermatic secretion, but changes the whole growth and appearance of the animal. This is accounted for by the hypothesis that such glands produce an internal secretion, which leaves the gland *viâ* the lymph or venous blood, and is then distributed to minister to parts elsewhere. Removal of such glands as the thyroid or suprarenal produces disease or death because this internal secretion can no longer be formed. In the case of the pancreas, the external secretion of the pancreas (that is, pancreatic juice) is formed by the cells lining the acini, and the internal secretion, stoppage of which in some way leads to diabetes, has been attributed to the islets of Langerhans.

Glycosuria (sugar in the urine) occurs in many conditions. It may be a temporary condition, as in alimentary glycosuria, which is due to excess of carbohydrate food, or a comparatively inactive liver which is incapable of dealing with the usual carbohydrate supply. It may be produced by injury to the floor of the fourth ventricle (Claude Bernard's celebrated puncture experiment), but only when the liver has within it a store of glycogen. The injury to the bulbar centre influences thus the nervous mechanism which regulates the glycogenic function of the liver. In diabetes mellitus, the body is unable to utilise sugar by burning it, and so liberating heat and energy; sugar therefore accumulates in the blood and overflows into the urine. In some cases rigid abstention from carbohydrate food makes little or no difference, and the sugar must come from the protein constituents of protoplasm, alanine being one of the most important of the intermediate products. When the pancreatic functions are in abeyance, the diabetic state is due to an impaired capacity to oxidise sugar down to its ultimate products carbonic acid and water. The destruction of sugar by the tissues is termed glycolysis, and one view advanced is that the internal secretion of the pancreas activates the glycolytic enzyme; therefore when the internal secretion of the pancreas is absent, the activating impulse is absent also. There are many difficulties, however, in accepting this view. Adrenaline, the secretion of the suprarenal body, produces an increased discharge of sugar from the liver, but under normal conditions this is inhibited by an antagonistic hormone secreted by the pancreas. The most satisfactory view of pancreatic diabetes is that it is due to the absence of this antagonistic hormone.

Many drugs and poisons produce glycosuria, but the most potent of them is phloridzin; this substance causes diabetes in animals which have no glycogen in their tissues, and phloridzin-diabetes is analogous to those severe forms of diabetes mellitus in man in which the sugar must come from protein katabolism. Curiously enough, in phloridzin-diabetes the blood shows no excess of sugar, but this is probably because the drug renders the kidney so permeable to sugar that the outflow into the urine occurs at such a rapid rate that the percentage in the blood is kept at a low figure.

Acidosis.—This condition is seen in diabetes; poisonous acids in the blood produce a state of coma, or deep unconsciousness, which may finally cause death. For a diabetic patient is not only unable to burn and so utilise carbohydrate, but he fails in a similar way in his utilisation of fat. Butyric acid and β-hydroxybutyric acid are probably normal intermediate products in fat katabolism, but a healthy man on a normal diet is able still further to oxidise them into carbonic acid and water. But on an abnormal diet, for instance, when carbohydrate food is absent, fat-cleavage largely stops short at the hydroxybutyric acid stage; consequently this and possibly other related fatty acids accumulate and cause acidosis; this condition is increased the more fat is given in the food, and the acidosis of diabetes is similarly increased by fatty food. These poisonous acids were once believed to originate from proteins; if that were so there ought to be an increase of other protein katabolites in the urine, which there is not. The acids decrease the alkalinity and carbonic acid of the blood, and the ammonia of the urine is increased; this indicates an attempt of the body to neutralise the acids.

The hydroxybutyric acid does not pass entirely unchanged into the urine. β-Hydroxybutyric acid is $CH_3.C\mathbf{H}O\mathbf{H}.CH_2.COOH$. By oxidation, the two hydrogen atoms in thick type are removed to form water, and this leaves $CH_3.CO.CH_2.\mathbf{COO}H$, which is aceto-acetic acid: when the COO in thick type is removed we get acetone ($CH_3.CO.CH_3$), which gives the breath and urine of such patients an apple-like smell.

In these changes the liver plays an important part by means of certain enzymes which Dakin has proved to exist. One enzyme, called *β-hydroxybutyrase*, is an oxidase; it oxidises the β-hydroxybutyric into aceto-acetic acid, and its action is increased by the addition of blood or oxyhæmoglobin, which furnishes the necessary oxygen. It probably is active in health as well as in disease, the aceto-acetic acid being finally burnt into carbonic acid and water. The other enzyme which forms acetone is not an oxidative one, and acetone formation probably never occurs in the healthy state.

The question of diabetes is an important one, and the foregoing paragraphs have treated it only in outline; the student should consult a general text-book on Physiology or Pathology for a full consideration of the subject.

THE BILE

The liver is an organ which has many functions; one of these, the glycogenic function, is referred to in the preceding section; it also plays a part in the metabolism of proteins (see formation of urea and uric acid) and of fats. In this place, however, we are specially concerned with its secretion, namely, the Bile.

Bile is the secretion of the liver which is poured into the duodenum: it has been collected in living animals by means of a biliary fistula; the same operation has occasionally been performed in human beings. At death the gall bladder yields a good supply of bile which is more concentrated than that obtained from a fistula.

Though the chief blood supply of the liver is by a vein (the portal vein), the amount of blood in the liver varies with its needs, being increased during the periods of digestion. This is due to the fact that in the area from which the portal vein collects blood—stomach, intestine, spleen, and pancreas—the arterioles are all dilated, and the capillaries are thus gorged with blood. Further, the active peristalsis of the intestine and the pumping action of the spleen are additional factors in driving more blood onwards to the liver.

The bile is secreted from the portal blood at a much lower pressure than one finds in glands, such as the salivary glands, the blood supply of which is arterial. Herring and Simpson have found that the pressure in the bile duct averages 30 mm. of mercury, which is considerably above that in the portal vein.

The increase in the flow of bile which occurs after the arrival of the semi-digested food (chyme) in the intestine has been explained by the circumstance that secretin is a stimulant of the liver as well as of the pancreas. The action of secretin on bile is not, however, a pronounced one. The most efficient cholagogue known consists of the bile salts themselves ; these, after entering the intestine, are reabsorbed and return to the liver, and once more stimulate that organ to activity.

The chemical processes by which the constituents of the bile are formed are obscure. We, however, know that the biliary pigment is produced by the decomposition of hæmoglobin. Bilirubin is, in fact, identical with the iron-free derivative of hæmoglobin called hæmatoidin, which is found in the form of crystals in the old blood-clots such as occur in the brain after cerebral hæmorrhage (see fig. 19). Moreover, bilirubin yields hæmopyrrol, a substance also obtained from blood-pigment.

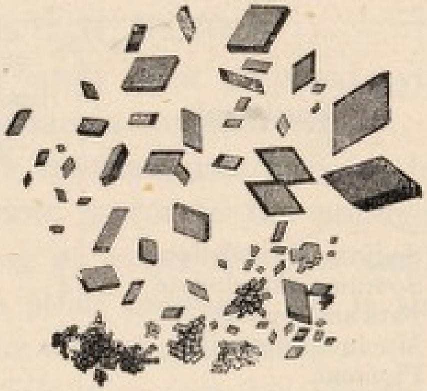


FIG. 19.—Hæmatoidin crystals.

An injection of hæmoglobin into the portal vein, or of substances such as water which liberate hæmoglobin from the red blood corpuscles, produces an increase of bile pigment. If the spleen takes any part in the elaboration of bile pigment, it does not proceed so far as to liberate hæmoglobin from the corpuscles. No free hæmoglobin is discoverable in the blood plasma in the splenic vein.

The amount of bile secreted is differently estimated by different observers; the amount secreted daily in man appears to vary from 500 c.c. to 1 litre (1000 c.c.).

THE CONSTITUENTS OF BILE

The constituents of the bile are the bile salts proper (taurocholate and glycocholate of soda), the bile pigments (bilirubin, biliverdin), a mucinoid substance, small quantities of fats, soaps, cholesterol, lecithin, urea, and mineral salts, of which sodium chloride and the phosphates of iron, calcium, and magnesium are the most important.

Bile is a yellowish, reddish-brown or green fluid, according to the relative preponderance of its two chief pigments. It has a musk-like odour, a bitter-sweet taste, and a neutral or faintly alkaline reaction.

The specific gravity of human bile from the gall-bladder is 1026 to 1032 ; that from a fistula, 1010 to 1011. The greater concentration of gall-bladder bile is partly but not wholly explained by the addition to it from the walls of that cavity of the mucinoid material.

The amount of solids in gall-bladder bile varies from 9 to 14 per cent., in fistula bile from 1·5 to 3 per cent. The following table shows that this low percentage of solids is almost entirely due to want of bile salts. This can be accounted for in the way first suggested by Schiff—that there is normally a bile circulation going on in the body; a large quantity of the bile salts which pass into the intestines is first split up, then reabsorbed and again secreted. Such a circulation would obviously be impossible in cases where all the bile is discharged to the exterior.

The following table gives some important analyses of human bile:—

Constituents.	Fistula Bile (healthy woman. Copeman and Winston).	Fistula Bile (case of cancer. Yeo and Herroun)	Normal Bile (Frerichs).
Sodium glycocholate .	0·6280	0·165	9·14
Sodium taurocholate .		0·055	
Fats and lipoids . .	0·0990	0·038	1·18
Mucinoid material .	0·1725	0·148	2·98
Pigment . . .	0·0725		
Inorganic salts . .	0·4510	0·878	0·78
Total solids . . .	1·4230	1·284	14·08
Water (by difference) .	98·5770	98·716	85·92
	100·0000	100·000	100·00

Bile Mucin.—There has been considerable diversity of opinion as to whether the bile mucin is really mucin. The most recent work in Hammarsten's laboratory shows that differences occur in different animals. Thus in the ox there is very little true mucin, but a great amount of nucleo-protein; in human bile, on the other hand, there is very little if any nucleo-protein; the mucinoid material present there is really mucin. (On the general characters of MUCIN and NUCLEO-PROTEINS, see pp. 62 to 65.)

The Bile Salts.—The bile contains the sodium salts of complex acids called the bile acids. The acids most frequently found are glycocholic and taurocholic acids. The former is more abundant in the bile of man and herbivora; the latter in carnivora such as the dog. The most important difference between the two acids is that taurocholic acid contains sulphur, and glycocholic acid does not.

Glycocholic acid ($C_{26}H_{43}NO_6$) is hydrolysed by the action of dilute acids and alkalis, and also in the intestine, and split into glycine or amino-acetic acid and cholalic acid :—

$$\underset{\text{[glycocholic acid]}}{C_{26}H_{43}NO_6} + H_2O = \underset{\text{[glycine]}}{CH_2.NH_2.COOH} + \underset{\text{[cholalic acid]}}{C_{24}H_{40}O_5}$$

The glycocholate of soda has the formula $C_{26}H_{42}NaNO_6$.

Taurocholic acid ($C_{26}H_{45}NO_7S$) similarly splits into taurine or amino-ethyl-sulphonic acid and cholalic acid :—

$$\underset{\text{[taurocholic acid]}}{C_{26}H_{45}NO_7S} + H_2O = \underset{\text{[taurine]}}{C_2H_4.NH_2.HSO_3} + \underset{\text{[cholalic acid]}}{C_{24}H_{40}O_5}$$

The taurocholate of soda has the formula $C_{26}H_{44}NaNO_7S$.

Glycocholic and taurocholic acids have lately been prepared synthetically from cholalic acid and glycine and taurine respectively.

The colour reaction called **Pettenkofer's reaction,** described in the practical exercises at the head of this lesson, is due to the presence of cholalic acid. The sulphuric acid acting on sugar forms a small quantity of furfuraldehyde, in addition to other products. It is the furfuraldehyde which gives the purple colour with cholalic acid.

The Bile Pigments.—The two chief bile pigments are bilirubin and biliverdin. Bile which contains chiefly the former (such as dog's bile) is of a golden or orange-yellow colour, while the bile of many herbivora, which contains chiefly biliverdin, is either green or bluish green. Human bile is generally described as containing chiefly bilirubin, but there have been some cases described in which biliverdin was in excess. The bile pigments show no absorption bands with the spectroscope.

Bilirubin has the formula $C_{32}H_{36}N_4O_6$: it is thus an iron-free derivative of hæmoglobin. The iron is apparently stored up in the liver cells, perhaps for future use in the manufacture of new hæmoglobin.

The bile contains only a trace of iron.

Biliverdin has the formula $(C_{16}H_{18}N_2O_4)_n$ (*i.e.* more oxygen than bilirubin): it may occur as such in bile; it may be formed by simply exposing red bile to the oxidising action of the atmosphere; or it may be formed, as in Gmelin's test, by the more vigorous oxidation produced by fuming nitric acid.

Gmelin's test consists of a play of colours—green, blue, red, and finally yellow—produced by the oxidising action of fuming nitric acid (that is, nitric acid containing nitrous acid in solution). The end or yellow product is called *choletelin*, $C_{32}H_{36}N_4O_{12}$. Occasionally in various animals these products of intermediate oxidation are found in the bile; thus bilicyanin (the blue pigment) and bilipurpurin (the purple one) may be present.

Hydrobilirubin.—If a solution of bilirubin or biliverdin in dilute alkali is treated with sodium amalgam or allowed to putrefy, a brownish pigment is formed called hydrobilirubin, $C_{32}H_{44}N_4O_7$. With the spectroscope it shows a dark absorption band between *b* and F, and a fainter band in the region of the D line.

Urobilin.—Hydrobilirubin is interesting because a similar substance is formed from the bile pigment by reduction processes in the intestine; this is *stercobilin*, the pigment of the fæces. Some of this is absorbed and ultimately leaves the body in the urine as one of its pigments called *urobilin*. A small quantity of urobilin is sometimes found preformed in the bile. The identity of urobilin and stercobilin has been frequently disputed, but the work of Garrod and Hopkins has confirmed the old statement that they are the same substance with different names. Urobilin has a well-marked absorption band in the region of the F line, and when partially precipitated from an alkaline solution by acidification, it also shows an absorption band in the region of the E line. Hydrobilirubin differs from urobilin in containing much more nitrogen in its molecule (9·2 instead of 4·1 per cent.), and is probably a product of less complete reduction than urobilin. (See further Lesson XXV.) Urobilin is also formed by the oxidation of hæmopyrrol (see Hæmoglobin, p. 148).

Cholesterol.—In bile this substance is normally present in small quantities only, but it may occur in excess, and so form the concretions known as gallstones, which are generally more or less tinged with bilirubin. Its chemical characters and physiological importance are discussed on pp. 38, 39; its colour reactions are given in to-day's practical exercises (p. 108).

THE USES OF BILE

Bile is doubtless, to a certain extent, excretory. In some animals it has a slight action on fats and starch, but it appears to be rather a coadjutor to the pancreatic juice (especially in the digestion of fat) than to have any independent digestive activity. Its auxiliary action in the digestion of fat and starch has been shown in our practical exercises (p. 107). It has a similar slight assisting power in the digestion of proteins.

Bile is said to be a natural antiseptic, lessening the putrefactive processes in the intestine. This is very doubtful. Though the bile salts are weak antiseptics, the bile itself is readily putrescible, and the power it has of diminishing putrescence in the intestine is due chiefly to the fact that by increasing absorption it lessens the amount of putrescible matter in the bowel.

When the bile meets the chyme the turbidity of the latter is increased, owing to the precipitation of unpeptonised protein. This is an action due to the bile salts, and it has been surmised that this conversion of the chyme into a more viscid mass is to hinder somewhat its progress through the intestine: it clings to the intestinal wall, thus allowing absorption to take place. The neutralisation of the acid gastric juice by the bile also allows the alkalinity of the pancreatic juice to have full play. Bile is a solvent of fatty acids, and assists the absorption of fat.

THE FATE OF THE BILIARY CONSTITUENTS

We have seen that fistula bile is poor in solids as compared with normal bile, and that this is explained on the supposition that the normal bile circulation is not occurring—the liver cannot excrete what it does not receive back from the intestine. Schiff was the first to show that if the bile is led back into the duodenum, or even if the animal is fed on bile, the percentage of solids in the bile excreted is at once raised. It is on these experiments that the theory of a bile circulation is mainly founded. The bile circulation relates, however, chiefly, if not entirely, to the bile salts: they are found but sparingly in the fæces; they are only represented to slight extent in the urine; hence it is calculated that seven-eighths of them are reabsorbed from the intestine. Small quantities of cholalic acid, taurine, and glycine are found in the fæces; the greater part of these products of the decomposition of the bile salts is taken by the portal vein to the liver, where they are once more synthesised into the bile salts. Some of the taurine is absorbed and excreted as tauro-carbamic acid ($C_2H_4NHCO\cdot NH_2HSO_3$) in the urine. Some of the absorbed glycine may be excreted as urea. The pigment is changed into stercobilin, a substance like hydrobilirubin. Some of the stercobilin is absorbed, and leaves the body as the urinary pigment, urobilin. The cholesterol in the fæces was formerly supposed to be a bile residue; but in some animals, especially those which feed on grass, the source of the fæcal cholesterol is the vegetable cholesterol (phytosterol) of the food. In some cases it is reduced to form a derivative termed *coprosterol*.

The fæces are alkaline in reaction, and on an ordinary mixed diet contain comparatively little food residues, and a small quantity is excreted even during starvation. Voit and Hermann showed independently that an intestinal loop which had been emptied and separated from the rest of the bowel contained, a few days later, material identical with fæces, and consisting of intestinal juice, desquamated epithelium cells, and bacteria. The increase in the amount of fæces which occurs when food is taken, even when the food is free from cellulose, is due to the mechanical and chemical stimulation which leads to an increase in the succus entericus, and in the shedding of epithelial cells. The fæces contain about 1 per cent. of nitrogen, but this is chiefly contained in the bodies of bacteria, and the disintegrated epithelial cells. Addition of protein to the diet makes practically no difference to the nitrogen in the fæces under normal conditions.

The addition of cellulose to the diet increases the bulk of the fæces, partly because most of the cellulose is unchanged, partly because it stimulates the mucous membrane to secrete more succus entericus, and finally because the larger food residue favours the development of bacteria. On an average, from one-third to one-fifth (varying with the diet) of the weight of dried fæces consists of bacteria. The average weight of dried bacteria excreted daily is 8 grammes; this contains 0·8 gramme of nitrogen, or about half the nitrogen of the fæces. Strasburger estimated that about 128,000,000,000,000 bacteria are evacuated in the fæces of a man every day. The vast majority of these are dead.

When cellulose is absent from the diet, the fæces contain from 65–75 per cent. of water; the dry residue contains about 7 per cent. of nitrogen, and the non-nitrogenous material consists of about equal quantities of ash and substances soluble in ether, with small quantities of stercobilin and other bile residues. The ash contains mainly calcium phosphate, with small amounts of iron and magnesium. The ethereal extract contains cholesterol, lecithin, fatty acids, soaps, and a very small amount of neutral fat. The proteins are chiefly mucin and nucleo-protein, and are derived not from the food, but from the intestinal wall, or are contained in the bacteria; no doubt a large part of the ethereal extract is also supplied by the bacteria.

Cellulose is thus the only important constituent of the food which is unaffected by the digestive juices, although a variable amount, which is largest in herbivorous animals, undergoes bacterial decomposition. The presence of cellulose also interferes with the absorption of proteins, for the digestive juices have difficulty in penetrating the cellulose membranes of vegetable cells. Thus Voit found that 42 per cent. of the nitrogen in the food were lost in the fæces of a vegetarian. This is due solely to the cellulose and not to any difference in the digestibility of animal and vegetable proteins, for if vegetable food is finely subdivided, and then thoroughly cooked and softened, this loss is lessened, and if vegetable protein is entirely freed from cellulose, it is as thoroughly absorbed as animal protein. Fifteen per cent. of the dry substance of green vegetables and brown bread, 20 per cent. of carrots and turnips, and a still larger amount of beans are lost in the fæcal residue.

The intestinal contents travel more rapidly when vegetables are present, for the indigestible cellulose stimulates peristalsis, and therefore a large quantity of water escapes absorption in the colon. Thus on an ordinary mixed diet 35 grammes of dry substance and 100 grammes of water are daily excreted in the fæces, whereas on a vegetable diet the quantities are 75 and 260 grammes respectively.

MECONIUM

Meconium is the name given to the greenish-black contents of the intestine of new-born children. It is chiefly concentrated bile, with *débris* from the intestinal wall. The pigment is a mixture of bilirubin and biliverdin ; it is not stercobilin.

ABSORPTION

Food is digested in order that it may be absorbed. It is absorbed in order that it may be assimilated—that is, become an integral part of the living material of the body.

Having now considered the action of digestive juices, we can study the absorption which follows. In the mouth and œsophagus the thickness of the epithelium and the quick passage of the food through these parts reduce absorption to a minimum. Absorption takes place to a small extent in the stomach ; the small intestine, with its folds and villi to increase its surface, is, however, the great place for absorption ; and, although the villi are absent from the large intestine, absorption occurs there also, but to a less extent.

Foods such as water and soluble salts like sodium chloride are absorbed unchanged. The organic foods, however, are considerably changed, colloid materials such as starch and protein being converted respectively into the diffusible materials sugar and amino-acids.

There are two channels of absorption, the blood-vessels (portal capillaries) and the lymphatic vessels or lacteals.

Absorption, however, is no mere physical process of diffusion and filtration. We must also take into account the fact that the cells through which the absorbed substances pass are living, and in virtue of their vital activity not only select materials for absorption, but also change those substances while in contact with them. These cells are of two kinds: (1) the columnar epithelium that covers the surface; and (2) the lymph cells in the lymphoid tissue beneath. It is now generally accepted that of the two the former, the columnar epithelium, is the more important. When these cells are removed, or rendered inactive by sodium fluoride, absorption practically ceases, though the opportunities for simple filtration or diffusion would be by such means increased.

Absorption of Carbohydrates.—Though the sugar formed from starch by ptyalin and amylase is maltose, that found in the blood is glucose. Under normal circumstances little if any is absorbed by the lacteals. The glucose is formed from the maltose by the succus entericus. Sucrose and lactose are also converted into mono-saccharides before absorption.

The carbohydrate food which enters the blood as glucose is taken to the liver, and there stored up in the form of glycogen—a reserve store of carbohydrate material for the future needs of the body. Glycogen, however, is found in animals which take no carbohydrate food. It must then be formed by the protoplasmic activity of the liver cells from their protein constituents. The carbohydrate store leaves the liver in the blood of the hepatic vein as glucose once more.

The above is a brief statement of the glycogenic functions of the liver as taught by Claude Bernard, and accepted by the majority of physiologists. It was strongly contested by Pavy, who held that the glycogen formed in the liver from the sugar of the portal blood is never during life reconverted into sugar, but is used in the formation of other substances such as fat and protein; in support of this he showed that proteins contain a carbohydrate radical; and it certainly is the case that carbohydrate food is fattening. He denied that the post-mortem formation of sugar from glycogen that occurs in an excised liver is a true picture of what occurs during life.

Absorption of Proteins.—It is possible that under abnormal conditions a certain amount of soluble protein is absorbed unchanged. Thus, after eating a large number of eggs, egg-albumin has been stated to be discoverable in the urine. Patients fed *per rectum* derive nourishment from protein food, although proteolytic enzymes are not present in this part of the intestine.

Under normal conditions, however, the food-proteins are broken up into substances with smaller molecules, and the ready diffusibility of peptones led most physiologists to consider that protein was usually absorbed as peptone, or as proteose and peptone. But proteose and peptone are absent from the blood and lymph in all circumstances, even from the portal blood during the most active digestion. It is fortunate that this is so, for proteose and peptone when introduced into the blood produce poisonous effects; the coagulability of the blood is lessened, blood pressure falls, secretion ceases, and in the dog 0·3 gramme of commercial peptone per kilogramme of body-weight is often sufficient to produce death.

This absence of " peptone " (using the word to include the proteoses) did not, however, absolutely negative the idea that "peptone" is the form in which proteins are absorbed, and the difficulty was met by supposing that during absorption the products of proteolysis were reconverted into native proteins (albumins and globulins). This synthesis was further considered to be accomplished by the epithelial cells that line the intestine.

This view has now had its day, and the change of opinion that has relegated it to the past is due (1) to our increased knowledge of the power of trypsin and erepsin; (2) to a more careful examination of the intestinal contents, and of the blood during absorption. We now know that in the intestine the proteins are, by the two enzymes trypsin and erepsin, broken down beyond the peptone stage into their final cleavage products, the amino-acids, and that these pass into the blood as such, for the amount of non-protein nitrogen in that fluid is increased during absorption. If an animal is fed on the cleavage products obtained from a pancreatic digest, nitrogenous equilibrium is still maintained. These amino-acids are partly utilised by the cells of the body to repair their waste, but partly and to a still greater extent converted by the liver into the waste substance urea, which is finally excreted by the kidneys. The view that the absorptive epithelium of the alimentary tract has any special power in building up proteins from these simple cleavage products has not been confirmed.

We thus see that the cells of the body possess the power of rebuilding the proteins peculiar to themselves from the fragments of the molecules of the food proteins. This accounts for the fact that the animal tissues retain their chemical individuality in spite of the great variations in the composition of the diet the animal takes.

If a man wishes to build a new house, and to employ for the purpose the bricks previously used in the building of another house, he takes the old house to pieces and uses the bricks and stones most appropriate for his purpose, rearranges them in such a way that the new house has its own special architectural features, and discards as waste the bricks and stones which are not suitable. This idea underlies the term so often used by German writers, who speak of the cleavage products of protein as Bausteine (building stones). Each tissue has special architectural features in its protein molecules, and these molecules are reconstructed by using the Bausteine that previously had been used in the building of other protein molecules, either in another animal or in vegetable structures. The Bausteine which are in excess or are unsuitable are simply got rid of as waste substance.

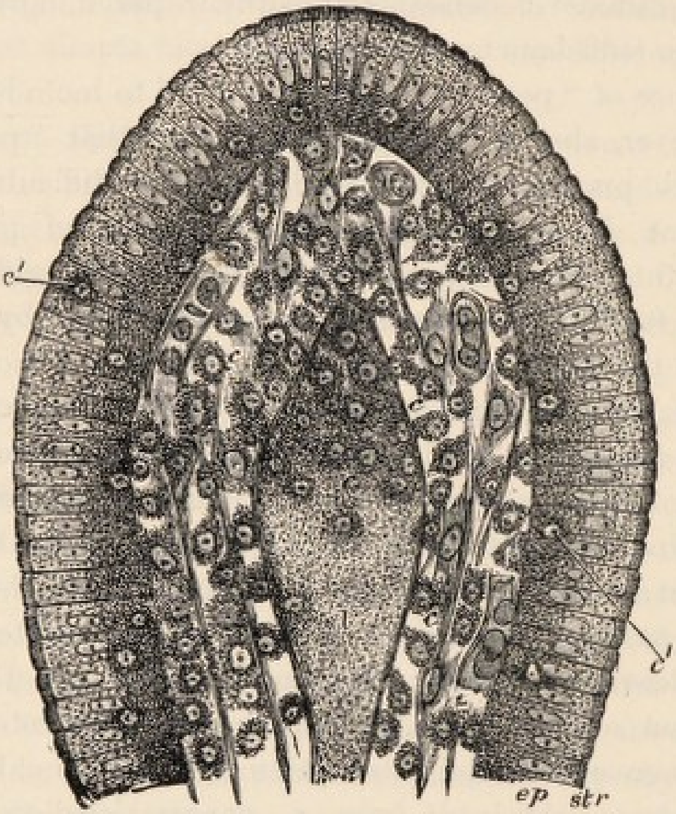


FIG 20.—Section of the villus of a rat killed during fat-absorption (E. A. Schafer): *ep*, epithelium; *str*, striated border; *c*, lymph cells; *c'*, lymph cells in the epithelium; *l*, central lacteal containing disintegrating lymph cells.

A large number of the Bausteine are never actually built into protoplasm, but are carried to the liver when the amino-group is removed; this is termed *deamidation*; the nitrogenous portion is then converted into urea, and the non-nitrogenous portion of the protein molecule is then available for calorific processes (see Urea formation). One can only conjecture at present which are the ones that on p. 72 we compared to diamonds, because they are unusually precious for the synthesis of protein by tissue cells; but probably phenyl-alanine and its near relations tyrosine and tryptophane come into this category, for if they are injected into the blood-stream they do not give rise to any increase in the urea formed; moreover, proteins destitute of these amino-acids are of inferior nutritive value.

Absorption of Fats.—The fats undergo in the intestine two changes: one a physical change (emulsification), the other a chemical change (saponification). The lymphatic vessels are the great channels for fat-absorption, and their name, lacteals, is derived from the milk-like appearance of their contents (chyle) during the absorption of fat.

The way in which the minute fat globules pass from the intestine into the lacteals has been studied by killing animals at varying periods after a meal of fat and making osmic acid microscopic preparations of the villi. Figs. 20 and 21 illustrate the appearances observed.

The columnar epithelium cells become first filled with fatty globules of varying size, which are generally larger near the free border. The globules pass down the cells, the larger ones breaking up into smaller ones during the journey; they are then transferred to the amœboid cells of the lymphoid tissue beneath; these ultimately penetrate into the central lacteal, where they either disintegrate or discharge their cargo into the lymph-stream. The globules are by this time divided into immeasurably small ones, the molecular basis of chyle. The chyle enters the blood-stream by the thoracic duct, and after an abundant fatty meal the blood plasma is quite milky; the fat droplets are so small that they circulate without hindrance through the capillaries. The fat in the blood after a meal is eventually stored up in the connective-tissue cells of adipose tissue. It must, however, be borne in mind that the fat of the body is not exclusively derived from the fat of the food, but it may originate also from carbohydrates and possibly, in the opinion of some physiologists, from protein as well.

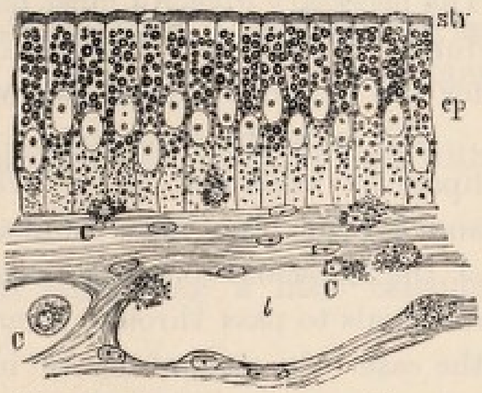


FIG. 21.—Mucous membrane of frog's intestine during fat-absorption (E. A. Schäfer): *ep*, epithelium; *str*, striated border; C, lymph cells; *l*, lacteal.

As the fat globules were never seen penetrating the striated border of the epithelial cells, there was a difficulty in understanding how they reached the interior of these cells; the cells will not take up other particles, and it is certain that they do not in the higher animals protrude pseudopodia from their borders (this, however, does occur in the endoderm of some of the lower invertebrates).

Recent research has solved this difficulty. In the first place, particles may be present in the epithelium and lymphoid cells while no fat is being absorbed. These particles are protoplasmic in nature, as they stain with reagents that stain protoplasmic granules; but as they also stain darkly with osmic acid, they are apt to be mistaken for fat. There is, however, no doubt that the particles found during fat-absorption are composed of fat. There is also no doubt that the epithelial cells have the power of again forming fat out of the fatty acids and glycerol into which it has been broken up in the intestine. These substances, being soluble, pass readily into the epithelium cells, and these cells perform the synthetic act of building them into fat once more; the fat so formed appears in the form of small globules, surrounding or becoming mixed with the protoplasmic granules that are ordinarily present. A remarkable fact which Munk made out is that after feeding an animal on fatty acids the chyle contains fat. The necessary glycerol must have been formed by protoplasmic activity during absorption. Preliminary emulsification, though advantageous for the action of lipase, is not essential.

Bile aids the digestion of fat by co-operating with the pancreatic lipase as explained on p. 115. It is also a solvent of fatty acids, and it probably assists fat-absorption by reducing the surface tension of the intestinal contents; membranes moistened with bile allow fatty materials to pass through them more readily than would otherwise be the case. In cases of disease in which bile is absent from the intestines a large proportion of fat in the food passes into the fæces.

In conclusion it must be mentioned that the lymphocytes are greatly increased in the blood during absorption, and some physiologists hold the view that these come from the intestinal mucous membrane, and share in the work of transporting absorbed materials.

LESSON IX

THE BLOOD AND RESPIRATION

BLOOD PLASMA

1. The coagulation of the blood has been prevented in specimen A by the addition of neutral salt (an equal volume of saturated sodium-sulphate solution, or a quarter of its volume of saturated magnesium-sulphate solution). The corpuscles have settled, and the supernatant salted plasma has been siphoned off.

2. The coagulation of the blood in specimen B has been prevented by the addition of an equal volume of a 0·4 per-cent. solution of potassium oxalate in normal saline solution.

3. Put a small quantity of A into three test-tubes and dilute each with about ten times its volume of liquid :

A 1. With distilled water.

A 2. With solution of fibrin-ferment containing a little calcium chloride.[1]

A 3. With the same.

4. Put A 1 and A 2 into the water-bath at 40° C. ; leave A 3 at the temperature of the air. A 1 coagulates slowly or not at all ; A 2 coagulates rapidly : A 3 coagulates less rapidly than A 2.

5. Add to some of B a few drops of dilute (2 per-cent.) calcium-chloride solution : it coagulates, and more quickly, if the temperature is 40° C.

BLOOD SERUM

Blood serum is the fluid residue of the blood after the separation of the clot ; it is blood plasma *minus* the fibrin which it yields. The general appearance of fibrin obtained by whipping fresh blood will already be familiar to the student, as he has used it in experiments on digestion.

Serum has a yellowish tinge due to serum lutein, but as generally obtained it is often contaminated with a small amount of oxyhæmoglobin, and so looks reddish. It contains proteins (giving the general tests

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[1] An easy way of preparing an impure but efficient solution of fibrin-ferment is to take 5 c.c. of blood serum and dilute it with a litre of distilled water. A partial precipitation of globulin takes place, and carries down the ferment with it. After a few hours pour off the supernatant fluid and dissolve the precipitate in half a litre of tap water to which a few drops of 2-per-cent. solution of calcium chloride have been added. The solution can be then given round to the class as fibrin-ferment.

already studied in Lesson V), extractives, and salts in solution. The proteins are serum albumin and serum globulin. Fibrin-ferment or thrombin is also present, and in the following experiments is precipitated with serum globulin.

SEPARATION OF THE SERUM PROTEINS.—(a) Dilute serum with fifteen times its volume of water. It becomes cloudy owing to the partial precipitation of serum globulin. Add a few drops of 2 per-cent. acetic acid; the precipitate becomes more abundant, but dissolves in excess of the acid.

(b) Pass a stream of carbonic acid through serum diluted with twenty times its bulk of water. A partial precipitation of serum globulin occurs.

(c) Saturate some serum with magnesium sulphate by adding crystals of the salt and grinding in a mortar. A precipitate of serum globulin is produced.

(d) Half saturate the serum with ammonium sulphate by adding to it an equal volume of a saturated solution of the salt. Serum globulin is precipitated.

(e) Completely saturate the serum with ammonium sulphate by adding crystals of the salt and grinding in a mortar; a precipitate is produced of both the globulin and the albumin. Filter through a dry filter paper; the filtrate contains no protein.

(f) Put some serum in a dialyser with distilled water in the outer vessel. In a day or two, especially if the water in the outer vessel is changed frequently, the salts dialyse out into the water; the proteins remain inside the dialyser; of these the serum albumin and a fraction of the serum globulin (called pseudo-globulin) remain in solution, but the other fraction of the serum globulin (called euglobulin) is precipitated, as it requires salt to hold it in solution.

(g) Whether serum albumin is a single protein or a mixture of several proteins is a matter of dispute. The only method by which it can be at present fractionated is the somewhat uncertain one of heat coagulation. If the globulin is removed by saturation with magnesium sulphate, the filtrate, which contains serum albumin, when heated after faint acidulation with 2 per-cent. acetic acid, deposits a flocculent precipitate at about 73° C. Filter this off, and on heating the filtrate a second coagulum is obtained at about 77° C., and similarly a third and very small fraction separates out at 86° C.

HÆMOGLOBIN

1. THE SPECTROSCOPE.—Direct the spectroscope to the window and carefully focus Fraunhofer's lines. Note especially D in the yellow, and E, the next well-marked line, in the green.

Direct the spectroscope to a luminous gas flame; these lines are absent. Place a little sodium chloride in the flame. Notice the bright yellow line in the position of the D line.

2. **SPECTROSCOPIC EXAMINATION OF BLOOD.**—Take a series of six test-tubes of about equal size. Fill the first with diluted defibrinated ox-blood (1 part of blood to 31 of water); then fill the second tube with the same mixture diluted with an equal bulk of water (1 in 64); half fill the third tube with this and fill up the tube with an equal bulk of water (1 in 128), and so on. The sixth tube will contain 1 part of blood to 1024 of water and will be nearly colourless.

3. Into another series of six test-tubes put a few drops of ammonium sulphide; then pour in some of the contents of each of the first series and warm *very* gently. Another reducing agent called Stokes's reagent may be employed in this experiment in the place of ammonium sulphide. It must always be freshly prepared; it is a solution of ferrous sulphate to which a little tartaric acid has been added, and then ammonia till the reaction is alkaline. The ammonia should not be added until just before it is used.

4. Examine the tubes with the spectroscope and map out on a chart the typical absorption bands of oxyhæmoglobin in the first series, and of (reduced) hæmoglobin in the second series. Notice that in the more dilute specimens of hæmoglobin the bands are no longer seen, whereas those of oxyhæmoglobin in specimens similarly diluted are still visible.

5. Take a tube which shows the single band of reduced hæmoglobin and shake it with the air; the bright red colour returns to it and it shows spectroscopically the two bands of oxyhæmoglobin for a short time. Continue watching the two bands, and note that they fade and are replaced by a single band as reduction again occurs.

6. **BLOOD CRYSTALS.**—Mix a drop of defibrinated rat's blood on a slide with a drop of water, or mount it in a drop of Canada balsam. Examine the crystals of oxyhæmoglobin as they form.

7. Smear a little blood, obtained by pricking the finger, on a slide, and allow it to dry; cover, and run glacial acetic acid under the cover slip, and boil. Examine microscopically for the dark brown crystals of hæmin.

8. **CHEMICAL TESTS FOR BLOOD.**—*Guaiacum Test.*—Take some tincture of guaiacum, and add a small quantity of blood to it; add to the mixture a little hydrogen peroxide (or most specimens of commercial turpentine will do as well) and a blue colour is developed. This test is due to the iron-containing radical in hæmoglobin, and is given even after the blood has been previously boiled: repeat the test using some boiled blood.

9. *Adler's Test.*—Take half a test-tube of diluted blood (so dilute as to be practically colourless); add a few drops of benzidine dissolved in glacial acetic acid, and a few drops of pure hydrogen peroxide: a blue colour develops immediately. Spectroscopically, this blue solution shows an absorption band in the yellow. This test is far more delicate than the guaiacum test, but its intensity is lessened if the blood has been previously boiled. It is a very convenient test for blood in urine.

COAGULATION OF BLOOD

Microscopic investigation of vertebrate blood shows it to consist of a fluid (the blood plasma or liquor sanguinis) which holds in suspension large numbers of corpuscles; the corpuscles are red or coloured (erythrocytes); white or colourless (leucocytes), and the blood platelets.

After blood is shed it rapidly becomes viscous and then sets into a firm red jelly. The jelly soon contracts and squeezes out a straw-coloured fluid called the serum, in which the shrunken clot ultimately floats. With the microscope, filaments of fibrin are seen forming a network throughout the fluid, many radiating from small clumps of blood platelets. It is the formation of fibrin which is the essential act of coagulation: this entangles the corpuscles and forms the clot. Fibrin is formed from the plasma, and may be obtained free from corpuscles when blood plasma is allowed to clot, the corpuscles having previously been removed. It may also be obtained from blood by whipping it with a bunch of twigs; the fibrin adheres to the twigs and entangles but few corpuscles. These may be removed by washing with water. Serum is plasma *minus* the fibrin which it yields. The relation of plasma, serum, and clot can be seen at a glance in the following scheme of the constituents of the blood :—

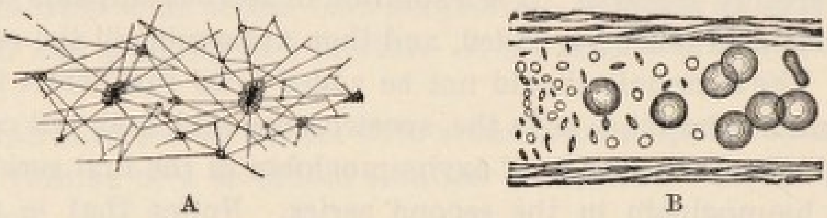


FIG. 22.—Fibrin filaments and blood platelets: A, network of fibrin shown after washing away the corpuscles from a preparation ol blood that has been allowed to clot. Many of the filaments radiate from small clumps of blood platelets. B (from Osler), blood corpuscles and blood platelets within a small vein.

$$\text{Blood}\left\{\begin{array}{l}\text{Plasma}\left\{\begin{array}{l}\text{Serum}\\ \text{Fibrin}\end{array}\right.\\ \text{Corpuscles}\end{array}\right.\Big\}\text{Clot}$$

It may be roughly stated that in 100 parts by weight of blood 60–65 parts consist of plasma and 35–40 of corpuscles.

The *buffy coat* is seen when blood coagulates slowly, as in horse's blood. The red corpuscles sink more rapidly than the white, and the upper stratum of the clot (buffy coat) consists mainly of fibrin and white corpuscles.

Coagulation is hastened by—

1. A temperature a little over that of the body.
2. Contact with foreign matter.

3. Injury to the vessel walls.

4. Agitation.

5. Addition of calcium salts.

6. Injection of nucleo-protein produces intravascular clotting (positive phase). Very minute doses, however, produce the opposite effect: namely, delay of coagulation (negative effect).

Coagulation is hindered or prevented by—

1. A low temperature. In a vessel cooled by ice, coagulation may be prevented for an hour or more.

2. The addition of a large quantity of neutral salts, such as sodium sulphate or magnesium sulphate.

3. Addition of a soluble oxalate, fluoride or citrate.

4. Injection of commercial peptone (which consists chiefly of proteoses) into the circulation of the living animal.

5. Addition of leech extract to the blood, or injection of leech extract into the circulation while the animal is alive.

6. Contact with the living vascular walls.

7. Contact with oil.

The cause of the coagulation of the blood may be briefly stated as follows:—

When blood is within the vessels one of the constituents of the plasma, a protein of the globulin class called *fibrinogen*, exists in a soluble form.

When the blood is shed the fibrinogen molecule is altered in such a way that it gives rise to the comparatively insoluble material *fibrin*. The statement has been made that the fibrinogen molecule is split into two parts: one part is a globulin (fibrinoglobulin), which remains in solution; the other and larger part is the insoluble substance fibrin. It is, however, doubtful if this really represents what occurs, for recent work seems to show that the fibrinoglobulin is not a product of fibrinogen, but exists in the blood plasma beforehand. At any rate, whether this is so or not, the fact remains that fibrin is the important product and the only one which need concern us.

The next question is, What causes the transformation of fibrinogen into fibrin? And the answer to that is, that the change is due to the activity of a special enzyme called *fibrin-ferment* or *thrombin*.

This enzyme does not exist in healthy blood contained in healthy blood-vessels, but is formed by the *disintegration* of the blood platelets and colourless corpuscles which occurs when the blood leaves the blood-vessels or comes into contact with foreign matter. Hence the blood does not coagulate during life. But, it will be said, disintegration of the blood corpuscles occurs during life; why, then, does the blood not coagulate? The reason is that although the formed elements do disintegrate in the living blood, such a phenomenon takes place very slowly and gradually, so that there can never in normal circumstances be any massive liberation of fibrin-ferment, and, further, that there are agencies at work to neutralise the fibrin-ferment as it is formed. The most noteworthy of these neutralising agencies is the presence in the blood of an anti-enzyme called *antithrombin*, analogous to the antipepsin and antitrypsin which, we have seen, are efficacious in preventing the stomach and intestines from undergoing self-digestion.

Nucleo-proteins (see pp. 62–65) obtained from most of the cellular organs of the body produce intravascular clotting when injected into the circulation of the living animal. In certain diseased conditions intravascular clotting, or thrombosis, sometimes occurs. This must be due to the entrance of nucleo-protein into the circulation from disintegrated cells. Nucleo-protein and thrombin are probably not identical; but it is possible that the nucleo-protein may hold in loose combination or admixture another material called thrombo-kinase, which we shall be considering immediately.

Thrombin is believed to originate chiefly from the blood platelets and in part from the leucocytes. Birds' blood clots very slowly, and the absence of blood platelets in this variety of blood will in part account for this. Lymph which contains colourless corpuscles, but no platelets, also clots, so in this case the colourless corpuscles must be the source of the enzyme. One should, however, be careful in speaking of the disintegration of leucocytes to remember that the word "disintegration" does not mean complete breakdown, leading to disappearance; the colourless corpuscles do not appreciably diminish in number when the blood is shed, but what occurs in the surviving leucocytes is a shedding out of certain products, among which fibrin-ferment is one.

We have now traced fibrin-formation, the essential cause of blood-clotting, to the activity of thrombin; it is next necessary to allude to what has been discovered in relation to the origin of thrombin. Like other enzymes, it is preceded by a mother substance or zymogen. This zymogen is called prothrombin, or *thrombogen*, and there appear to be two necessary agents concerned in the conversion of thrombogen into thrombin: one of these is the action of *calcium salts*, the other is the presence of an activating agent, analogous to entero-kinase (see p. 90), and called *thrombo-kinase*. The exact rôle played by each is still a matter of speculation.

The part played by calcium salts is well illustrated by the fact that coagulation is prevented by the decalcification of the blood. This can be accomplished by the addition of a small amount of soluble oxalate or fluoride to the blood immediately it is shed. The calcium of the blood plasma is then immediately precipitated as insoluble calcium oxalate or fluoride, and is thus not available for the transformation of thrombogen into thrombin. The addition of the oxalate or fluoride must be rapidly performed, otherwise time will be given for the conversion of thrombogen into thrombin, and thrombin, when formed, will act upon fibrinogen whether the calcium has been removed or not. In other words, calcium is only necessary for the formation of fibrin-ferment, and not for the action of fibrin-ferment on fibrinogen. Fibrin is thus not a compound of calcium and fibrinogen.

The action of a soluble citrate is also in a certain sense a decalcifying action, for although calcium citrate is a soluble salt it does not ionise in solution so as to liberate the free calcium ions which are essential for thrombin formation.

Oxalated blood (or oxalated plasma) will clot when calcium is once more restored by the addition of a small amount of calcium chloride, but such an addition to fluoride plasma will not induce clotting; thrombin must be added also. In some way sodium fluoride interferes with the formation of thrombin, probably by preventing the liberation of thrombo-kinase from the formed elements of the blood.

The other activating agent, thrombo-kinase, is in part liberated from the formed elements of the blood, but it is also obtained from many other tissues. If a hæmorrhage takes place under ordinary conditions the blood as it flows from the wound passes over the muscles and skin which have been cut, and rapidly clots owing to the thrombo-kinase supplied by those tissues. If blood is obtained by drawing it off through a perfectly clean cannula into a clean vessel without allowing it to touch the tissues, it remains unclotted for a long time; in the case of birds' blood this time may extend to many days; but the addition of a small piece of tissue such as muscle, or of an extract of such a tissue, produces almost immediate clotting. If a solution of fibrinogen is prepared and calcium added it will not clot; if thrombin or a fluid such as serum which contains thrombin is added also it will clot. It will not clot if birds' plasma obtained as above is added to it; nor if tissue extract is added to it; but if both are added it will. In other words, the thrombogen of the birds' plasma *plus* the thrombo-kinase of the tissue extract have the same effect as thrombin.

The next point to consider is why blood obtained after the previous injection of proteoses (or commercial peptone) into the circulation should not clot. It certainly contains calcium salts, and probably both thrombogen and thrombo-kinase, for it can be made to clot without the addition of either; for instance, by dilution or the passage of a stream of carbon dioxide through it. There must be something in peptone blood which antagonises the action of thrombin. This something is an excess of antithrombin. Peptone will not hinder blood coagulation, or only very slightly if it is added to the blood after it is shed. The antithrombin must therefore have been added to the blood while it was circulating in the body. We can even go further than this and say what part of the body it is which is concerned in the production of antithrombin: it is the liver, for if the liver is shut off from the circulation, peptone is ineffective in its action. The converse experiment confirms this conclusion, for if a solution of peptone is artificially perfused through an excised surviving liver, a substance is formed which has the power of hindering or preventing the coagulation of shed blood.

We are thus justified in two conclusions:—

(1) That the antithrombin which is normally present in healthy blood in sufficient quantities to prevent intravascular clotting, is formed in the liver.

(2) That commercial peptone in virtue of the proteoses it contains stimulates this action of the liver to such an extraordinary degree, that the accumulation of antithrombin in the blood becomes sufficiently great to prevent the blood from clotting even after it is shed.

It should be noted, however, that this effect upon the liver varies in different animals, and is most marked in the dog. Peptone blood is very poor in leucocytes, but the cause of their disappearance is not clear.

We shall conclude by considering only one more of the hindrances to coagulation, and that by no means the least interesting. The leech lives by sucking the blood of other animals; from the leech's point of view it is therefore necessary that the blood should flow freely and not clot. The glands at the head end of the leech, often spoken of roughly as the salivary glands, secrete something which hinders the blood from coagulating, and everyone who has been treated by leeches knows by experience how difficult it is to prevent a leech-bite from bleeding after the leech has been removed; complete cleansing is necessary to wash away the leech's secretion from the wound. Now if an extract of leeches' heads is made with salt solution and filtered, that fluid will prevent coagulation, whether it is injected into the blood-stream or added to shed blood. The substance in the extract is called *hirudin*, and this is believed to be antithrombin itself.

We may summarise our present knowledge of the causes of coagulation in the following tabular way:—

From the platelets, and to a lesser degree from the leucocytes, a material is shed out called—

THROMBOGEN.

From the formed elements of the blood, but also from the tissues over which the escaping blood flows, is shed out an activating agent called

THROMBO-KINASE.

In the blood plasma a protein substance exists called

FIBRINOGEN.

In the presence of CALCIUM salts, thrombo-kinase activates thrombogen in such a way that an active enzyme is produced, which is called

THROMBIN.

Thrombin or fibrin-ferment acts on fibrinogen in such a way that it is transformed into the insoluble stringy material which is called

FIBRIN.

The views enumerated in the preceding paragraphs are in the main those of Morawitz. Howell agrees that the lack of coagulation seen in birds' blood and peptone blood is due to excess of antithrombin, but holds that thrombo-kinase (or thrombo-plastin, as he terms it) brings about clotting not by activating thrombogen, but by neutralising antithrombin. He further thinks that thrombo-plastin is a lipoid.

THE PLASMA AND SERUM

The liquid in which the corpuscles float may be obtained by employing one or other of the methods already described for preventing the blood from coagulating. The corpuscles, being heavy, sink, and the supernatant plasma can then be removed by a pipette or siphon; the separation can be effected more thoroughly and rapidly by the use of a centrifugal machine.

On counteracting the influence which has prevented the blood from coagulating, the plasma then itself coagulates. The plasma obtained by the use of cold, clots on warming gently; plasma which has been decalcified by the action of soluble oxalate clots on the addition of a calcium salt; plasma obtained by the use of a strong solution of salt coagulates when this is diluted by the addition of water, the addition of thrombin being necessary in most cases; where coagulation occurs without the addition of thrombin, no doubt some is present from the partial disintegration of the corpuscles which has already occurred. Pericardial and hydrocele fluids resemble pure plasma very closely in composition. As a rule, however, they contain few or no white corpuscles, and do not clot spontaneously, but after the addition of thrombin or liquids such as serum which contain thrombin they always yield fibrin.

Pure plasma may be obtained from horse's veins by what is known as the "living test-tube" experiment. If the jugular vein is ligatured in two places, so as to include a quantity of blood within it, then removed from the animal and hung in a cool place, the blood will not coagulate for many hours. The corpuscles settle, and the supernatant plasma can be removed with a pipette.

The plasma is alkaline, yellowish in tint, and its specific gravity is about 1026 to 1029.

Its chief constituents may be enumerated as follows:—

1000 parts of plasma contain—

Water		902·90
Solids		97·10
Proteins:	1, yield of fibrin	4·05
	2, other proteins	78·84
Extractives (including fat)		5·66
Inorganic salts		8·55

In round numbers plasma contains 10 per cent. of solids, of which 8 are protein in nature.

Serum contains the same three classes of constituents—proteins, extractives, and salts. The extractives and salts are the same in the two liquids. The proteins differ, as is shown in the following table:—

Proteins of Plasma.	*Proteins of Serum.*
Fibrinogen.	Serum globulin.
Serum globulin.	Serum albumin.
Serum albumin.	Thrombin.

The gases of the plasma and serum are small quantities of oxygen, nitrogen, and carbonic acid. The greater part of the oxygen of the blood is combined in the red corpuscles with hæmoglobin; the carbonic acid is chiefly combined as carbonates (see RESPIRATION).

We may now consider one by one the various constituents of the plasma and serum.

A. **Proteins.**—*Fibrinogen.*—This is the parent substance of fibrin. It is a globulin. It differs from serum globulin, and may be separated from it by the fact that half saturation with sodium chloride precipitates it. It coagulates by heat at the low temperature of 56° C. As judged from the yield of fibrin, it is the *least* abundant of the proteins of the plasma (see table on p. 142).

Serum Globulin and Serum Albumin.—These substances, which are typical of the globulin and albumin groups of proteins, are considered in the practical exercises at the head of this lesson; see also Lesson V, p. 59. Both serum globulin and serum albumin probably consist of more than one protein substance (see practical exercises *f* and *g* in to-day's lesson).

Thrombin.—Schmidt's method of preparing it is to take serum and add excess of alcohol. This precipitates all the proteins, and also thrombin. After some weeks the alcohol is poured off; the serum globulin and serum albumin have been by this means rendered insoluble in water; an aqueous extract is, however, found to contain thrombin, which is not so easily coagulated by alcohol as the proteins are. A simpler method of preparing fibrin ferment in an impure but efficient form is given in the footnote on p. 133.

B. **Extractives.**—These are non-nitrogenous and nitrogenous. The non-nitrogenous are sugar (0·12 per cent.), fats, soaps, cholesterol and cholesterol esters; and the nitrogenous are urea (0·02 to 0·04 per cent.) and still smaller quantities of uric acid, creatine, creatinine, xanthine, hypoxanthine, and amino-acids.

C. **Salts.**—The most abundant salt is sodium chloride; it constitutes between 60 and 90 per cent. of the total mineral matter. Potassium chloride is present in much smaller amount. It constitutes about 4 per cent. of the total ash. The other salts are phosphates and sulphates.

Schmidt gives the following table :—

1000 parts of plasma yield—

Mineral matter	8·550
Chlorine	3·640
SO_3	0·115
P_2O_5	0·191
Potassium	0·323
Sodium	3·341
Calcium phosphate	0·311
Magnesium phosphate	0·222

THE WHITE BLOOD CORPUSCLES

These corpuscles are typical animal cells. Their nucleus consists of nuclein; their cell-protoplasm yields protein belonging to the nucleo-protein and globulin groups. The protoplasm of these cells also contains small quantities of fat, lipoids, and glycogen.

THE RED BLOOD CORPUSCLES

The red blood corpuscles are much more numerous than the white, averaging in man 5,000,000 per cubic millimetre, or 400 to 500 red to each white corpuscle. The method of enumeration of the corpuscles is described in the Appendix.

They vary in size and structure in different groups of vertebrates. In mammals they are biconcave (except in the camel tribe, where they are biconvex) non-nucleated discs, in man averaging $\frac{1}{3200}$ inch in diameter; during fœtal life nucleated red corpuscles are, however, found. In birds, reptiles, amphibians, and fishes they are biconvex oval discs with a nucleus: they are largest in the amphibia.

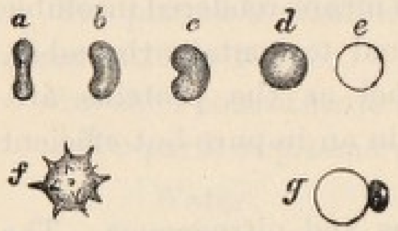


FIG. 23.—*a–e*, successive effects of water on a red blood corpuscle; *f*, a red corpuscle crenated by salt solution; *g*, action of tannin on a red corpuscle.

Water causes the corpuscles to swell up, and dissolves out the red pigment (oxyhæmoglobin), leaving a globular colourless membrane or stroma. This is termed laking the blood. Laking or *hæmolysis* is also produced by soaps, bile salts, and other substances. *Strong salt solution* causes the corpuscles to shrink: they become crenated or wrinkled. The action of water and salt solution is explained by the existence of a membrane on the surface of the corpuscles through which osmosis takes place. *Physiological salt solution* (0·9 per-cent. sodium chloride) produces no change as it has the same osmotic pressure as blood plasma. *Dilute alkalis* (0·2 per-cent. potash) dissolve the corpuscles. *Dilute acids* (1 per-cent. acetic acid) act like water, and in nucleated corpuscles render the nucleus distinct. *Tannic acid* causes a discharge of hæmoglobin from the stroma, but this is immediately altered and precipitated. It remains adherent to the stroma as a brown globule, consisting probably of hæmatin. *Boric acid* acts similarly, but in nucleated red corpuscles the pigment collects chiefly round the nucleus, which may then be extruded from the corpuscles.

Composition.—1000 parts of red corpuscles contain—

Water		688	parts
Solids	organic	303·88	„
	inorganic	8·12	„

100 parts of dried corpuscles contain—

Protein	5 to 12	parts
Hæmoglobin	86 „ 94	„
Phosphatides calculated as lecithin	1·8	part
Cholesterol	0·1	„

The protein present appears to be identical with the nucleo-protein of white corpuscles. The mineral matter consists chiefly of chlorides of potassium and sodium, and phosphates of calcium and magnesium. In most animals, including man, potassium chloride is more abundant than sodium chloride.

Oxygen is contained in combination with the hæmoglobin to form oxyhæmoglobin. The corpuscles also contain a certain amount of carbonic acid (see RESPIRATION, at the end of this lesson).

The Pigment of the Red Corpuscles.—The pigment is by far the most abundant and important of the constituents of the red corpuscles. It differs from most other proteins in containing the element iron; it is also readily crystallisable.

It exists in the blood in two conditions: in arterial blood it is combined loosely with oxygen, is of a bright red colour, and is called oxyhæmoglobin; the other condition is the deoxygenated or reduced hæmoglobin (better called simply hæmoglobin). This is found in the blood after asphyxia. It also occurs in all venous blood—that is, blood which is returning to the heart after it has supplied the tissues with oxygen. Venous blood, however, always contains a considerable quantity of oxyhæmoglobin also. Hæmoglobin is the oxygen-carrier of the body, and it may be called a respiratory pigment.

Crystals of oxyhæmoglobin may be obtained with readiness from the blood of such animals as the rat, guinea-pig, or dog; with difficulty from other animals, such as man, ape, and most of the common mammals. The following methods are the best:—

1. Mix a drop of defibrinated blood of the rat on a slide with a drop of water; put on a cover glass; in a few minutes the corpuscles are rendered colourless, and then the oxyhæmoglobin crystallises out from the solution so formed.

2. Microscopical preparations may also be made by Stein's method,

which consists in using Canada balsam instead of water in the above experiment.

3. On a larger scale the crystals may be obtained by laking the blood by shaking it with one-sixteenth of its volume of ether. After a period, varying from a few minutes to days, abundant crystals are deposited. The laking of blood by ether and similar reagents is due to their solvent effects on the lipoids of the cell membrane. The accompanying illustrations (fig. 24) represent the form of the crystals so obtained.

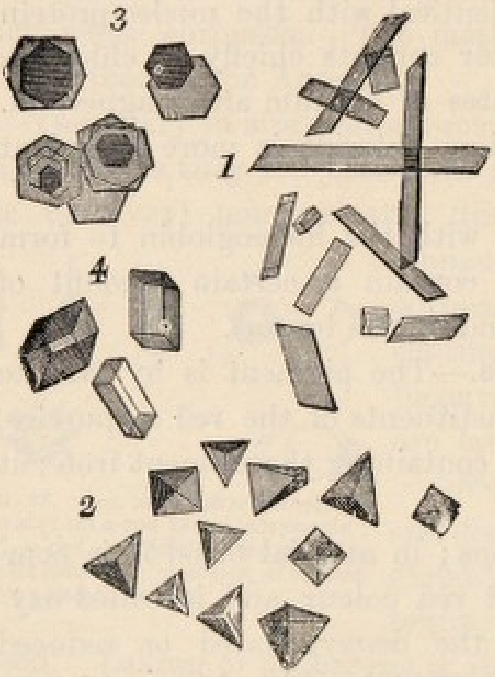


FIG. 24.—Oxyhæmoglobin crystals magnified: 1, from human blood; 2, from the guinea-pig; 3, squirrel; 4, hamster.

In nearly all animals the crystals are rhombic prisms; but in the guinea-pig they are rhombic tetrahedra (four-sided pyramids); in the squirrel, hexagonal plates; and in the hamster, rhombohedra and hexagonal plates.

The crystals also contain a varying amount of water of crystallisation: this may explain their different crystalline forms and solubilities. The varying form of the crystals does not therefore prove that the hæmoglobin of different animals has a different composition, for it has been shown that the crystals of one form can be transformed on recrystallisation into those of another form and then back again into the original form. The crystalline form of hæmoglobin is, however, no guarantee of the purity of the substance, for after many recrystallisations, although the crystalline form remains unaltered, the cleavage products obtained on hydrolysis are different; for instance, no glycine is found after many recrystallisations (Abderhalden). The hæmoglobin molecule contains carbon, hydrogen, nitrogen, oxygen, sulphur, and iron. The percentage of iron is about 0·4, but varies in different preparations. Oxyhæmoglobin may be estimated in the blood (1) by the amount of iron in the ash, or (2) by certain colorimetric methods which are described in the Appendix.

Hæmoglobin is a conjugated protein, and on the addition of an acid or alkali it is broken up into two parts, a protein called *globin*, and a brown pigment called *hæmatin*, which contains all the iron of the original substance.

Globin is coagulable by heat, soluble in dilute acids, and precipitable from such solutions by ammonia. It is a member of the group of proteins called *histones* (see p. 59).

Hæmatin is not crystallisable: its formula is $C_{34}H_{33 \text{ or } 35}O_5N_4Fe$. It presents different spectroscopic appearances in acid and alkaline solutions, and yields several products under the influence of certain reagents. We shall mention only four of these: hæmochromogen, hæmin, hæmatoporphyrin, and hæmopyrrol.

Hæmochromogen or reduced hæmatin is obtained by adding a reducing agent to an alkaline solution of hæmatin; it has recently been obtained in crystalline form. Its absorption spectrum forms the most delicate spectroscopic test for blood pigment.

Hæmin is of great importance, as the obtaining of this substance in a crystalline form is the best chemical test for blood. Hæmin crystals, sometimes called Teichmann's crystals, are prepared for microscopic examination by boiling a fragment of dried blood with a drop of glacial acetic acid on a slide; on cooling, dark-brown plates and prisms belonging to the triclinic system, often in star-shaped clusters and with rounded angles (fig. 25), separate out.

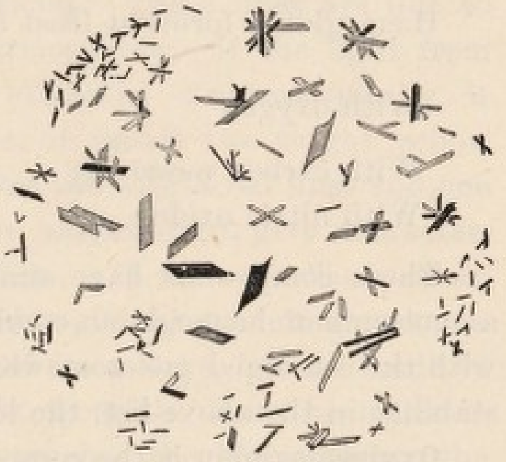
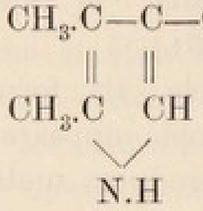


FIG. 25.—Hæmin crystals magnified. (Preyer.)

In the case of an old blood-stain it is necessary to add a crystal of sodium chloride. Fresh blood contains sufficient sodium chloride in itself. The action of the acetic acid is to split the hæmoglobin into hæmatin and globin. A hydroxyl group of the hæmatin is then replaced by chlorine. It is similarly easily replaceable by an atom of bromine or iodine. Nencki has further shown that, when prepared in this way, hæmin also contains the acetyl group. It has the empirical formula $C_{34}H_{32 \text{ or } 34}O_4N_4FeCl$.

Hæmatoporphyrin ($C_{34}H_{38}O_6N_4$) is iron-free hæmatin: it may be prepared by mixing blood with strong sulphuric acid; the iron is taken out as ferrous sulphate. This substance is also found sometimes in nature; it occurs in certain invertebrate pigments, and may also be found in certain forms of pathological urine. Even normal urine contains traces of it. It shows well-marked spectroscopic bands, and so is not identical with the iron-free derivative of hæmoglobin called hæmatoidin which is formed in extravasations of blood in the body (see p. 121).

Hæmopyrrol is dimethyl-ethyl-pyrrol, with the formula



and is obtained by reduction from hæmatoporphyrin. It is also similarly obtained from the derivative of chlorophyll called phylloporphyrin, a fact which illustrates the near relationship of the principal animal and vegetable pigments.

COMPOUNDS OF HÆMOGLOBIN WITH GASES

Hæmoglobin forms at least four compounds with gases :—

With oxygen	1. Oxyhæmoglobin. 2. Methæmoglobin.
With carbon monoxide	3. Carbonic oxide hæmoglobin.
With nitric oxide	4. Nitric oxide hæmoglobin.

These compounds have similar crystalline forms: each consists of a molecule of hæmoglobin combined with one of the gas. They part with the combined gas somewhat readily, and are arranged in order of stability in the above list, the least stable first.

Oxyhæmoglobin is the compound that exists in arterial blood. The oxygen linked to the hæmoglobin, which is removed by the tissues through which the blood circulates, may be called the *respiratory oxygen of hæmoglobin.* The processes that occur in the lungs and tissues, resulting in the oxygenation and deoxygenation respectively of the hæmoglobin, may be imitated outside the body, using either blood or pure solutions of hæmoglobin. The respiratory oxygen can be removed, for example, in the Torricellian vacuum of a mercurial air-pump, or by passing a neutral gas such as hydrogen through the blood or by the use of reducing agents such as ammonium sulphide or Stokes's reagent (an ammoniacal solution of ferrous tartrate). One gramme of hæmoglobin will combine with 1·34 c.c. of oxygen.

If any of these methods for reducing oxyhæmoglobin is used, the bright red (arterial) colour of oxyhæmoglobin changes to the purplish (venous) tint of hæmoglobin. On once more allowing oxygen to come into contact with the hæmoglobin, as by shaking the solution with the air, the bright arterial colour returns.

These colour-changes may be more accurately studied with the spectroscope, and the constant position of the absorption bands seen constitutes an important test for blood pigment.

The Spectroscope.—When a ray of white light is passed through a prism, it is refracted or bent at each surface of the prism; the whole ray is, however, not equally bent, but it is split into its constituent colours, which may be allowed to fall on a screen. The band of colours beginning with the red, passing through orange, yellow, green, blue, and ending with violet, is called a *spectrum*: this is seen in nature in the rainbow.

The spectrum of sunlight is interrupted by numerous dark lines crossing it vertically called Fraunhofer's lines. These are perfectly constant in position, and serve as landmarks in the spectrum. The most prominent are A, B, and C, in the red; D, in the yellow; E, *b*, and F, in the green; G and H, in the violet. These lines are due to certain volatile substances in the solar atmosphere. If the light from burning sodium or its compounds is examined spectroscopically, it will be found to give a bright yellow line, or rather two bright yellow lines very close together. Potassium gives two bright red lines and one violet line; and the other elements, when incandescent, give characteristic lines, but none so simple as sodium. If now the flame of an ordinary lamp be examined, it will be found to give a continuous spectrum like that of sunlight in the arrangement of its colours, but unlike it in the absence of dark lines; but if the light from the lamp be made to pass through sodium vapour before it reaches the spectroscope, the bright yellow light will be found absent, and in its place a dark line, or rather two dark lines very close together, occupy the same position as the two bright lines of the sodium spectrum. The sodium vapour thus absorbs the same rays as those which it itself produces at a higher temperature. Thus the D line, as we term it, in the solar spectrum is due to the presence of sodium vapour in the solar atmosphere. The other dark lines are similarly accounted for by other elements.

The large form of spectroscope (fig. 26) consists of a tube A, called the collimator, with a slit at the end S, and a convex lens at the end L. The latter makes the rays of light passing through the slit from the source of light parallel: they fall on the prism P, and then the spectrum so formed is focussed by the telescope T.

A third tube, not shown in the figure, carries a small transparent scale of wave-lengths, so that the position of any point in the spectrum may be given in terms of the corresponding wave-lengths.

If we now interpose between the source of light and the slit S a piece of coloured glass (H in fig. 26), or a solution of a coloured substance contained in a vessel with parallel sides, the spectrum is found to be no longer continuous, but is interrupted by a number of dark shadows, or *absorption bands*, corresponding to the light absorbed by the coloured medium. Thus a solution of oxyhæmoglobin of a certain strength gives two bands between the D and E lines; hæmoglobin gives only one; and other red solutions, though to the naked eye similar to oxyhæmoglobin, will give characteristic bands in other positions.

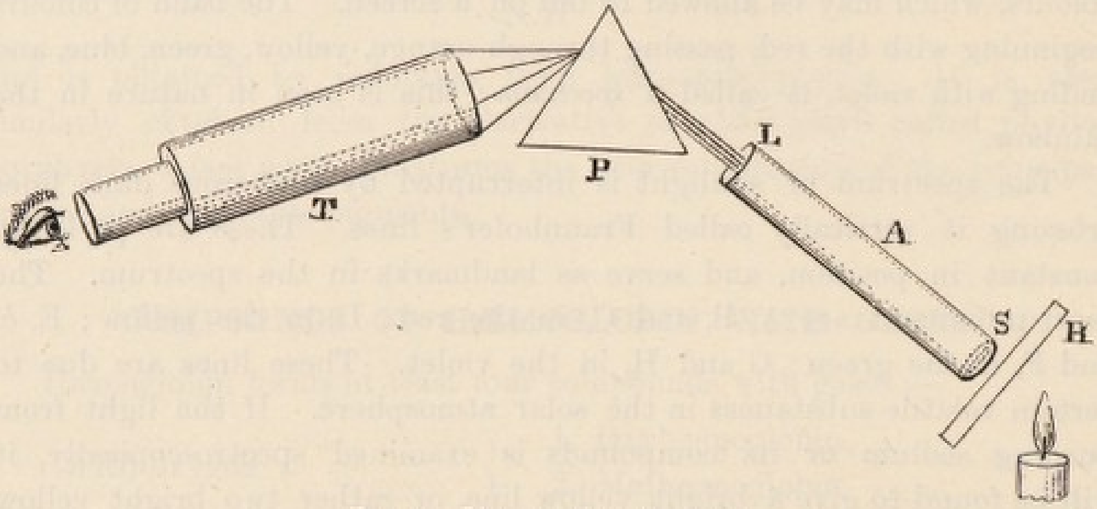


FIG. 26.—Diagram of spectroscope.

A convenient form of small spectroscope is the *direct-vision spectroscope*, in which, by an arrangement of alternating prisms of crown and flint glass (see fig. 27), the spectrum is observed by the eye in the same line as the tube furnished with the slit. Such small spectroscopes may for convenience be mounted on a stand provided with a gas-burner and a receptacle for the test-tube (see fig. 28). In the examination of the spectrum of small coloured objects, a combination of the microscope and direct-vision spectroscope, called the *micro-spectroscope*, is used.

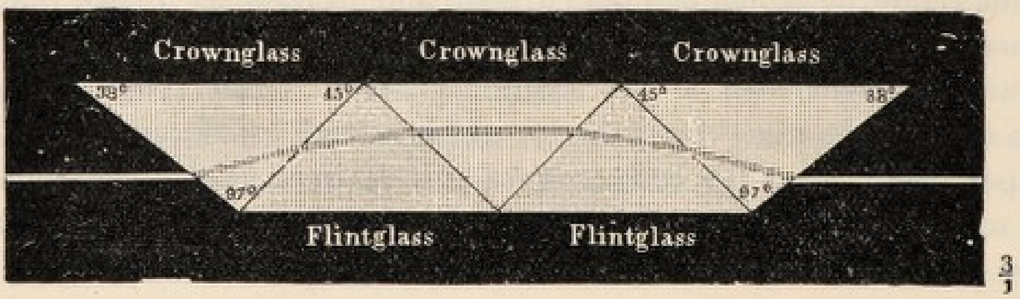


FIG. 27.—Arrangement of prisms in direct-vision spectroscope.

Fig. 29 illustrates a method of representing absorption spectra diagrammatically. The solution was examined in a layer 1 centimetre thick. The base line has on it at the proper distances the chief Fraunhofer lines, and along the right-hand edges are the percentage amounts of oxyhæmoglobin present in I, of hæmoglobin in II. The width of the

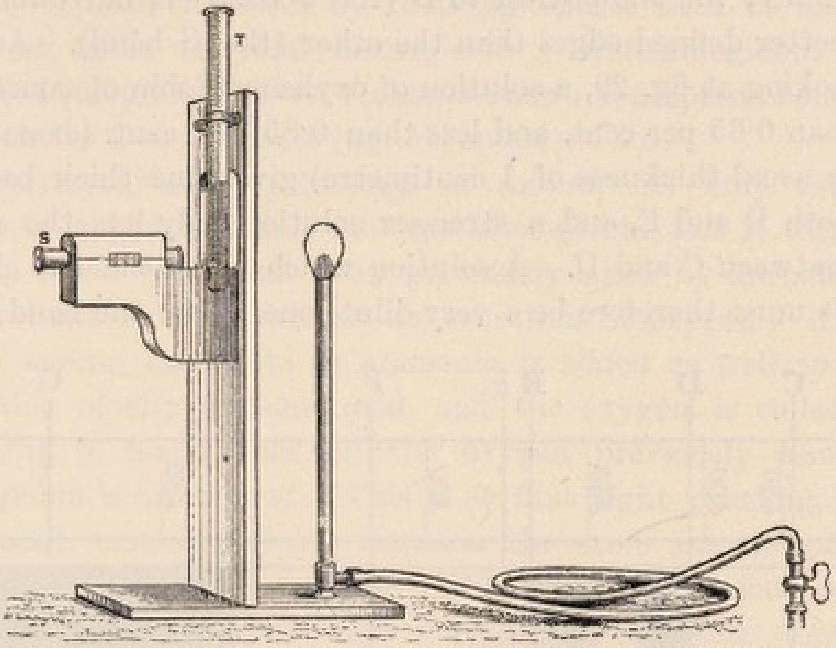


FIG. 28.—Stand for direct-vision spectroscope: S, spectroscope; T, test-tube for coloured substance under investigation.

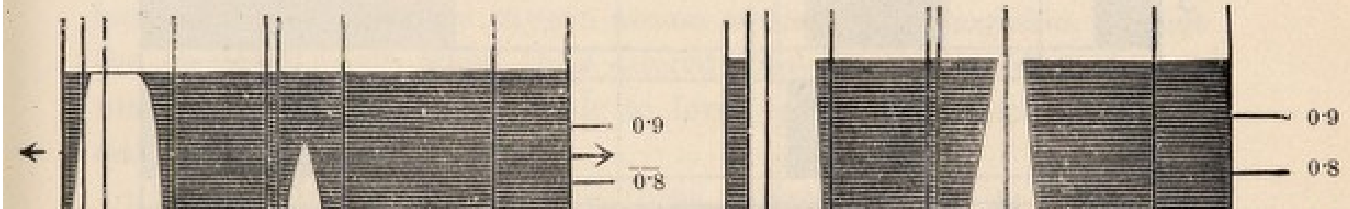


FIG. 29.—Graphic representations of the amount of absorption of light by solution (I) of oxyhæmoglobin; (II) of hæmoglobin, of different strengths. The shading indicates the amount of absorption of the spectrum; the figures on the right border express percentages. (Rollett.)

shadings at each level represents the position and amount of absorption corresponding to the percentages.

The characteristic spectrum of oxyhæmoglobin, as it actually appears through the spectroscope, is seen in the next figure (fig. 30, spectrum 2). There are two distinct absorption bands between the D and E lines; the one nearest to D (the α band) is narrower, darker, and has better defined edges than the other (the β band). As will be seen on looking at fig. 29, a solution of oxyhæmoglobin of concentration greater than 0·65 per cent. and less than 0·85 per cent. (examined in a cell of the usual thickness of 1 centimetre) gives one thick band overlapping both D and E, and a stronger solution only lets the red light through between C and D. A solution which gives the two characteristic bands must therefore be a very dilute one. The one band (γ band) of hæmoglobin (fig. 30, spectrum 3) is not so well defined as the α and β bands. On dilution it fades rapidly, so that in a solution of such a strength that both bands of oxyhæmoglobin would be quite distinct the single band of hæmoglobin has disappeared from view. The oxyhæmoglobin bands can be distinguished in a solution which contains only one part of the pigment to 10,000 of water, and even in more dilute solutions which seem to be colourless the α band is still visible.

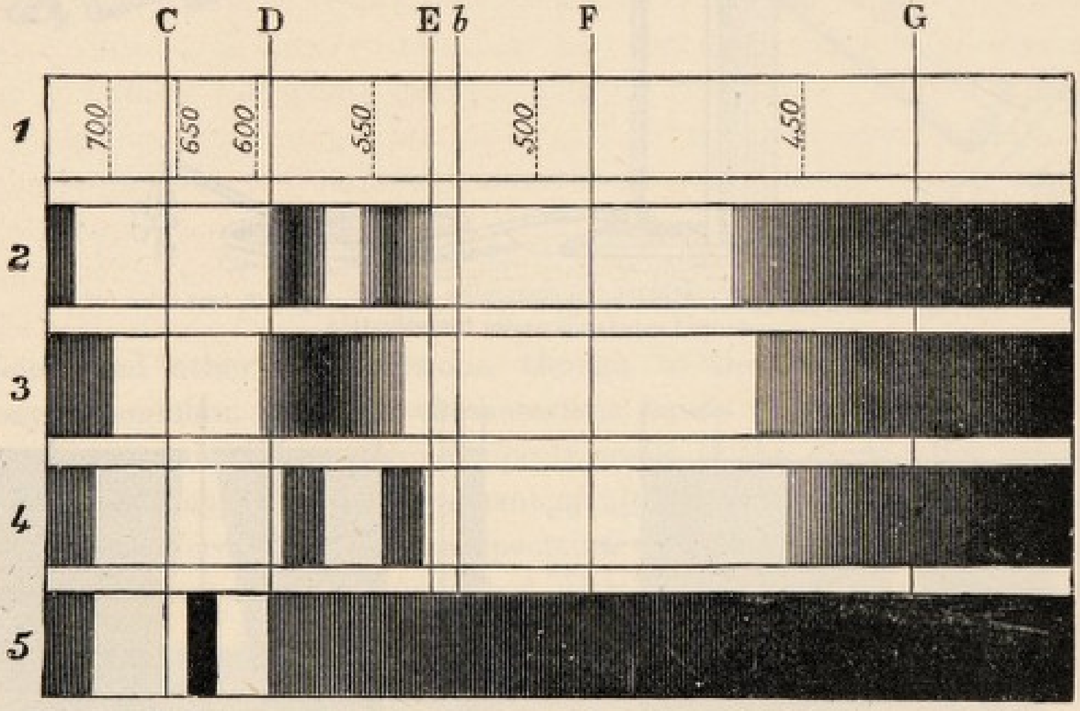


FIG. 30.—1, Solar spectrum; 2, spectrum of oxyhæmoglobin (0·37 per cent. solution); 3, spectrum of hæmoglobin; 4, spectrum of CO-hæmoglobin; 5, spectrum of methæmoglobin (concentrated solution).

Methæmoglobin.—This may be produced artificially by adding such reagents as potassium ferricyanide or amyl nitrite to a solution of oxyhæmoglobin; it may also occur in certain diseased conditions in the urine; it is therefore of considerable practical importance. It can be crystallised, and is found to contain the same amount of oxygen as oxyhæmoglobin, only combined differently. The oxygen is not removable by the air-pump, nor by a stream of a neutral gas such as hydrogen. It can, however, by reducing agents such as ammonium sulphide, be made to yield hæmoglobin. Methæmoglobin is of a brownish-red colour, and gives a characteristic absorption band in the red between the C and D lines (fig. 30, spectrum 5).

Bac

The ferricyanide of potassium or sodium not only causes the conversion of oxyhæmoglobin into methæmoglobin, but if the reagent is added to blood which has been previously laked by the addition of twice its volume of water there is an evolution of oxygen. If a small amount of sodium carbonate or ammonia is added as well to prevent the evolution of any carbonic acid, and the oxygen is collected and measured, it is found that all the oxygen previously combined in oxyhæmoglobin is discharged. This is at first sight puzzling, because, as just stated, methæmoglobin contains the same amount of oxygen that is present in oxyhæmoglobin What occurs is that, after the oxygen is discharged from oxyhæmoglobin, an equal quantity of oxygen takes its place from the reagents added. The oxygen atoms of the methæmoglobin must be attached to a different part of the hæmatin group from the oxygen atoms of the oxyhæmoglobin, so that the hæmatin group when thus altered loses its power of combining with oxygen and carbonic oxide to form compounds which are dissociable in a vacuum.

Haldane, to whom we owe these interesting results, gives the following provisional equation to represent what occurs :—

$$\underset{\substack{\text{[oxyhæmo-}\\\text{globin]}}}{HbO_2} + \underset{\substack{\text{[sodium ferri-}\\\text{cyanide]}}}{4Na_3Cy_6Fe} + \underset{\substack{\text{[sodium bicar-}\\\text{bonate]}}}{4NaHCO_3} = \underset{\substack{\text{[methæmo-}\\\text{globin]}}}{HbO_2} + \underset{\substack{\text{[sodium ferro-}\\\text{cyanide]}}}{4Na_4Cy_6Fe}$$

$$+ \underset{\substack{\text{[carbonic}\\\text{acid]}}}{4CO_2} + \underset{\text{[water]}}{2H_2O} + \underset{\text{[oxygen]}}{O_2}$$

Carbonic Oxide Hæmoglobin may be readily prepared by passing a stream of carbonic oxide or coal gas through blood or through a solution of oxyhæmoglobin. It has a peculiar cherry-red colour. Its absorption spectrum is very like that of oxyhæmoglobin, but the two bands are slightly nearer the violet end of the spectrum (fig. 30, spectrum 4). Reducing agents, such as ammonium sulphide, do not change it ; the gas is more firmly combined than the oxygen in oxyhæmoglobin. CO-hæmoglobin forms crystals like those of oxyhæmoglobin : it resists putrefaction for a very long time.

Carbonic oxide is given off during the imperfect combustion of carbon such as occurs in charcoal stoves ; it is a powerful poison combining with the hæmoglobin of the blood, and thus interfering with normal respiratory processes. The colour of the blood and its resistance to reducing agents are in such cases characteristic.

Nitric Oxide Hæmoglobin.—When ammonia is added to blood, and then a stream of nitric oxide is passed through it, this compound is formed. It may be obtained in crystals isomorphous with oxy- and CO-hæmoglobin. It also has a similar spectrum. It is even more stable than CO-hæmoglobin; it has little practical interest, but is of theoretical importance as completing the series.

CO_2 ?

TESTS FOR BLOOD

These may be gathered from preceding descriptions. Briefly, they are microscopic, spectroscopic, and chemical. The best chemical test is the formation of hæmin crystals. The old test with tincture of guaiacum and hydrogen peroxide, the blood causing the tincture to become blue, is not very trustworthy, as it is also given by many other organic substances. The test for instance is given by milk, and is there due to the presence of an enzyme called a *peroxidase*, which is destroyed by boiling. Boiled blood, however, gives the test as well as fresh blood, and the reaction is due to the presence of the iron-containing radical of hæmoglobin.

In medico-legal cases it is often necessary to ascertain whether a red fluid or stain upon clothing is or is not blood. In any such case it is advisable not to rely upon one test only, but to try every means of detection at one's disposal. To discover whether it is blood or not is by no means a difficult problem, but to distinguish human blood from that of the common mammals is possible only by the "biological" test described at the end of the next section.

IMMUNITY

The chemical defences of the body against injury and disease are numerous. The property of coagulating which the blood possesses is a defence against hæmorrhage; the acid of the gastric juice is a protection against harmful bacteria introduced with food. Bacterial activity in urine is inhibited by the acidity of that secretion.

Far more important and widespread in its effects than any of the foregoing is the bactericidal (*i.e.* bacteria-killing) action of the blood and lymph; a study of this question has led to many interesting results, especially in connection with the important problem of immunity.

It is a familiar fact that one attack of many of the infective maladies protects us against another attack of the same disease. The person is said to be *immune*, either partially or completely, against that disease. Vaccination produces in a patient an attack of cowpox or vaccinia. This disease is either closely related to smallpox, or may be it is smallpox modified and rendered less malignant by passing through the body of a calf. At any rate, an attack of vaccinia renders a person immune to smallpox for a certain number of years. Vaccination is an instance of what is called *protective inoculation*, which is now practised with more or less success in reference to other diseases, such as plague and typhoid fever. The study of immunity has also rendered possible what may be called *curative inoculation*, or the injection of antitoxic material as a cure for diphtheria, tetanus, snake-poisoning, etc.

The power the blood possesses of slaying bacteria is not limited to the colourless corpuscles or *phagocytes*, but is also a property of the fluid part of the blood, at any rate in the case of some micro-organisms. The chemical characters of the substances which kill the bacteria are not fully known; but they appear to be protein in nature. The bactericidal powers of blood are destroyed by heating it for an hour to 55° C. The substances, whatever be their source or their chemical nature, are called *bacterio-lysins*.

Closely allied to the bactericidal power of blood, or blood serum, is its globulicidal power. By this one means that the blood serum of one animal has the power of dissolving the red blood corpuscles of another species. If the serum of one animal is injected into the blood-stream of an animal of another species, the result is a destruction of its red corpuscles, which may be so excessive as to lead to the passing of the liberated hæmoglobin into the urine (hæmoglobinuria). The substances in the serum that possess this property are called *hæmolysins*, and though there is some doubt whether bacterio-lysins and hæmolysins are absolutely identical, there is no doubt that they are closely related.

Normal blood thus possesses not only *phagocytes*, which eat up bacteria, but also a certain amount of chemical substances which are inimical to the life of our bacterial foes. But suppose a person gets "run down"; everyone knows he is then more liable to "catch anything." This coincides with a diminution in the bactericidal power of his blood. But even a perfectly healthy person has not an unlimited supply of bacterio-lysins, and if the bacteria are sufficiently numerous he will fall a victim to the disease they produce. Here, however, comes in the remarkable part of the defence. In the struggle he will produce more and more bacterio-lysin, and if he gets well it means that the bacteria are finally vanquished, and his blood remains rich in the particular bacterio-lysin he has produced, and so will render him immune to further attacks from that particular species of bacterium. Every bacterium seems to cause the development of a specific anti-substance.

Immunity can more conveniently be produced gradually in animals, and this applies, not only to the bacteria, but also to the toxins they form. If, for instance, the bacilli which produce diphtheria are grown in a suitable medium, they produce the diphtheria poison, or toxin, much in the same way that yeast-cells will produce alcohol when grown in a solution of sugar. Diphtheria toxin is associated with a proteose, as is also the case with the poison of snake venom. If a certain small dose called a "lethal dose" is injected into a guinea-pig the result is death. But if the guinea-pig receives a smaller dose it will recover; a few days after it will stand a rather larger dose; and this may be continued until, after many successive gradually increasing doses, it will finally stand an amount equal to many lethal doses without any ill effects. The gradual introduction of the toxin has called forth the production of an antitoxin. If this is done in the horse instead of the guinea-pig the production of antitoxin is still more marked, and the serum obtained from the blood of an immunised horse may be used for injecting into human beings suffering from diphtheria, and it rapidly cures the disease. The two actions of the blood, antitoxic and antibacterial, are frequently associated, but may be entirely distinct.

The antitoxin is also a protein probably of the nature of a globulin; at any rate it is a protein of larger molecular weight than a proteose. This suggests a practical point. In the case of snake-poisoning the poison gets into the blood rapidly owing to the comparative ease with which it diffuses, and so it is quickly carried all over the body. In treatment with the antitoxin or antivenin, speed is everything if life is to be saved; injection of this material under the skin is not much good, for the diffusion into the blood is too slow. It should be injected straight away into a blood-vessel.

There is no doubt that in these cases the antitoxin neutralises the toxin much in the same way that an acid neutralises an alkali. If the toxin and antitoxin are mixed in a test-tube, and time allowed for the interaction to occur, the result is an innocuous mixture. The toxin, however, is merely neutralised, not destroyed; for if the mixture in the test-tube is heated to 68° C., the antitoxin is coagulated and destroyed, and the toxin remains as poisonous as ever.

The substances which on injection provoke the appearance of antidotes of this nature are either proteins, or are protein-like. They are called *antigens*.

Immunity is distinguished into *active* and *passive*. Active immunity is produced by the development of protective substances in the body; passive immunity by the injection of a protective serum. Of the two the former is the more permanent.

Ricin, the poisonous protein of castor-oil seeds, and *abrin*, that of the Jequirity bean, also produce when gradually given to animals an immunity, due to the production of antiricin and antiabrin respectively.

Ehrlich's hypothesis to explain such facts is usually spoken of as the *side-chain theory* of immunity. He considers that the toxins are capable of uniting with the protoplasm of the living cells by possessing groups of atoms like those by which nutritive proteins are united to cells during normal assimilation. He terms these *haptophor* groups, and the groups to which these are attached in the cells he terms *receptor* groups. The introduction of a toxin stimulates an excessive production of receptors, which are finally thrown out into the circulation, and the free circulating receptors constitute the antitoxin. The comparison of the process to assimilation is justified by the fact that non-toxic substances such as milk or egg-white introduced gradually by successive doses into the blood-stream cause the formation of anti-substances capable of coagulating them.

Up to this point I have spoken only of the blood, but workers are steadily bringing forward evidence to show that other cells of the body may by similar measures be rendered capable of producing a corresponding protective mechanism.

One further development of the theory must be mentioned. At least two different substances are necessary to render a serum bactericidal or globulicidal. The bacterio-lysin or hæmolysin consists of these two substances. One of these is called the *amboceptor*, the other the *complement*. We may illustrate the use of these terms by an example. The repeated injection of the blood of one animal (*e.g.* the goat) into the blood of another animal (*e.g.* a sheep) after a time renders the latter animal immune to further injections, and at the same time causes the production of a serum which dissolves readily the red blood corpuscles of the first animal. The sheep's serum is thus hæmolytic towards goat's blood corpuscles. This power is destroyed by heating to 56° C. for half an hour, but returns when fresh serum of any animal is added. The specific immunising substance formed in the sheep is called the amboceptor; the enzyme-like substance destroyed by heat is the complement. The latter is not specific, since it is furnished by the blood of non-immunised animals, but it is nevertheless essential for hæmolysis. Ehrlich believes that the immune body has two side groups—one which unites with the receptor of the red corpuscles, and one which unites with the haptophor group of the complement, and thus renders possible the enzyme-like action of the complement on the red corpuscles.

To put it another way: the cell-dissolving substances cannot act on their objects of attack without an intermediate substance to anchor them on the substance in question. This intermediary substance, known as the amboceptor, is specific, and varies with the substance to be attacked (red corpuscles, bacterium, toxin, etc.). The complement may be compared to a person who wants to unlock a door; to do this effectively he must be provided with the proper key (amboceptor).

Many antigens in small doses cause an increased sensitiveness of an animal to the foreign protein; so that a second small dose injected a few weeks later may produce death. This is called *anaphylaxis.*

Quite distinct from the bactericidal, globulicidal, and antitoxic properties of blood is its agglutinating action. This is another result of infection with many kinds of bacteria or their toxins. The blood acquires the property of rendering immobile and clumping together the specific bacteria used in the infection. The test applied to the blood in cases of typhoid fever, and generally called Widal's reaction, depends on this fact. The substances that produce this effect are called *agglutinins.* They also are probably protein-like in nature, but are more resistant to heat than the lysins. Prolonged heating to over 60° C. is necessary to destroy their activity.

We thus see that the means the body possesses of combating bacterial invasion are numerous. In some cases the bacteria are killed by bacterio-lysins, and in other cases they are directly attacked and devoured by the phagocytes. Bacteria which are destroyed in this way produce no evil results, whereas those which are not destroyed are called *pathogenic*, or disease-producing organisms. There is still another line of defence, for if the bacteria are not destroyed the poisons or toxins they produce are in certain other cases neutralised by antitoxins.

Metschnikoff's view, which is very widely accepted by bacteriologists, is that the most stress should be laid upon phagocytosis as the principal factor in the resistance of the body to bacteria; and the discovery of *opsonins* by Sir A. E. Wright not only emphasises this opinion, but shows how the body fluids co-operate with the phagocytes in the process. The word "opsonin" is derived from a Greek word which means "to prepare the feast." Washed bacteria from a culture are distasteful to leucocytes, and would therefore, other things being equal, be pathogenic if injected into an animal's body. But if the bacteria have been previously soaked in serum, especially if that serum has been obtained from the blood of an animal previously immunised against that special bacterium, then the leucocytes devour them eagerly. It was at first supposed that something had been added to the bacterium to make it tasty, and that each kind of bacterium requires its own special sauce or opsonin. It is, however, equally possible that the serum has not added anything to the bacterium, but removed from it something that previously made it distasteful. At any rate the ultimate effect is the same, and the bacterium is rendered *non-pathogenic.* When a person is attacked with some invading organism, say the tubercle bacillus, if that person's blood is naturally rich in the proper kind of opsonin he will not be troubled with tuberculosis; but if the opsonic power of his blood is low the bacillus will produce the disease. The modern treatment of tuberculosis aims at increasing the opsonic power of the blood by improving the general condition of the patient by good food and pure air, and also by the injection of the appropriate opsonin into his blood.

Lastly, we come to a question which more directly appeals to the physiologist than the preceding, because experiments in relation to immunity have furnished us with what has hitherto been lacking, a means of distinguishing human blood from the blood of other animals.

The discovery was made by Tchistovitch (1899), and his original experiment was as follows:—Rabbits, dogs, goats, and guinea-pigs were inoculated with eel-serum, which is toxic; he thereby obtained from these animals an antitoxic serum. But the serum was not only antitoxic; it also produced a precipitate when added to eel-serum, though not when added to the serum of any other animal. In other words, not only has a specific antitoxin been produced, but also a specific *precipitin*. Numerous observers have since found that this is a general rule throughout the animal kingdom, including man. If, for instance, a rabbit is treated with human blood, the serum ultimately obtained from the rabbit contains a specific precipitin for human blood; that is to say, a precipitate is formed on adding such a rabbit's serum to human blood, but not when added to the blood of any other animal.[1] The great value of the test is its delicacy: it will detect the specific blood when it is greatly diluted, after it has been dried for weeks, or even when it is mixed with the blood of other animals.

The lipoids contained in the membrane of cells play some part in the relationship of such cells to toxins. The matter has been mainly studied in relation to red corpuscles, and the toxins (such as saponin and the hæmolysin of snake venom) which attack them. There is some evidence that the cholesterol in the envelope of the red corpuscles is a protective agent (see also p. 38). A few years ago, Preston Kyes stated that lecithin is the amboceptor which anchors the hæmolysin on to the red cells. But more recent research has failed to substantiate this view, and the compounds which Kyes described and called lecithides are impure mixtures of several substances. It is much more probable that the real agent at work in hæmolysis is a lipolytic or fat-splitting enzyme; this splits up the lecithin of the cell-wall, liberating oleic acid and desoleolecithin (that is, lecithin *minus* its oleic acid radical), and it is these cleavage products which dissolve out the hæmoglobin and so destroy the corpuscles.

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[1] There may be a slight reaction with the blood of allied animals; for instance, with monkey's blood in the case of man.

CHEMISTRY OF RESPIRATION

The consideration of the blood, and especially of its pigment, is so closely associated with respiration that a brief account of that process follows conveniently here.

The air in the alveoli of the lungs, and the blood in the pulmonary capillaries are only separated by the thin capillary and alveolar walls. The blood parts with its excess of carbonic acid and watery vapour to the alveolar air; the blood at the same time receives from the alveolar air the oxygen which renders it arterial.

The intake of oxygen is the commencement, and the output of carbonic acid the end, of the series of changes known as respiration. The intermediate steps take place all over the body, and constitute what is known as *internal* or *tissue respiration*. The exchange of gases which occurs in the lungs is sometimes called in contradistinction *external respiration*. We have already seen that the oxyhæmoglobin is only a loose compound, and in the tissues it parts with its oxygen. The oxygen does not necessarily undergo immediate union with carbon to form carbonic acid, and with hydrogen to form water, but in most cases, as in muscle, is held in reserve by the tissue itself. Ultimately, however, these substances pass into the venous blood, and the carbonic acid and a portion of the water find an outlet by the lungs.

Inspired and Expired Air.—The composition of the inspired and expired air may be compared in the following table :—

	Inspired or Atmospheric Air.	Expired Air.
Oxygen	20·96 vols. per cent.	16·03 vols. per cent.
Nitrogen	79 ,, ,,	79 ,, ,,
Carbonic acid	0·04 ,, ,,	4·4 ,, ,,
Watery vapour . . .	variable	saturated
Temperature	,,	that of body (37° C.)

The nitrogen remains unchanged. The recently discovered gases, argon, crypton, etc., are in the above table reckoned in with the nitrogen. They are, however, only present in minute quantities. The chief change is in the proportion of oxygen and carbonic acid. The loss of oxygen is about 5, the gain in carbonic acid 4·5. If the inspired and expired airs are carefully measured at the same temperature and barometric pressure, the volume of expired air is thus rather less than that of the inspired. The conversion of oxygen into carbonic acid would not cause any change in the volume of the gas, for a molecule of oxygen (O_2) would give rise to a molecule of carbonic acid (CO_2) which would occupy the same volume (Avogadro's law). It must, however, be remembered that carbon is not the only element which is oxidised. Fats contain a number of atoms of hydrogen which during metabolism are oxidised to form water; a certain small amount of oxygen is also used in the formation of urea. Carbohydrates contain sufficient oxygen in their own molecules to oxidise their hydrogen; hence the apparent loss of oxygen is least when a vegetable diet (that is, one consisting largely of starch and other carbohydrates) is taken, and greatest when much fat and protein are eaten. The quotient $\frac{CO_2 \text{ given off}}{O_2 \text{ absorbed}}$ is called the *respiratory quotient*. Normally it is $\frac{4{\cdot}5}{5} = 0{\cdot}9$, but this varies considerably with diet, as just stated.

THE GASES OF THE BLOOD

Before we can understand either the chemistry of respiration or its regulation, which is in part a chemical process, it is necessary that we should study the fundamental laws which regulate the retention of oxygen and carbonic acid in the blood; and as the blood presents many complications, it will be best at the outset to consider the solution of gases in such a simple medium as water.

Solution of Gases in Water

If water is shaken up with oxygen, a certain definite amount of oxygen will become dissolved in the water. Under the same conditions the same quantity of oxygen would always be dissolved, and in the following argument it is assumed throughout that the temperature remains constant. The amount dissolved, then, depends upon two circumstances, each of which can be measured. The first is the pressure of the oxygen to which the water is exposed when shaken; the second is a property of the oxygen itself, namely, its solubility in water. The solubilities of different gases differ very much; some (for instance, oxygen) are not readily soluble in water, whilst others, such as carbonic acid, are very soluble.

If a cubic centimetre of water is introduced into a large air-tight bottle containing pure oxygen at the atmospheric pressure, and another cubic centimetre of water is similarly placed in a bottle containing pure carbonic acid at the same pressure, the former would be found to have dissolved 0·04 c.c. of oxygen, the latter 1 c.c. of carbonic acid.

These figures represent the degrees to which the two gases are soluble in water under similar circumstances, and are called their coefficients of solubility. The *coefficient of solubility* of gas in a liquid is therefore the amount of a gas which 1 c.c. of liquid will dissolve at 760 mm. of mercury, that is, at atmospheric pressure.

The quantity of gas which a liquid will dissolve depends also upon the pressure of the gas to which the liquid is exposed. Thus, in the instance given above, if the oxygen had been rarefied in the bottle until it only exerted a pressure of one-fifth of an atmosphere, the water would have only taken up not 0·04 c.c. of oxygen, but only one-fifth of that amount, 0·008 c.c. If we represent the coefficient of solubility of a gas by K, and the pressure of the gas to which the liquid is exposed by P′, and the atmospheric pressure by P; then the quantity (Q) of the gas dissolved by 1 c.c. of the liquid may be found by the following formula :—

$$Q = K \times \frac{P'}{P}.$$

Dalton-Henry Law.—What has been said above is as true of gases which are mixed together as of pure gases. For instance, we have seen that a cubic centimetre of water shaken up with oxygen at one-fifth of an atmosphere (152 mm. pressure) will absorb $0·04 \times \frac{1}{5} = 0·008$ c.c.; if it is shaken with nitrogen at a pressure of four-fifths of an atmosphere, it will dissolve $0·02 \times \frac{4}{5} = 0·016$ c.c. If now a c.c. of water is shaken with air (a mixture of one part of oxygen to four of nitrogen), it will dissolve 0·008 c.c. of oxygen and 0·016 c.c. of nitrogen. This fact has been stated as the Dalton-Henry Law in the following words :—When two or more gases are mixed together, they each produce the same pressure as if they separately occupied the entire space and the other gases were absent. The *total* pressure of the mixture is the sum of the *partial* pressures of the individual gases in the mixture.

The Tension of Gases in Fluids

In the cases which have been discussed up to this point, a condition of equilibrium exists between the gas dissolved in the fluid and the gas in the atmosphere to which the fluid is exposed, so that as many molecules of the gas leave the surface of the fluid as enter it. The gas dissolved in the fluid therefore exercises a pressure which is the same as that of the gas in the atmosphere when equilibrium exists. For the sake of convenience the word *Tension* is applied to the pressure of the gas in the fluid.

Definition of Tension.—The tension of a gas dissolved in a fluid is equal to the pressure of the same gas in an atmosphere with which the gas in the fluid would be in equilibrium. Above, we have called the pressure which the gas exerts on the liquid, P'. If we call the tension of the gas in the liquid T, we find that when equilibrium exists $P' = T$. In the case of all true solutions, therefore, we may replace P' in our previous equation by T; therefore $Q = K \times \frac{T}{P}$. We thus arrive at a relation between two separate things, which must be most carefully distinguished from one another—the quantity of the gas dissolved in the liquid and its tension.

Measurement of Tension in Fluids — Aërotonometer. — Numerous instruments have been invented for measuring tension. They are called *tonometers.* Krogh's (fig. 31) is the best. It consists of a T-shaped cannula (A) introduced into the blood-vessel, say the carotid artery. The blood fills the cavity B and leaves it at C, so that a constant stream of blood is kept flowing. Into it a small bubble of air (D) is introduced. Exchange of gases takes place between the bubble and the blood, and the former very soon gets into equilibrium with the latter. When it has done so, the bubble is withdrawn up the capillary tube E, taken away, and analysed.

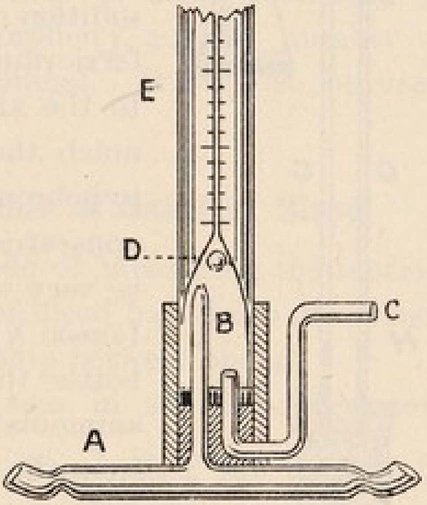

FIG. 31.—Krogh's Tonometer.

As an example, suppose the bubble on analysis proved to consist of 4 per cent. carbonic acid and 12 per cent. oxygen, together with nitrogen and aqueous vapour. The gas in the instrument was compressed by the pressure of the arterial blood (say 120 mm. of mercury) in addition to the atmospheric pressure of 760 mm. of mercury, and therefore its total pressure was $120 + 760 = 880$ mm. of mercury. Four per cent. of this would have been due to the carbonic acid; 4 per cent. of 880 is 35·2. Twelve per cent. would have been due to the oxygen; 12 per cent. of 880 is 105·6. That is, the carbonic acid and oxygen tensions would have been in round figures 35 and 106 mm. of mercury respectively.

Measurement of the Quantity of a Gas in a Fluid

The most general method of determining the quantity of gas in a fluid is by boiling a measured quantity of the fluid in a vacuum. The gas is all given off; it may be collected and measured. In the case of blood, which is the only fluid that need be considered, this process is carried out by means of a mercurial air-pump known as the blood-gas pump. One of the numerous forms of mercury pump is described in the Appendix.

The total gas obtained is first measured; the carbonic acid is removed by caustic potash, and the gas that remains, which consists of oxygen and nitrogen, is measured; the oxygen is then removed by pyrogallic acid, and the residual gas is nitrogen. Haldane's apparatus for carrying this out is described in the Appendix. Another method is the following :—

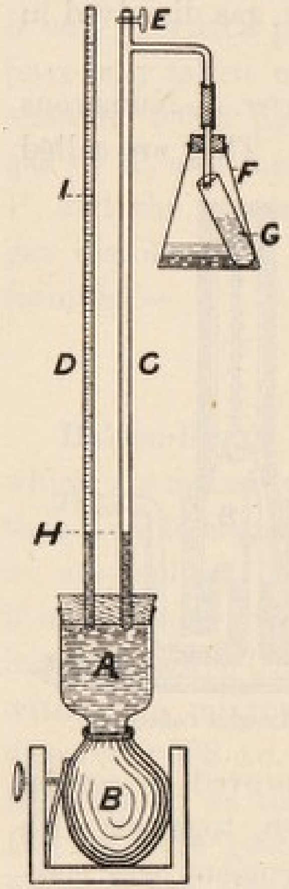

Fig. 32.—Apparatus for obtaining the blood gases.

Chemical Method of Blood-gas Analysis.—When a solution of oxyhæmoglobin is shaken with potassium ferricyanide, it yields the same amount of oxygen to the air as it would if boiled in a vacuum. In much the same way urea when treated with sodium hypobromite yields up all its nitrogen, and the apparatus used for determining the oxygen in blood is very similar to a Dupré's urea apparatus (see Lesson X). The blood (5 c.c.) is placed in the large bottle (fig. 32, F) underneath a layer of dilute ammonia solution. The blood is thus protected from the air whilst the apparatus becomes equal in temperature to the bath in which it is placed. The blood is shaken with the ammonia solution, which lakes it thoroughly; the ferricyanide solution is then spilt into the laked blood from the tube G, and the oxygen is shaken out of the solution. When the oxygen has been determined the bottle is opened and tartaric acid is placed in the small tube G; this is subsequently spilt into the mixture of blood, ammonia, and ferricyanide; it liberates the carbonic acid which is also shaken out of the fluid. The carbonic acid does not come completely out, however, and a correction has to be introduced for the quantity which remains in solution. The gas (oxygen or carbonic acid, as the case may be) passes over into the tube C, which was previously filled up to the zero mark (H) with water, and connected to a reservoir (A); this would drive water out of A into the open tube D, and the water will therefore rise in D; but in practice it is convenient to keep the gas always at the same volume; this may be done by raising the pressure in the open limb (D) of the pressure gauge by squeezing some of the water, with which the gauge is filled, out of a rubber reservoir (B) which forms the base of the gauge, thus the level of the water in C is maintained at the zero mark, while that in D rises from H to I. The actual measurement then is the increase of pressure (*i.e.* the height of the column of water H I) which is necessary to keep the gas at the same volume after the oxygen or carbonic acid has been shaken off as it previously occupied. From this the quantity coming off can be calculated. The apparatus just described illustrates the principle of the method. Barcroft's most recent improved instrument is described in the Appendix.

The chemical method is not quite so accurate as the vacuum pump, but it is much more convenient for the study of many problems, as it requires less blood, and, owing to its simplicity, a great number of observations can be made upon a single animal. It can also be used for observations on human blood.

Relation between Quantity and Tension of Gases in Blood

In the preceding paragraphs the methods of measuring the tension and the quantity of gas in a given sample of blood have been described. It is now necessary to consider the relationship between them.

On p. 163 we have seen that for gases in solution in water, $Q = K \times \frac{T}{P}$, where Q is the quantity of gas dissolved, T the tension, K the coefficient of solubility, and P the atmospheric pressure. Since K and P are constant, it follows that Q varies directly in proportion to T; that is to say, if the tension is doubled, the quantity of gas dissolved is also doubled; if the tension is trebled, the quantity of gas is trebled, and so on. These results might be plotted out on a curve in which the quantities are placed on the ordinate, and the tensions on the abscissa. Such a curve would give the quantity of gas dissolved at any given tension, and, in the case of water, it would turn out to be a straight line.

But in the case of both the oxygen and the carbonic acid in blood, the curve showing the relationship between the tension of gas and the volume which can be pumped off is not a straight line.

Oxygen in Blood.—From every 100 c.c. of arterial blood, rather more than 20 c.c. of oxygen can be removed by the air-pump. Nearly all of this oxygen is chemically combined with hæmoglobin; the amount in actual solution in the blood is 0·7 c.c. for every 100 c.c. of blood. Hæmoglobin owes its value as a respiratory pigment to two principal facts. (1) It can unite with a large quantity of oxygen, and therefore blood can carry about thirty times as much oxygen to the tissues as plasma would under the same circumstances. (2) The interaction between hæmoglobin and oxygen is a reversible one; the two unite in the lungs, where the pressure of oxygen is high; but when oxygen is absent or at a low pressure, as in the tissues, the hæmoglobin parts with its store of oxygen.

We will now consider the nature of this union, and the conditions under which it takes place.

The reaction between hæmoglobin and oxygen is a chemical one. At most, 1 gramme of hæmoglobin can unite with 1·34 c.c. of oxygen. This figure is not quite constant, probably on account of slightly different forms of globin (the protein constituent of hæmoglobin) uniting with the hæmatin (the iron-containing constituent) in different animals. The relation between the respiratory oxygen and the iron of the hæmoglobin is, however, quite constant, and is called the "specific oxygen capacity." Each gramme of iron in hæmoglobin unites with 400 c.c. of oxygen; these figures are in the relation of one atom of iron to two atoms of oxygen. The reversible nature of the reaction may therefore be expressed by the equation $Hb + O_2 \rightleftarrows HbO_2$. A reversible reaction means that it will go in either direction according to the concentration of the substances present; thus if the concentration of oxygen *in solution* is increased, more of the hæmoglobin will become oxyhæmoglobin; and if it is diminished, oxyhæmoglobin will break up into hæmoglobin (sometimes called reduced hæmoglobin) and oxygen.

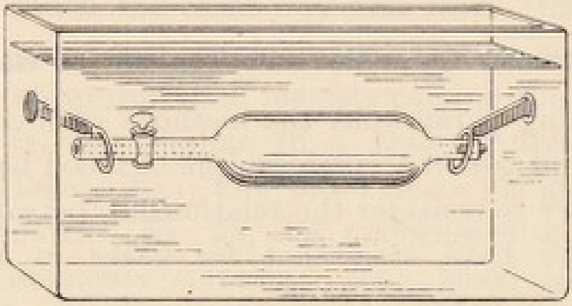

FIG. 33.—Barcroft's Tonometer, suspended horizontally in warm bath in which it is rotated.

The quantity of gas *in solution* (that is, not united with hæmoglobin) varies in proportion to the oxygen pressure to which the hæmoglobin solution is exposed; therefore the problem before us is to ascertain the relative quantities of oxy- and reduced hæmoglobin when a hæmoglobin solution is shaken up with oxygen at different pressures.

This can be done by means of Barcroft's tonometer (fig. 33). Suppose we have six of these tubes, and each contains the same amount (a few c.c.) of hæmoglobin solution, and gases of the following composition :—

No. 1. Nitrogen and no oxygen.
No. 2. Nitrogen and enough oxygen to give 5 mm. oxygen pressure.
No. 3. ,, ,, ,, 10 ,, ,,
No. 4. ,, ,, ,, 20 ,, ,,
No. 5. ,, ,, ,, 50 ,, ,,
No. 6. ,, ,, ,, 100 ,, ,,

Each tonometer is rotated round and round in a bath at body temperature until the hæmoglobin and the oxygen are in equilibrium ; this will take about fifteen minutes ; the solution is then withdrawn and examined in order to ascertain the relative quantities of oxy- and reduced hæmoglobin in each of the six vessels.

The figures for a pure solution of hæmoglobin would be :—

	No. 1.	No. 2.	No. 3.	No. 4.	No. 5.	No. 6.
Percentage of hæmoglobin	100	63	45	28	13	6
Percentage of oxyhæmoglobin	0	37	55	72	87	94
	100	100	100	100	100	100

The same answer may be expressed graphically ; if the pressures of oxygen are plotted horizontally, and the percentages of oxy- and reduced hæmoglobin in the solution are plotted vertically, we get the curve shown in the accompanying diagram (fig. 34), which is called the *dissociation curve of hæmoglobin.*

A solution of pure hæmoglobin is, however, not the same thing as blood, and the dissociation of hæmoglobin in the latter fluid during life is influenced by various conditions, especially by (1) temperature, (2) the presence of salts, and (3) the presence of acids, especially carbonic acid. These factors make the oxyhæmoglobin molecules break down more rapidly, and form more rapidly. It is clearly necessary that the two processes (the union of hæmoglobin with oxygen, and the liberation of oxygen from oxy-hæmoglobin) should occur at the same rate, that is under one second, which is about the time occupied by any given portion of blood in travelling along the capillaries.

It would be futile to have an oxygen carrier in the blood which took a fraction of a second to acquire its oxygen in the lungs, and a fraction of an hour to release it in the tissues. Yet a solution of pure hæmoglobin is just such a substance. In the actual blood, however, the three factors just mentioned, the salts, especially the potassium salts of the red corpuscles, the high temperature, and the presence of carbonic acid at 40 mm. pressure, increase the rate of dissociation of hæmoglobin so greatly that it equals the rate at which the union of oxygen and hæmoglobin occurs in the lungs. Nature has adapted the conditions of life so admirably that the needs of the body are served by a substance hæmoglobin, which by itself is inefficient for oxygen transport.

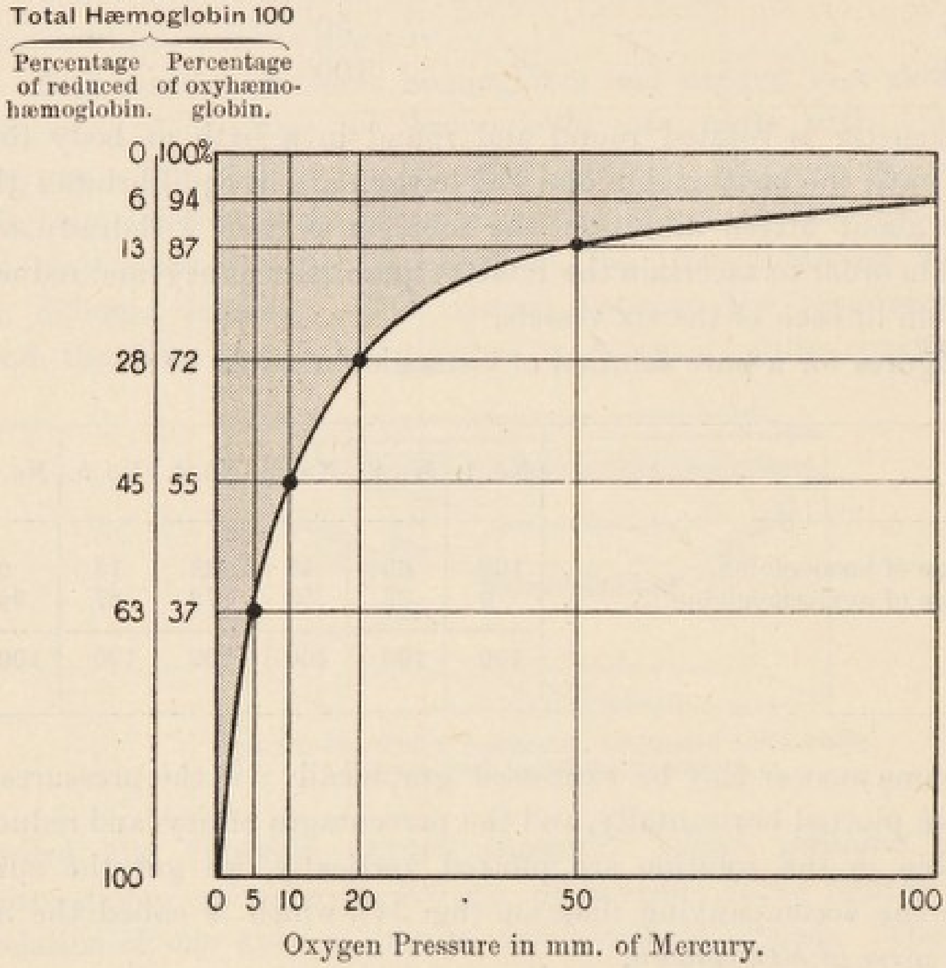

FIG. 34.—Dissociation curve of hæmoglobin at 37° C. The shaded area represents reduced hæmoglobin, the white, oxyhæmoglobin.

The next figure (fig. 35) shows the dissociation curve in the actual blood, and it should be carefully compared with fig. 34. The two present to the eye graphically the superiority of hæmoglobin as an oxygen carrier when it is present in the living blood over that which it possesses in a pure solution.

In the second curve, that of the blood itself, it will be seen that at an oxygen pressure of over 60 mm. of mercury (the pressure in the lung alveoli is about 100) the blood will nearly saturate itself with oxygen, and that at pressures below 50 the blood loses its oxygen rapidly, whilst at 10 mm. pressure it is nearly completely reduced. As the rate at which oxygen can diffuse out of the capillaries into the surrounding tissues depends upon the pressure it exerts in the plasma, it is important that the blood should be capable of a considerable degree of reduction when it is in contact with fluid containing oxygen at a pressure such as one finds in the tissues (20 to 30 mm. of mercury).

Carbonic Acid in Blood.—If blood is divided into plasma and corpuscles, it will be found that both yield carbonic acid, but the yield from the plasma is the greater. If we place blood in a vacuum it bubbles and gives out all its gases; addition of a weak acid causes no further liberation of carbonic acid. When plasma or serum is similarly treated the gas also comes off, but about 5 per cent. of the carbonic acid is fixed—that is, it requires the addition of some stronger acid, such as phosphoric or tartaric acid, to displace it.

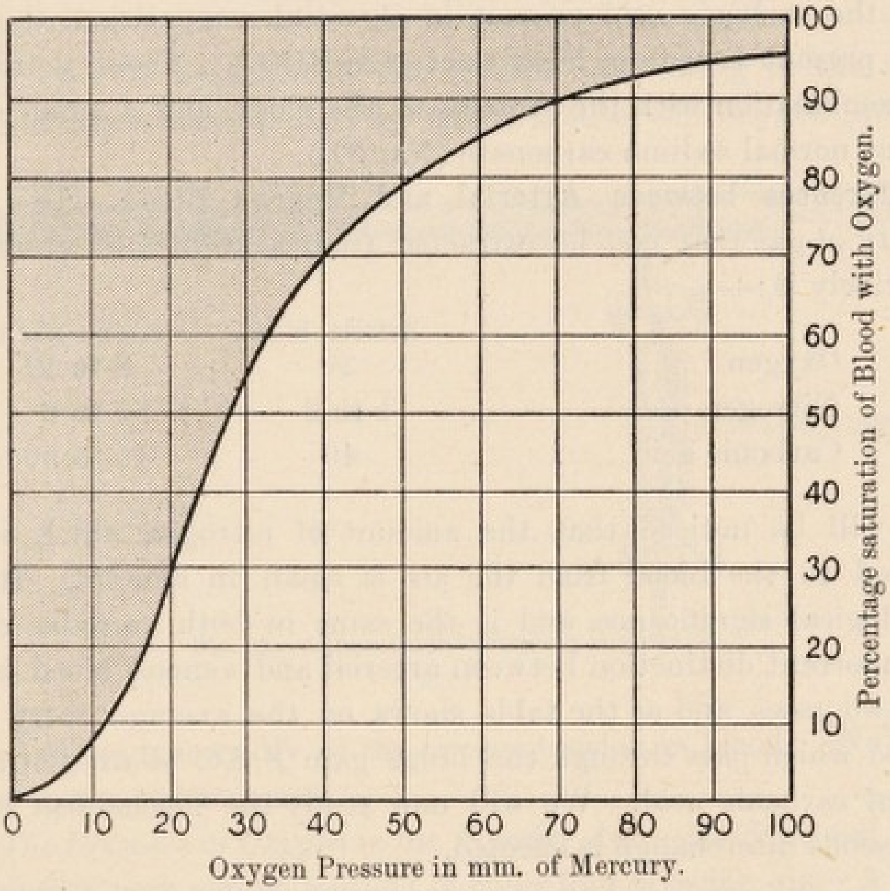

FIG. 35.—Dissociation curve of hæmoglobin in the blood. The shaded area as before represents reduced hæmoglobin, the white area, oxyhæmoglobin.

One hundred volumes of venous blood contain about forty-six volumes of carbonic acid. Whether this is in solution or in chemical combination is determined by ascertaining the tension of the gas in the blood. One hundred volumes of blood plasma would dissolve about an equal volume of the gas from an atmosphere of carbonic acid, if its solubility in plasma were equal to that in water. If, then, the carbonic acid were in a state of solution, its tension would be very high, but, as we have seen, it proves to be only equal to about 5 per cent. of an atmosphere. This means that when venous blood is brought into an atmosphere containing 5 per cent. of carbonic acid, the blood neither gives off any carbonic acid nor takes up any from that atmosphere. Hence the remainder of the gas, 41 per cent., is in a condition of chemical combination.

Of the carbonic acid present in chemical composition, the greater part is present as sodium bicarbonate ($NaHCO_3$). About a third is in loose combination with the proteins of the blood, and a small quantity exists as normal sodium carbonate (Na_2CO_3).

Differences between Arterial and Venous Blood.—The average quantity of gas that can be extracted from arterial and venous blood respectively is :—

	Arterial blood.	Venous blood.
Oxygen . . .	20	8 to 12
Nitrogen . . .	1 to 2	1 to 2
Carbonic acid . .	40	46 to 50

It will be noticed that the amount of nitrogen which is simply dissolved in the blood from the air is small in amount. It has no physiological significance, and is the same in both varieties of blood. The important distinction between arterial and venous blood is in the other two gases, and as the table shows, on the average every 100 c.c. of blood which pass through the lungs gain 8 c.c. of oxygen and lose 6 c.c. of carbonic acid. We will now study the mechanism by which this gaseous interchange is effected.

THE MECHANISM OF GASEOUS EXCHANGE IN THE LUNG

1. **Oxygen.**—The simplest explanation of the passage of oxygen from the alveolar air into the blood is that the process is one of diffusion. This view can be maintained if it can be proved that the pressure of oxygen in the alveolar air is as great or greater than the tension of oxygen in the arterial blood, and therefore *a fortiori* greater than that of oxygen in the venous blood.

The conception of respiration based upon this view would be that the oxygen in the air of the alveoli, though less than that in the atmosphere, is greater than that in venous blood ; hence oxygen passes from the alveolar air into the blood plasma ; the oxygen immediately combines with the hæmoglobin, and thus leaves the plasma free to absorb more oxygen; and this goes on until the hæmoglobin is entirely, or almost entirely, saturated with oxygen. The reverse change occurs in the tissues when the partial pressure of oxygen is lower than in the plasma, or in the lymph that bathes the tissue elements; the plasma parts with its oxygen to the lymph, the lymph to the tissues; the oxyhæmoglobin then undergoes dissociation to supply more oxygen to the plasma and lymph, and thus in turn to the tissues once more. This goes on until the oxyhæmoglobin loses about half its store of oxygen.

This view is regarded as the correct one, owing to the accurate determinations which can now be made, first, of the oxygen pressure in alveolar air, and, secondly, of the oxygen tension in blood. We will take the two in the order named.

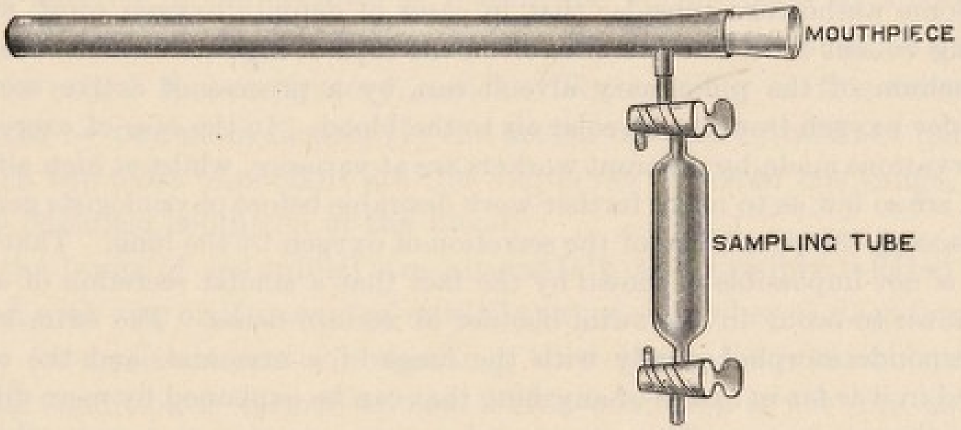

FIG. 36.—Apparatus for obtaining alveolar air.

i. *The Pressure of Oxygen in the Alveolar Air.*—Haldane and Priestley introduced a very simple method of collecting alveolar air. A piece of rubber tubing is taken about 1 inch in diameter and about 4 feet long (fig. 36). A mouthpiece is fitted into one end. About 2 inches from the mouthpiece a small hole is made into which is inserted the tube of a gas-receiver, or sampling-tube, as in the figure (fig. 36). The gas-receiver is fitted at the upper end with a three-way tap, and the lower end is also closed by a tap. Before it is used, it is filled with mercury. The subject of the experiment breathes normally through the tube for a time, and then, at the end of a normal inspiration, he expires quickly and very deeply through the mouthpiece and instantly closes it with his tongue. The lower tap of the receiver is then turned, and as the mercury runs out, a sample of the air takes its place and fills the receiver; this sample is then analysed. A second experiment is then done, in which the subject expires deeply at the end of a normal expiration, and another sample obtained. The mean result of the two analyses gives the composition of alveolar air.

It is found on analysis that the normal oxygen pressure in the alveoli is approximately equal to 100 mm. of mercury, and this is equivalent to 13 per cent. of an atmosphere.

ii. *The Tension of Oxygen in the Blood.*—This is estimated by Krogh's tonometer (fig. 31, p. 163), and the experiments show that the tension of oxygen in the blood is lower than the alveolar oxygen pressure. If the latter is artificially raised or lowered, the oxygen tension in the blood rises and falls in the same way, but always remains lower than the oxygen pressure in the alveoli.

Some authorities consider that in cases of definite oxygen want, such as during violent muscular exercise, or on the tops of high mountains, the lining epithelium of the pulmonary alveoli can, by a process of active secretion, transfer oxygen from the alveolar air to the blood. In the case of exercise the observations made by different workers are at variance, whilst at high altitudes they are so few as to make further work desirable before physiologists generally can accept the possibility of the secretion of oxygen by the lung. That secretion is not impossible is shown by the fact that a similar secretion of oxygen is known to occur in the swim-bladder of certain fishes. The swim-bladder corresponds morphologically with the lungs of a mammal, and the oxygen stored in it is far in excess of anything that can be explained by mere diffusion from the sea-water. This storage of oxygen, moreover, ceases when the vagus nerves which supply the swim-bladder are divided.

2. **Carbonic Acid.**—Here, again, the same two measurements are necessary and are obtained in the same way. The alveolar tension of this gas is always lower than that of the arterial blood; the pressure differences are less than in the case of oxygen; this coincides with the ease with which carbonic acid passes out through the membrane which separates the blood from the air.

The tension of the carbonic acid in the tissues is high, but one cannot give exact figures; we can measure the tension of the gas in certain secretions: in the urine it is 9, in the bile 7 per cent. The tension in the cells themselves must be higher still.

The following figures (from Fredericq) give the tension of carbonic dioxide in percentages of an atmosphere:—

Tissues	5 to 9	in dog.
Venous blood	3·8 to 5·4	
Alveolar air	2·8	
External air	0·04	

The arrow indicates the direction in which the gas passes, namely, in the direction of pressure from the tissues to the atmosphere.

In view of such figures, and of Krogh's experiments, and having regard to the very slight changes in the tension of carbonic acid in the alveolar air, which are capable of affecting the respiratory centre (a subject we shall immediately pass to), we shall adhere to the view that diffusion explains the passage of that gas from the blood to the alveolar air, and that it is unnecessary to call to our assistance the hypothesis that secretory activity of the alveolar epithelium is at work.

CAUSE AND REGULATION OF RESPIRATION

The activity of the muscles of respiration is controlled by a specialised small district of grey matter in the floor of the fourth ventricle, which is called the respiratory centre. The activity of this centre is regulated by two main factors (1) the action upon it by afferent nerves, of which the most important are the vagus nerves from the lungs, and (2) the chemical condition of the blood.

If the lungs of an animal are alternately and forcibly inflated and deflated with air, or if a man voluntarily takes a number of deep breaths rapidly for a minute or two, breathing then ceases for a variable time, and this condition is termed *apnœa*. This condition is not due, as was at one time supposed, to an over-oxygenation of the blood, nor is it produced as Head considered, purely as a result of the excitation of the vagus nerve-endings in the lung, for it occurs after the vagus nerves are severed. Fredericq has always maintained that apnœa has a chemical rather than a nervous origin; he attributed it, however, not to over-oxygenation of the blood, but to a lessening of the carbonic acid which is swept out of the body by the powerful respiratory efforts. Haldane and Priestley corroborated this view by their important researches.

They found that, under constant atmospheric pressure, in man the alveolar air contains a nearly constant percentage of carbon dioxide in the same person. In different individuals this percentage varies somewhat, but averages 5·1 per cent. of an atmosphere in men, and 4·7 in women and children.

With varying atmospheric pressures, the percentage varies inversely as the atmospheric pressure, so that the pressure or tension of the carbon dioxide remains constant. The oxygen pressure, however, varies widely under the same conditions.

These observations and the next to be immediately described furnish the chemical key to the cause of the amount of pulmonary ventilation, and play an important part in conjunction with the respiratory nervous system in the regulation of breathing. For the respiratory centre is not only affected by the impulses reaching it by the vagi and other afferent nerves, but it is also very sensitive to any rise in the tension of carbon dioxide in the blood that supplies it. The changes in the tension of this gas in the arterial blood are normally proportional to the changes in the carbon dioxide pressure in the alveoli, and the changes in the lung alveoli are transmitted to the respiratory centre by the blood. They found that a rise of 0·2 per cent. in the alveolar carbon dioxide pressure is sufficient to double the amount of alveolar ventilation during rest. During work the alveolar carbon dioxide pressure increases slightly, and the pulmonary ventilation is consequently increased.

Changes in the oxygen pressure within wide limits have no such influence; the normal chemical stimulus to respiration is, therefore, an increase of carbon dioxide, and not diminution of oxygen. If these limits are exceeded, as when the oxygen pressure falls below 13 per cent. of an atmosphere, the respiratory centre begins to be excited for want of oxygen. Physiologists now agree that fatigue products, such as sarcolactic acid, assist the carbon dioxide in stimulating the respiratory centre. The important stimulus is not any particular acid, but the total hydrogen ion concentration.

In connection with the relative importance of the nervous and chemical factors in breathing, F. H. Scott has shown that the principal respiratory nerves (the pneumo-gastrics) regulate the rate or rhythm of the respiratory movements, whilst the chemical factor specially regulates the amount of pulmonary ventilation, that is, the depth of the individual respiratory efforts; for when these nerves are divided, a rise in the alveolar tension of carbon dioxide (or *great* diminution of the oxygen in the respired air) increases the depth, but not the rate of breathing.

In a normal respiration the chemical and nervous factors would, therefore, appear to be related somewhat as follows:—The inspiratory centre makes an effort, the degree of exaltation of the centre, and, therefore, the magnitude of the effort, more especially in the matter of depth, is governed by the tension of carbonic acid in the blood, but it is cut short by an inhibitory impulse passing up the vagus, only to begin again when the effects of this inhibitory impulse are removed.

TISSUE RESPIRATION

It must be borne in mind that pulmonary respiration is but the means of supplying the tissues with oxygen and removing from the body the waste products of tissue activity such as carbon dioxide. The amount of respiratory exchange which takes place in the tissues is connected with the degree of metabolism which occurs there.

Tissue respiration consists in the passage of oxygen from the blood of the capillaries to the cells of the tissues, and the passage of carbonic acid in the reverse direction. This gaseous interchange is no doubt brought about by a simple process of diffusion. The oxygen passes out of the plasma of the blood through the capillary wall, and then through the lymph until it reaches the cell in which it is going to be used. In order that a constant stream of oxygen may pass from the blood to the cell, there must be a difference of oxygen pressure between the oxygen dissolved in the plasma and that dissolved in the lymph, and the latter must be at a greater pressure than that dissolved in the cell. The amount of oxygen which passes will, other things being equal, be directly proportional to these pressure differences, and as the amount varies greatly at different times, it is obvious that the pressure differences vary greatly also. When, for instance, a muscle is at rest, the oxygen pressure in the capillaries is very near to that in the muscle fibre; when the muscle is active and using large quantities of oxygen, the intra-capillary oxygen pressure is much greater than the intra-muscular oxygen pressure. Such a change might be brought about by a rise in the intra-capillary oxygen pressure, or a fall in the intra-muscular oxygen pressure, or by both taking place simultaneously. Let us therefore inquire what is known about these quantities.

The tension of oxygen in muscle has recently been calculated as being at most equal to 19 mm. of mercury; from this it may vary down to zero. Within these limits the conditions for diffusion can be increased by a drop in the intra-muscular oxygen pressure.

There is, in addition, a mechanism for raising the intra-capillary oxygen pressure. This is the increased quantity of acid (carbonic and sarco-lactic acids) which is thrown into the blood as the result of the metabolism in the muscles and other tissues.

In glandular structures the oxygen pressure is higher than in muscle; probably owing to the relatively more copious blood-supply of glands, equilibrium is more readily established between the blood and the gland cells, the oxygen pressure in the cells being almost that present in venous blood.

The quantity of oxygen used by different tissues varies not only with the degree of their activity, but also with the nature of the tissues. On the whole it may be said that, weight for weight, glandular tissue uses most oxygen; next in order come the muscular tissues, and last of all, the connective tissues. There are some important tissues, notably the nervous system, about which little is known in this connection. The amount of oxygen used by an organ or tissue per gramme per minute is called its *coefficient of oxidation*.

In order to obtain the coefficient of oxidation, it is necessary:—(1) to estimate the gases in the blood going to and emerging from the organ; (2) to determine the amount of blood passing through the organ in a given time, say one minute; and (3) at the conclusion of the experiment the organ is weighed so that its gaseous exchange can be calculated.

In order to measure the gaseous exchange of an organ over a long period the organ is supplied with blood which alternately traverses the organ and aerates itself in a closed chamber. The amount of oxygen in the chamber is kept constant by the addition of that gas to the air of the chamber at the same rate at which the circulation acquires it. The amount of oxygen so added is measured. The method has recently been applied with conspicuous success to the gaseous exchange of the heart.

Relation of Tissue Respiration to Functional Activity. — In all organs, so far as is known, increased activity is accompanied by increased oxidation.

Much interest centres about the question of the order of time in which these events take place. This matter has been investigated in the case of skeletal muscle and the submaxillary gland, both of which organs can be thrown into profound activity for a short space of time; in each case most of the oxidation follows upon the activity, and not the activity upon the oxidation. The important inference is drawn that the contraction or secretion, as the case may be, is not caused by the oxidation in the sense that the machinery of a locomotive is driven by the energy derived from the oxidation of the coal; rather is the mechanism like that of a spring which is liberated at the moment of doing the work, and has to be rewound subsequently; the process of rewinding involves oxidation. In the case of muscle, the heat-formation which occurs in the period following activity only takes place if the muscle is supplied with oxygen. The output of carbonic acid, in its turn, follows upon the intake of oxygen. The order of events is therefore (1) increase of functional activity, (2) increase of heat formation and oxygen taken in, and (3) increase of carbonic acid put out.

The following table shows the coefficients of oxidation for resting organs, and the extent to which they are increased in activity.

Organ.	Condition of Rest.	Oxygen used per minute per gramme of organ.	Condition of Activity.	Oxygen used per minute per gramme of organ.
Voluntary muscle.	Nerves cut. Tone absent.	0·003 c.c.	Tone existing in rest. Gentle contraction. Active contraction.	0·006 c.c. 0·020 c.c. 0·080 c.c.
Unstriped muscle.	Resting.	0·004 c.c.	Contracting.	0·007 c.c.
Heart.	Very slow and feeble contractions.	0·007 c.c.	Normal contractions. Very active.	0·05 c.c. 0·08 c.c.
Submaxillary gland.	Nerves cut.	0·03 c.c.	Chorda stimulation.	0·10 c.c.
Pancreas.	Not secreting.	0·03 c.c.	Secretion after injection of secretin.	0·10 c.c.
Kidney.	Scanty secretion.	0·03 c.c.	After injection of diuretic.	0·10 c.c.
Intestines.	Not absorbing.	0·02 c.c.	Absorbing peptone.	0·03 c.c.
Liver.	In fasting animal.	0·01 to 0·02 c.c.	In fed animal.	0·03 to 0·05 c.c.
Suprarenal gland.	Normal.	·045 c.c.	...	...

Intensity of Respiration.—Most of the figures relating to gaseous metabolism given in the preceding paragraph were obtained from the examination of the tissues and organs of the dog. If all the tissues were examined in turn and their relative weights known, an average might be struck which would give the gaseous metabolism for the body taken as a whole, and this might be expressed as the amount of oxygen used per minute per gramme of body-weight. An easier and more practicable method would be to weigh the animal, and then from the composition of the inspired and expired air and the amount of oxygen taken in, and given out, calculate how much is retained and utilised. In the dog, the amount is about 0·016 c.c. of oxygen per minute per gramme of body-weight. This figure, however, is not the same in all animals, and the size of the figure will indicate what we may term the intensity of respiration. Thus in cold-blooded animals, especially fishes with their small supply of oxygen, the figure is very much smaller. But in warm-blooded animals great variations are seen; the intensity of respiration, for instance, is much greater in birds than in mammals. Among the mammals, the intensity of respiration varies, roughly, inversely with the size of the animal; thus, in the mouse, an animal that breathes with extreme rapidity, the intensity is probably ten to fifteen times greater than in the dog, and in the elephant very much less. In man, the average is about half that in the dog, that is, 0·008 c.c. of oxygen per gramme of body-weight per minute.

I have not included any practical exercises on Respiration at the head of this lesson, because I am aware how variable are the resources of physiological laboratories for this purpose. I would only suggest that as far as practicable the following experiments might be performed or shown by way of demonstration.

1. The use of the mercurial pump.
2. Analysis of blood gases by the chemical method.
3. Collection of alveolar air by the Haldane-Priestley method.
4. An analysis of a mixture of gases, say in expired air, by Haldane's apparatus described in the Appendix.
5. The use of the aërotonometer.

LESSON X

URINE

1. Test the reaction of urine with litmus paper.

2. Determine its specific gravity by the urinometer.

3. Test for the following INORGANIC SALTS :—

(a) *Chlorides.*—**Acidulate with nitric acid and add silver nitrate ; a white precipitate of silver chloride, soluble in ammonia, is produced. The object of acidulating with nitric acid is to prevent phosphates being precipitated by the nitrate of silver.**

(b) *Sulphates.*—**Acidulate with hydrochloric acid and add barium chloride. A white precipitate of barium sulphate is produced. Hydrochloric acid is added first, to prevent precipitation of phosphates.**

(c) *Phosphates.*—**i. Add ammonia ; a white crystalline precipitate of** *earthy* **(that is, calcium and magnesium) phosphates is produced. This becomes more apparent on standing. The** *alkaline* **(that is, sodium and potassium) phosphates remain in solution.**

ii. Mix another portion of urine with half its volume of nitric acid ; add ammonium molybdate, and boil. A yellow crystalline precipitate of ammonium phospho-molybdate falls. This test is given by both kinds of phosphates.

4. UREA.—Take some urea crystals. Observe that they are readily soluble in water, and that effervescence occurs when fuming nitric acid (*i.e.* **nitric acid containing nitrous acid in solution) is added to the solution. The effervescence is due to the breaking up of the urea. Carbonic acid and nitrogen come off. A similar bubbling, due to evolution of nitrogen, occurs when an alkaline solution of sodium hypobromite is added to another portion of the solution.**

5. Heat some urea crystals in a dry test-tube. Biuret is formed, and ammonia comes off.

$$\underset{\text{[urea]}}{2CON_2H_4} = \underset{\text{[biuret]}}{C_2O_2N_3H_5} + \underset{\text{[ammonia]}}{NH_3}$$

After cooling add a drop of copper-sulphate solution and a few drops of 20 per-cent. potash. A rose-red colour is produced.

6. Quantitative estimation of urea.

For this purpose Dupré's apparatus (fig. 37) is the most convenient. It consists of a bottle united to a measuring tube by indiarubber tubing. The measuring tube (an inverted burette will do very well) is placed within a cylinder of water, and can be raised and lowered at will. Measure 25 c.c. of an alkaline solution of sodium hypobromite (made by mixing 2 c.c. of bromine with 23 c.c. of a 40 per-cent. solution of caustic soda) into the bottle. Measure 5 c.c. of urine into a small tube, and lower it carefully, so that no urine spills, into the bottle. Close the bottle securely with a stopper perforated by a glass tube: this glass tube[1] is connected to the measuring tube by indiarubber tubing and a T-piece. The third limb of the T-piece is closed by a piece of indiarubber tubing and a pinch-cock, seen at the top of the figure. Open the pinch-cock and lower the measuring tube until the surface of the water is at the zero point of the graduation. Close the pinch-cock, and raise the measuring tube to ascertain whether the apparatus is air-tight. Then lower it again. Tilt the bottle so as to upset the urine, and shake well for a minute or so. During this time there is an evolution of gas. Then immerse the bottle in a large beaker containing water of the same temperature as that in the cylinder. After two or three minutes raise the measuring tube until the surfaces of the water inside and outside it are at the same level. Read off the amount of gas evolved. This is nitrogen. The carbonic acid resulting from the decomposition of urea has been absorbed by the excess of soda in the bottle. 354 c.c. of nitrogen are yielded by 1 gramme of urea. From this the quantity of urea in the 5 c.c. of urine and the percentage of urea can be calculated. If the total urea passed in the twenty-four hours is to be ascertained, the twenty-four hours' urine must be carefully measured and thoroughly

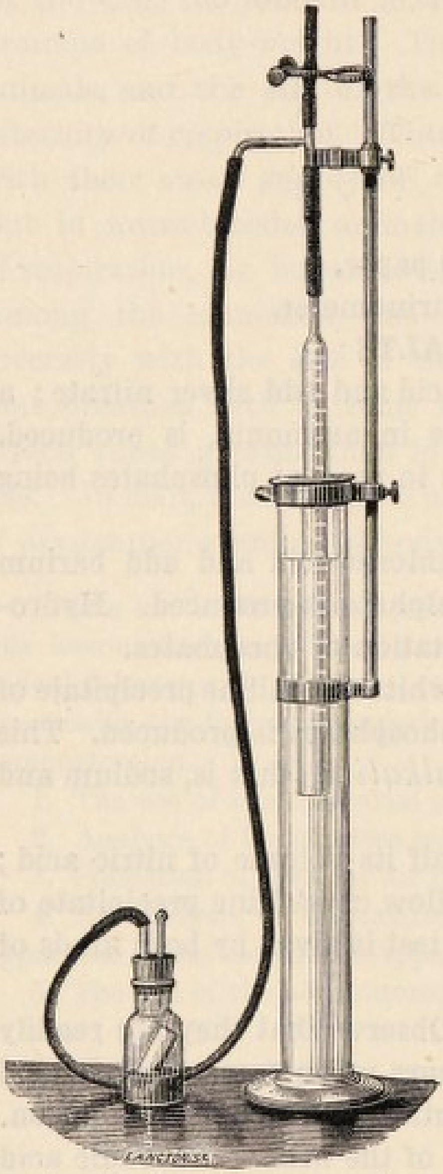
FIG. 37.—Dupré's urea apparatus.

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[1] The efficiency of the apparatus is increased by having a glass bulb blown on this tube to prevent froth passing into the rest of the apparatus. This is not shown in the figure.

mixed. A sample is then taken from the total for analysis; and then, by a simple sum in proportion, the total amount of urea is ascertained. More accurate methods for estimating urea are described in Lesson XXII.

7. CREATININE.—(a) *Weyl's Test.*—Add a little sodium nitroprusside and caustic soda to the urine. A red colour develops, which fades on boiling.

(b) *Jaffé's Test.*—Add picric acid and a few drops of strong potash; a deep red colour results. This method may be employed quantitatively (see Lesson XXIII.). If the urine contains sugar, the fluid becomes so dark as to be opaque.

The kidney is a compound tubular gland, the tubules of which differ much in the character of the epithelium that lines them in various parts of their course. The obviously secreting part of the kidney is the glandular epithelium that lines the convoluted portion of the tubules; there is in addition to this what is usually termed the filtering apparatus: tufts of capillary blood-vessels called the Malpighian glomeruli are supplied with afferent vessels from the renal artery; the efferent vessels that leave these have a smaller calibre, and thus there is high pressure in the Malpighian capillaries. Certain constituents of the blood, especially water and salts, pass through the thin walls of these vessels into the surrounding Bowman's capsule, which forms the commencement of each renal tubule. Bowman's capsule is lined by a flattened epithelium, which is reflected over the capillary tuft. Though the process which occurs here is generally spoken of as a filtration, many physiologists contest the correctness of this view. During the passage of this dilute urine through the rest of the renal tubule it gains the constituents, urea, urates, etc., which are poured into it by the secreting cells of the convoluted tubules.

GENERAL CHARACTERS OF URINE

Quantity.—A man of average weight and height passes from 1400 to 1600 c.c., or about 50 oz., daily. This contains about 50 grammes (1½ oz.) of solids. The urine should be collected in a tall graduated glass vessel capable of holding 3000 c.c., which should have a smooth-edged neck accurately covered by a ground-glass plate to exclude dust and avoid evaporation. A few drops of chloroform should be added as an antiseptic. From the total quantity thus collected in the twenty-four hours, samples should be drawn off for examination.

Colour.—This is some shade of yellow which varies considerably in health with the concentration of the urine. It appears to be due to a mixture of pigments: of these *urobilin* is the one of which we have the most accurate knowledge. Urobilin has a reddish tint and is ultimately derived from the blood pigment, and, like bile pigment, is an iron-free derivative of hæmoglobin. The bile pigment (and possibly also the hæmatin of the food) is in the intestines converted into stercobilin; most of the stercobilin leaves the body with the fæces; but some is reabsorbed and is excreted with the urine as urobilin. Urobilin is very like the artificial reduction product of bilirubin called hydrobilirubin (see p. 124). Normal urine, however, contains very little urobilin. The actual body present is a chromogen or mother substance called urobilinogen, which by oxidation (such as occurs when the urine stands exposed to the air) is converted into the pigment proper. In certain diseased conditions the amount of urobilin is considerably increased.

The most abundant urinary pigment is a yellow one called *urochrome.* Some regard it as a derivative of urobilin, but it probably is not related to that substance. (See Lesson XXV.)

Reaction.—The reaction of normal urine is acid to litmus. This acidity is mainly due to acid salts, especially acid sodium phosphate. In certain circumstances the urine becomes less acid and even alkaline; the most important of these are as follows:—

1. During digestion. Here there is a formation of free acid in the stomach, and a corresponding liberation of bases in the blood which passing into the urine diminish its acidity, or even render it alkaline. This is called *the alkaline tide*; the opposite condition, *the acid tide*, occurs after a fast—for instance, before breakfast.

2. In herbivorous animals and vegetarians. The food here contains excess of alkaline salts of acids such as tartaric, citric, malic, etc. These acids are oxidised into carbonates, which passing into the urine give it an alkaline reaction.

The true meaning of acidity and alkalinity is, however, more fully described in the Appendix.

Specific Gravity.—This should be taken in a sample of the twenty-four hours' urine with a good urinometer.

The specific gravity varies inversely as the quantity of urine passed under normal conditions from 1015 to 1025. A specific gravity below 1010 should excite suspicion of hydruria; one over 1030 of a febrile condition, or of diabetes, a disease in which it may rise to 1050. The specific gravity has, however, been known to sink as low as 1002 (after large potations, *urina potus*), or to rise as high as 1035 (after great sweating) in perfectly healthy persons.

Composition.—The following table gives the average amounts of the urinary constituents passed by a man (taking an ordinary diet containing about 100 grammes of protein) in the twenty-four hours:—

Total quantity of urine	1500·00 grammes
Water	1440·00 ,,
Total solids	60·00 ,,
Urea	35·00 ,,
Uric acid	0·75 ,,
Hippuric acid	1·05 ,,
Creatinine	0·91 ,,
Sodium chloride	16·5 ,,
Sulphuric acid	2·01 ,,

Phosphoric acid	3·16	grammes
Chlorine	11·00	,,
Ammonia	0·65	,,
Potassium	2·50	,,
Sodium	5·50	,,
Calcium	0·26	,,
Magnesium	0·21	,,

The most abundant constituents of the urine are water, urea, and sodium chloride. In the foregoing table the student must not be misled by seeing the names of the acids and metals separated. The acids and the bases are combined to form salts: urates, chlorides, sulphates, phosphates, etc.

UREA

Urea or **Carbamide**, $CO(NH_2)_2$, is isomeric (that is, has the same empirical, but not the same structural formula) with ammonium cyanate $(NH_4)CNO$, from which it was first prepared synthetically by Wöhler in 1828. Since then it has been prepared synthetically in other ways. Wöhler's observation derives interest from the fact that this was the first organic substance which was prepared synthetically by chemists.

When separated from the urine, it is found to be crystalline and readily soluble both in water and in alcohol: it has a saltish taste, and is neutral to litmus paper.

When treated with nitric acid, nitrate of urea $(CON_2H_4.HNO_3)$ is formed; this crystallises in octahedra, lozenge-shaped tablets, or hexagons (fig. 38, *a*). When treated with oxalic acid, prismatic crystals of urea oxalate $(CON_2H_4.H_2C_2O_4 + H_2O)$ are formed (fig. 38, *b*).

These crystals may be readily obtained in an impure form by adding excess of the respective acids to urine which has been concentrated to a third or a quarter of its bulk.[1]

Under the influence of an enzyme secreted by certain micro-organisms, such as the micrococcus ureæ, which grows readily in stale urine, urea takes up water, and is converted into ammonium carbonate $[CON_2H_4 + 2H_2O = (NH_4)_2CO_3]$. Hence the ammoniacal odour of putrid urine.

By means of nitrous acid, urea is broken up into carbonic acid, water, and nitrogen, $CON_2H_4 + 2HNO_2 = CO_2 + 3H_2O + 2N_2$. This may be used as a test for urea. Add fuming nitric acid (*i.e.* nitric acid containing nitrous acid in solution) to a solution of urea, or to urine; an abundant evolution of gas bubbles takes place.

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[1] The preparation of urea nitrate and urea oxalate is postponed to the next lesson, when other microscopic crystals will also be under examination.

Hypobromite of soda decomposes urea in the following way:—

$$\underset{\text{[urea]}}{CON_2H_4} + \underset{\text{[sodium hypobromite]}}{3NaBrO} = \underset{\text{[carbonic acid]}}{CO_2} + \underset{\text{[nitrogen]}}{N_2} + \underset{\text{[water]}}{2H_2O} + \underset{\text{[sodium bromide]}}{3NaBr}$$

This reaction is important, for on it one of the readiest methods for estimating urea depends. There have been various pieces of apparatus invented for rendering the analysis easy; but the one described in the practical exercise at the head of this lesson is the best. If the experiment is performed as directed, nitrogen is the only gas that comes off, the carbonic acid being absorbed by excess of soda. The amount of nitrogen is a measure of the amount of urea.

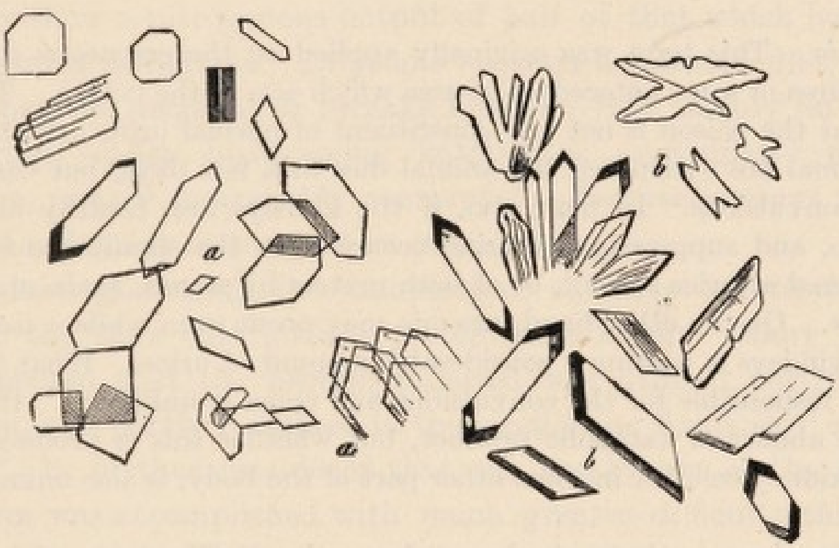


FIG. 38.—*a*, nitrate; *b*, oxalate of urea.

The quantity of urea excreted is somewhat variable, the chief cause of variation being the amount of protein food ingested. In a man taking the usual Voit diet containing about 100 grammes of protein (which will contain about 16 grammes of nitrogen) the quantity of urea excreted daily averages 33 grammes (500 grains). The percentage in human urine would then be 2 per cent.; but this also varies, because the concentration of the urine varies considerably in health. The excretion of urea is usually at a maximum three hours after a meal, especially after a meal rich in protein.

Muscular exercise has but little effect on the amount of nitrogen discharged. This is strikingly different from what occurs in the case of carbonic acid; the more the muscles work, the more carbonic acid do they send into venous blood, which is rapidly discharged by the expired air. Muscular energy is derived normally from the combustion of non-nitrogenous material; this is very largely carbohydrate. If the muscles, however, are not supplied with the proper amount of carbohydrate and fat, or if the work done is very excessive, then they consume some of their more precious protein material.

Where is Urea formed?—The older authors considered that it was formed in the kidneys, just as they also erroneously thought that carbonic acid was formed in the lungs. Prévost and Dumas were the first to show that after complete extirpation of the kidney the formation of urea and other waste products goes on, and these accumulate in the blood and tissues. Similarly, in those cases of disease in which the kidneys cease work, urea is still formed and accumulates. This condition is called *uræmia* (or urea in the blood), and unless the waste substances are discharged from the body the patient dies.

Uræmia.—This term was originally applied on the erroneous supposition that it is urea or some antecedent of urea which acts as the poison. There is no doubt that the poison is not any constituent of normal urine; if the kidneys of an animal are extirpated the animal dies in a few days, but there are no uræmic convulsions. In man also, if the kidneys are healthy or approximately so, and suppression of urine occurs from the simultaneous blocking of both renal arteries by clot, or of both ureters by stones, again uræmia does not follow. On the other hand, uræmia may occur even while a patient with diseased kindeys is passing a considerable amount of urine. What the poison is that is responsible for the convulsions and coma is unknown. It is doubtless some abnormal katabolic product, but whether this is produced by the diseased kidney cells, or in some other part of the body, is also unknown.

Where, then, is the seat of urea formation? The facts of experiment and of pathology point very strongly in support of the theory that urea is formed in the liver. The principal are the following:—

1. After removal of the liver in such animals as frogs, urea formation almost ceases, and ammonia is found in the urine instead.

2. In mammals, the extirpation of the liver is such a serious operation that the animals die. But the liver of mammals can be very largely thrown out of gear by the operation known as Eck's fistula, which consists in connecting the portal vein directly to the inferior vena cava. In these circumstances the liver receives blood only by the hepatic artery. The amount of urea is lessened, and its place is taken by ammonia.

3. When degenerative changes occur in the liver, as in *cirrhosis* of that organ, the urea formed is much lessened, and its place is taken by ammonia. In *acute yellow atrophy*, urea is almost absent from the urine, and, again, there is considerable increase in the ammonia. In

this disease amino-acids such as leucine and tyrosine are also found in the urine; these arise from the disintegration of the proteins of the liver cells, but they may in part originate in the intestine, and, escaping further decomposition in the degenerated liver, pass as such into the urine.

We have to consider next the intermediate stages between protein and urea. In order that the student may grasp the meaning of urea formation it would be advisable for him to turn again to p. 71 and read the paragraph there relating to Chittenden's views on diet, and to pp. 128 and 131, which treat of protein absorption, for the question, What is a normal diet? is intimately bound up with the question, What is a normal urine? If, for instance, the diet of the future is to contain only half as much protein as in the past, the urine of the future will naturally show a nitrogenous output of half of that which has hitherto been regarded as normal. In people on such a reduced diet, Folin has shown that the decrease in urinary nitrogen falls mainly on the urea, but certain other nitrogenous katabolites, particularly one called creatinine, remain remarkably constant in absolute amount in spite of the great reduction in the protein ingested.

The laws governing the composition of urine are obviously the effect of the laws that govern protein katabolism. Many years ago Voit supposed that the protein ingested was utilised partly in tissue formation, and partly remained in the circulating fluids as "circulating protein"; he further considered that the breakdown of the protein in the tissues was accomplished with much greater difficulty than that in blood and lymph, and that the small amount of "tissue-protein" which disintegrates as the result of the wear and tear of the tissues was dissolved and added to the "circulating protein," in which alone the formation of final katabolic products such as urea was supposed to occur. As time went on, it was shown that many facts were incompatible with this theory, and so it was largely displaced by Pflüger's view, in which it was held that the food protein must first be assimilated, and become part and parcel of living cells, before katabolism occurs. We now know that neither of these views is correct, and that nitrogenous katabolism is of two kinds: one kind varies with the food; it is therefore variable in amount, and occurs almost immediately or within a few hours after the food is absorbed; the amino-acids absorbed from the intestine are in great measure never built into living protoplasm at all, and are simply taken to the liver, where they are deamidised, and their nitrogenous part converted into urea. This variety of katabolism is called *exogenous*. The other kind of metabolism is constant in quantity and smaller in amount, and is due to the actual breakdown of protein matter in the body cells and tissues, which had been built into them previously. This form of metabolism is called *endogenous* or *tissue* metabolism, and the final product is in part urea, but the waste nitrogen finds its way out of the body in other substances also, of which creatinine appears to be important. This form of metabolism sets a limit to the lowest level of nitrogenous requirement attainable; the protein sufficient to maintain it is indispensable. Whether the amount of protein which is exogenously metabolised can be entirely dispensed with is at present questionable, and those who seek to replace it entirely by non-nitrogenous food are living dangerously near the margin. A point of considerable importance in this connection is, that the nitrogen of the protein is split off from it by hydrolysis without oxidation. There is thus very little loss of potential energy, the energy of the products being nearly equal to that of the original protein; it is, however, the non-nitrogenous residue which is mainly available for oxidation, and thus for calorific processes. The fact that muscular work does not normally increase nitrogenous metabolism becomes intelligible in the light of the consideration that protein katabolism, in so far as its nitrogen is concerned, is independent of the oxidations which give rise to heat, or to the energy which is converted into work. The body is very economical in so far as protein is concerned, and tissue or endogenous katabolism is kept at a low level.

What is the proportion between exogenous and endogenous nitrogen katabolism? It is very difficult to give any exact estimate. We do know that in ordinary diets, the former is far in excess, and probably in a man excreting 16 grammes of nitrogen daily (that is, the amount corresponding to an intake of 100 grammes of protein), only a quarter of this or even less represents tissue breakdown.

The view we have advanced concerning urea formation, then, is that it is mainly the result of the conversion, by the liver, of amino-acids absorbed from the intestine into that substance. This view receives confirmation from experiments in which certain amino-acids, such as glycine, leucine, and arginine, have been injected direct into the blood-stream. The result is an increased formation of urea. In the case of arginine the exact chemical decomposition which takes place is known. We have already seen that arginine is a compound of a urea radical and a substance called ornithine (di-amino-valeric acid, see p. 48); the liver is able to hydrolyse arginine, and so urea is liberated. This power is due to the action of a special enzyme called *arginase*, which, although it is also found in other organs, is specially abundant in the liver. In addition to this the ornithine itself is further broken up, and so an extra quantity of urea formed. On the other hand, there are some amino-acids (*e.g.* tyrosine) which on injection do not lead to any increase in urea formation.

If, however, we glance at the formula of ornithine, we see that it has one point in common with other amino-acids, such as glycine and leucine, to take simple examples :—

Ornithine $C_5H_{12}N_2O_2$
Glycine $C_2H_5NO_2$
Leucine $C_6H_{13}NO_2$

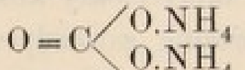
That is, in all cases the carbon atoms are more numerous than the nitrogen atoms. In urea, CON_2H_4, the reverse is the case. The amino-acids must therefore be split into simpler compounds, which unite with one another to form urea. Urea formation is thus in part synthetic. These simpler compounds are ammonium salts. Schröder's work proves that ammonium carbonate is one of the urea precursors, if not the principal one. The equation which represents the reaction is as follows :—

$$\underset{\text{[ammonium carbonate]}}{(NH_4)_2CO_3} = \underset{\text{[urea]}}{CON_2H_4} + \underset{\text{[water]}}{2H_2O}$$

Schröder's principal experiment was this : a mixture of defibrinated blood and ammonium carbonate was injected into the liver by the portal vein ; the blood leaving the liver by the hepatic vein was found to contain urea in abundance. This does not occur when the same experiment is performed with any other organ of the body, so that Schröder's experiments also prove the great importance of the liver in urea formation. Similar results were obtained by Nencki with ammonium carbamate.

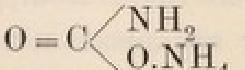
We must further remember that ammonia itself is one of the products of digestion of protein in the intestine, and it may possibly, to a small extent, be a result of tissue katabolism. This ammonia passes into the blood, where it unites with carbonic acid to form either the carbamate or carbonate of ammonium. Thus ammonia, whether formed directly or by the breakdown of amino-acids, is the principal immediate precursor of urea.

The following structural formulæ show the relationship between ammonium carbonate, ammonium carbamate, and urea :—



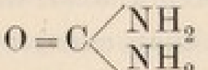
$$O=C\begin{cases}O.NH_4\\O.NH_4\end{cases}$$

[ammonium carbonate]



$$O=C\begin{cases}NH_2\\O.NH_4\end{cases}$$

[ammonium carbamate]



$$O=C\begin{cases}NH_2\\NH_2\end{cases}$$

[urea or carbamide]

The loss of one molecule of water from ammonium carbonate produces ammonium carbamate; the loss of a second molecule of water produces urea.

AMMONIA

The urine of man and carnivora contains small quantities of ammonium salts. The reason that some ammonia always slips through into the urine is that a part of the ammonia-containing blood passes through the kidney before reaching organs, such as the liver, which are capable of synthesising urea. In man the daily amount of ammonia excreted varies between 0·3 and 1·2 gramme: the average is 0·7 gramme. The ingestion of ammonium carbonate does not increase the amount of ammonia in the urine, but increases the amount of urea, into which substance the ammonium carbonate is easily converted. But if a more stable salt, such as ammonium chloride, is given, it appears as such in the urine.

In normal circumstances the amount of ammonia depends on the adjustment between the production of acid substances in metabolism and the supply of bases in the food. Ammonia formation is the physiological remedy for deficiency of bases.

When the production of acids is excessive (as in diabetes), or when mineral acids are given by the mouth or injected into the blood-stream, the result is an increase of the physiological remedy, and excess of the ammonia passes over into the urine. Under normal conditions ammonia is kept at a minimum, being finally converted into the less toxic substance urea, which the kidneys easily excrete. The defence of the organism against acids which are very toxic is an increase of ammonia formation, or, to put it more correctly, less of the ammonia formed is converted into urea.

Under the opposite conditions—namely, excess of alkali, either in food or given as such—the ammonia disappears from the urine, all being converted into urea. Hence the diminution of ammonia in the urine of man on a vegetable diet, and its absence in the urine of herbivorous animals.

Not only is this the case, but if ammonium chloride is given to a herbivorous animal like a rabbit, the urinary ammonia is but little increased. It reacts with sodium carbonate in the tissues, forming ammonium carbonate (which is excreted as urea) and sodium chloride. Herbivora also suffer much more from, and are more easily killed by, acids than carnivora, their organisation not permitting a ready supply of ammonia to neutralise excess of acids.

CREATINE AND CREATININE

Creatine is an abundant constituent of muscle; its chemical structure is very like that of arginine; it contains a urea radical, and by boiling it with baryta it splits into urea and sarcosine (methylglycine), as shown in the following equation:—

$$\underset{\text{[creatine]}}{C_4H_9N_3O_2} + \underset{\text{[water]}}{H_2O} = \underset{\text{[urea]}}{CON_2H_4} + \underset{\text{[sarcosine]}}{C_3H_7NO_2}$$

The same decomposition is shown graphically on p. 48.

Creatine is absent from normal urine, but it is present in the urine during starvation, in acute fevers, in women during involution of the uterus, and in certain other conditions in which there is rapid loss of muscular material.

Its normal fate in the body is unknown; it may be converted into urea as in the foregoing equation, but injection of creatine into the blood-stream does not cause any increase in urea formation; the creatine injected is almost wholly excreted unchanged.

It also is not converted into creatinine; although it has been generally assumed that this conversion does occur. The transformation of creatine into creatinine is shown in the following equation:—

$$\underset{\text{[creatine]}}{C_4H_9N_3O_2} - \underset{\text{[water]}}{H_2O} = \underset{\text{[creatinine]}}{C_4H_7N_3O}$$

Recent researches have entirely failed to substantiate the view that the urinary creatinine originates from the muscular creatine. If creatine (an innocuous neutral substance) were converted by the loss of water in the muscles into creatinine (a strongly basic substance), it would be contrary to all that is known of the chemical changes that occur in the body.

Creatinine is present in the urine. Amongst all the inconstancies of urinary composition, it appears to be the substance most constant in amount, diet and exercise having no effect on it. Folin's view, that its amount is a criterion of the extent of endogenous nitrogenous metabolism, has steadily gained ground, and the work of the past few years has shown that the liver and not the muscles is the seat of its formation. Some observers have supposed that certain tissue enzymes, termed creatase and creatinase, are agents in its formation and destruction; others have failed to discover the presence of these enzymes in the liver. On this and on other points there are differences of opinion, but without discussing the pros and cons of minor details, the following view of Mellanby may be taken as a working hypothesis of the metabolic history of the substances in question. Mellanby took as his starting-point the investigation of the contradictory data relating to the proportion of creatine and creatinine in muscle, and by improved methods showed that creatinine is never present in muscle at all, even after prolonged muscular work. He then studied in the developing bird the amount of creatine at different stages, and found that it is entirely absent in the chick's musculature up to the twelfth day of incubation; after this date the liver and the muscular creatine develop *pari passu*. After hatching, the liver still continues to grow rapidly, and the creatine percentage in the muscles increases also, although the development in the size of the muscles occurs very slowly. This and other experiments on the injection of creatine and creatinine into the blood-stream finally led Mellanby to the following hypothesis: Certain products of protein katabolism, the nature of which is uncertain, are carried by the blood to the liver, and from these the liver forms creatinine; this is transported to the muscles and there stored as creatine; when the muscles are saturated with creatine, excess of creatinine is then excreted by the kidneys. The small amount of creatinine excreted in diseases of the liver also supports the view that that organ is responsible for creatinine formation.

These views will no doubt be subjected to the usual tests of criticism and renewed research; they certainly appear to explain some of our previous difficulties, though the ultimate fate of the muscular creatine is still unsolved.

THE INORGANIC CONSTITUENTS OF URINE

The inorganic or mineral constituents of urine are chiefly chlorides, phosphates, sulphates, and carbonates; the metals with which these are in combination are sodium, potassium, ammonium, calcium, and magnesium. The total amount of these salts excreted varies from 19 to 25 grammes daily. The most abundant is sodium chloride, which averages in amount 10 to 16 grammes per diem. These substances are derived from two sources—first from the food, and secondly as the result of metabolic processes. The chlorides and most of the phosphates come from the food; the sulphates and some of the phosphates are a result of metabolism. The sulphates are derived from the changes that occur in the proteins; the nitrogen of proteins leaves the body chiefly as urea; the sulphur of the proteins is oxidised to form sulphuric acid, which passes into the urine in the form of sulphates. The excretion of sulphates, moreover, runs parallel to that of urea Sulphates, like urea, are the result of exogenous protein metabolism;

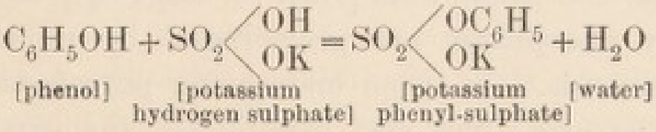
endogenous metabolism so far as sulphur is concerned is represented in the urine chiefly by less fully oxidised compounds of sulphur. The chief tests for the various salts have been given in the practical exercises at the head of this lesson.

Chlorides.—The chief chloride is that of sodium. The ingestion of sodium chloride is followed by its appearance in the urine, some on the same day, some on the next day. Some is decomposed to form the hydrochloric acid of the gastric juice. The salt, in passing through the body, fulfils the useful office of stimulating metabolism and excretion.

Sulphates.—The sulphates in the urine are principally those of potassium and sodium. They are derived from the metabolism of proteins in the body. Only the smallest trace enters the body with the food. Sulphates have an unpleasant bitter taste (for instance, Epsom salts); hence we do not take food that contains them. The sulphates vary in amount from 1·5 to 3 grammes daily.

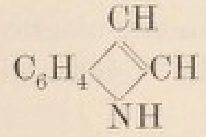
In addition to these sulphates there is a small quantity of sulphuric acid, comprising about one-tenth of the total which is combined with organic radicals; the compounds are known as **ethereal sulphates**, and they originate mainly from putrefactive processes occurring in the intestine. The most important of these ethereal sulphates are phenyl sulphate of potassium and indoxyl sulphate of potassium. The latter originates from the indole formed in the intestine, and as it yields indigo when treated with certain reagents it is sometimes called *indican*. It is very important to remember that the indican of urine is not the same thing as the indican of plants. Both yield indigo, but there the resemblance ceases.

The equation representing the formation of potassium phenylsulphate is as follows:—



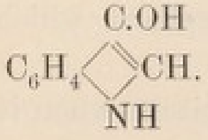
$$\underset{\text{[phenol]}}{C_6H_5OH} + \underset{\text{[potassium hydrogen sulphate]}}{SO_2{<}\begin{matrix}OH\\OK\end{matrix}} = \underset{\text{[potassium phenyl-sulphate]}}{SO_2{<}\begin{matrix}OC_6H_5\\OK\end{matrix}} + \underset{\text{[water]}}{H_2O}$$

The formation of potassium indoxyl-sulphate may be represented as follows:— Indole,



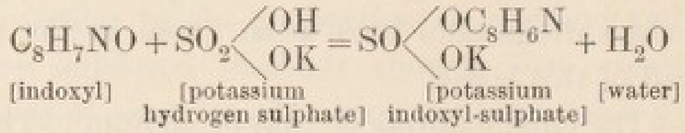
$$C_6H_4{<}\overset{CH}{\underset{NH}{\diamond}}{>}CH$$

on absorption is converted into indoxyl:



$$C_6H_4{<}\overset{C.OH}{\underset{NH}{\diamond}}{>}CH.$$

Indoxyl then interacts with potassium hydrogen sulphate as follows:—



$$\underset{\text{[indoxyl]}}{C_8H_7NO} + \underset{\text{[potassium hydrogen sulphate]}}{SO_2\left\langle\begin{matrix}OH\\OK\end{matrix}\right.} = \underset{\text{[potassium indoxyl-sulphate]}}{SO\left\langle\begin{matrix}OC_8H_6N\\OK\end{matrix}\right.} + \underset{\text{[water]}}{H_2O}$$

The formation of such sulphates is important; the aromatic substances liberated by putrefactive processes in the intestine are poisonous, but their conversion into ethereal sulphates renders them innocuous. (For tests for indoxyl in urine see Advanced Course, Lesson XXV.)

Carbonates.—Carbonates and bicarbonates of sodium, calcium, magnesium, and ammonium are present in alkaline urine only. They arise from the carbonates of the food, or from vegetable acids (malic, tartaric, etc.) in the food. They are, therefore, found in the urine of herbivora and vegetarians, whose urine is thus rendered alkaline. Urine containing carbonates becomes, like saliva, cloudy on standing, the precipitate consisting of calcium carbonate, and also phosphates.

Phosphates.—Two classes of phosphates occur in normal urine:—

(1) Alkaline phosphates—that is, phosphates of sodium (abundant) and potassium (scanty).

(2) Earthy phosphates—that is, phosphates of calcium (abundant) and magnesium (scanty).

The composition of the phosphates in urine is liable to variation. In acid urine the acidity is due to the acid salts. These are chiefly sodium dihydrogen phosphate, NaH_2PO_4, and calcium dihydrogen phosphate, $Ca(H_2PO_4)_2$.

In neutral urine, in addition, disodium hydrogen phosphate (Na_2HPO_4), calcium hydrogen phosphate, $CaHPO_4$, and magnesium hydrogen phosphate, $MgHPO_4$, are found. In alkaline urine there may be instead of, or in addition to the above, the normal phosphates of sodium, calcium, and magnesium [Na_3PO_4, $Ca_3(PO_4)_2$, $Mg_3(PO_4)_2$].

The earthy phosphates are precipitated by rendering the urine alkaline by ammonia. In urine undergoing putrefaction, ammonia is formed from the urea: this also precipitates the earthy phosphates. The phosphates most frequently found in the white creamy precipitate which occurs in decomposing urine are—

(1) Triple phosphate or ammonio-magnesium phosphate ($NH_4MgPO_4 + 6H_2O$). This crystallises in "coffin-lid" crystals (see fig. 39) or feathery stars.

(2) Stellar phosphate, or calcium phosphate, which crystallises in star-like clusters of prisms.

As a rule, normal urine gives no precipitate when it is boiled; but sometimes neutral, alkaline, and occasionally faintly acid urine gives a precipitate of calcium phosphate when boiled; this precipitate is amorphous, and is liable to be mistaken for albumin. It may be distinguished readily from albumin, as it is soluble in a few drops of acetic acid, whereas coagulated protein does not dissolve.

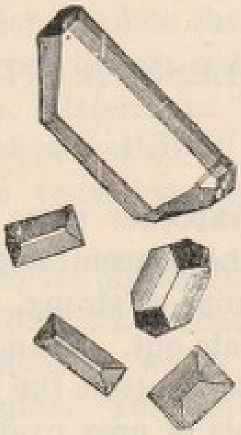


FIG. 39.—Ammonio-magnesium or triple phosphate.

The phosphoric acid in the urine chiefly originates from the phosphates of the food, but is partly a decomposition product of the phosphorised organic materials in the body, such as lecithin and nuclein. The amount of P_2O_5 in the twenty-four hours' urine varies from 2·5 to 3·5 grammes, of which the earthy phosphates contain about half (1 to 1·5 gr.).

LESSON XI

URINE (*continued*)

1. UREA NITRATE.—Evaporate some urine in a capsule to a quarter of its bulk. Pour the concentrated urine into a watch-glass; let it cool, and add a few drops of strong, but not fuming, nitric acid. Crystals of urea nitrate separate out. Examine these microscopically.

2. UREA OXALATE.—Concentrate the urine as in the last exercise, and add oxalic acid. Crystals of urea oxalate separate out. Examine these microscopically.

3. URIC ACID.—Examine microscopically the crystals of uric acid in some urine, to which 5 per cent. of hydrochloric acid has been added twenty-four hours previously. Note that they are deeply tinged with pigment, and to the naked eye look like granules of cayenne pepper.

When microscopically examined, the crystals are seen to be large bundles, principally in the shape of barrels, with spicules projecting from the ends, and whetstones. If oxalic acid is used instead of hydrochloric acid in this experiment, the crystals are smaller, and more closely resemble those observed in pathological urine in cases of uric acid gravel (see fig. 40).

Dissolve the crystals in caustic potash and then carefully add excess of hydrochloric acid. Small crystals of uric acid again form.

Murexide Test.—Place a little uric acid, or a urate (for instance, serpent's urine), in a capsule; add a little dilute nitric acid and evaporate to dryness. A yellowish-red residue is left. Add a little ammonia carefully. The residue turns to violet. This is due to the formation of murexide or purpurate of ammonia. On the addition of potash the colour becomes bluer.

Schiff's Test.—Dissolve some uric acid in sodium carbonate solution. Put a drop of this on blotting paper, add a drop of silver nitrate, and warm gently; the black colour of reduced silver is seen on the paper.

Folin's Test.—Suspend a minute quantity of uric acid in a few c.c. of water and add two or three drops of a saturated solution of sodium carbonate to dissolve it. To the clear solution add 1 or 2 c.c. of Folin's phosphotungstic acid reagent (see Lesson XXIII.), and enough saturated sodium carbonate solution (or a few crystals of sodium carbonate) to render the mixture alkaline. A blue colour develops. This test may be employed for the estimation of uric acid in urine (see Lesson XXIII.).

4. DEPOSITS OF URATES OR LITHATES (LATERITIOUS DEPOSIT).—The specimen of urine from the hospital contains excess of urates, which have become deposited on the urine becoming cool. They are tinged with pigment (uroerythrin), and have a pinkish colour, like brickdust; hence the term "lateritious." Examine microscopically. The deposit is usually amorphous—that is, non-crystalline. Sometimes crystals of calcium oxalate (envelope crystals—octahedra) are seen also; these are colourless.

The deposit of urates dissolves on heating the urine.

5. DEPOSIT OF PHOSPHATES.—Another specimen of pathological urine contains excess of phosphates, which have formed a white deposit on the urine becoming alkaline. This precipitate does not dissolve on heating; it may be increased. It is, however, soluble in acetic acid. Examine microscopically for coffin-lid crystals of triple phosphate (ammonio-magnesium phosphate), or crystals of stellar (calcium) phosphate, and for mucus. Mucus is flocculent to the naked eye, amorphous to the microscope.

N.B.—On boiling neutral, alkaline, or even faintly acid urine it may become turbid from deposition of phosphates. The solubility of this deposit in a few drops of acetic acid distinguishes it from albumin, for which it is liable to be mistaken.

Some of the facts described in the foregoing exercises have been already dwelt upon in the preceding lesson. They are, however, conveniently grouped together here, as all involve the use of the microscope.

URIC ACID

Uric acid ($C_5N_4H_4O_3$) is in mammals the medium by which only a small quantity of nitrogen is excreted from the body. It is, however, in birds and reptiles the principal nitrogenous constituent of their urine. It is not present in the free state, but is combined with bases to form urates.

It may be obtained from human urine by adding 5 c.c. of hydrochloric acid to 100 c.c. of the urine, and allowing the mixture to stand for twelve to twenty-four hours. The crystals which form are deeply tinged with urinary pigment, and though by repeated solution in caustic soda or potash, and reprecipitation by hydrochloric acid, they may be obtained free from pigment, pure uric acid is more readily obtained from the solid urine of a serpent or bird, which consists principally of the acid ammonium urate. This is dissolved in soda, and then the addition of hydrochloric acid produces as before the crystallisation of uric acid from the solution.

The pure acid crystallises in colourless rectangular plates or prisms. In striking contrast to urea it is a most insoluble substance; at 37° C. uric acid dissolves in pure water in the proportion of 1 : 15000 (Gudzent), and at 18° in the proportion 1 : 39500 (His and Paul). The forms which uric acid assumes when precipitated from human urine, either by the addition of hydrochloric acid or in certain pathological processes, are very various, the most frequent being the whetstone shape; there are also bundles of crystals resembling sheaves, barrels, and dumb-bells (see fig. 40).

The murexide test which has just been described among the practical exercises is the principal test for uric acid. The test has received the name on account of the resemblance of the colour to the purple of the ancients, which was obtained from certain snails of the genus *Murex*.

Another reaction that uric acid undergoes (though it is not applicable as a test) is that on treatment with certain oxidising reagents urea and oxalic acid can be obtained from it. It is, however, doubtful whether a similar oxidation occurs in the normal metabolic processes of the body.

Uric acid does not contain the carboxyl group (COOH) which is typical of organic acids, and in aqueous solution its reaction is neutral. Nevertheless one of its hydrogen atoms is replaceable by a metallic radical, and it acts therefore in aqueous solutions as a monobasic acid, and forms *primary salts* (also called mono-, bi-, or acid urates). In the presence of strong bases it forms *secondary salts* (also called neutral, normal, or di-urates). The secondary salts, however, only exist in the solid condition or in the presence of strong alkali. By water, they are decomposed at once into primary salt and alkali; by carbonic acid, they are decomposed into primary salt and alkali carbonate. A third series of salts (quadri-urates or hemi-urates) were formerly assumed to exist, but it has been shown that these substances are merely mixtures of uric acid and primary urate.

In water, in urine, and in the blood we have only to deal with primary urates. It has been recently shown that the primary or mono-sodium urate ($C_5H_3NaN_4O_3$) occurs in two modifications, the α and the β forms. The unstable α-salt is gradually transformed in aqueous solution into the stable β-salt owing to an intra-molecular change. It is assumed that the two salts correspond to the two tautomeric modifications of uric acid (see p. 201), the lactam form giving rise to the unstable and the lactim form to the stable salt. The solubility of the unstable α form at 37° C. is about 34 per cent. greater than that of the β form. These facts have a bearing on the pathological conditions occurring in gout; normally the small amount of urate in the blood is held in solution; in gout the amount is increased, and the excess is probably in the unstable α form: the conversion of this into the stable β form gives rise to the deposition of urates in the tissues which occurs during the course of the attack.

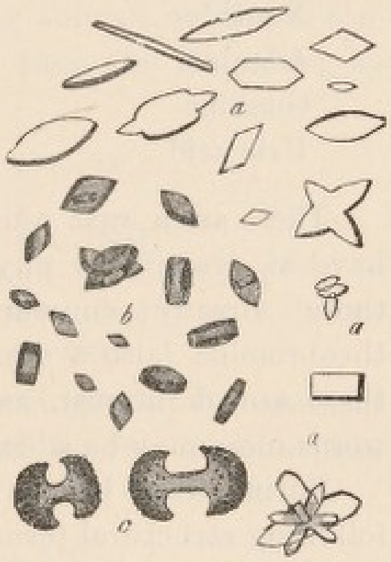


FIG. 40.—Uric acid crystals.

The quantity of uric acid excreted by an adult varies from 7 to 10 grains (0·5 to 0·75 gramme) daily. The method used for estimating uric acid is based on the discovery made by Hopkins, that when the urine is saturated with ammonium chloride, all the uric acid is precipitated in the form of ammonium urate; the precipitate is collected and the uric acid in it is then determined. Details of the Folin-Shaffer method founded on this reaction and of Folin's new colorimetric method with phosphotungstic acid are given in Lesson XXIII.

Origin of Uric Acid.—Uric acid is not formed in the kidney. When the kidneys are removed uric acid continues to be formed and accumulates in the organs, especially in the liver and spleen. The liver has been removed from birds, and uric acid is then hardly formed at all, its place being taken by ammonia and lactic acid. It is therefore probable that in these animals ammonia and lactic acid are normally synthesised in the liver to form uric acid.

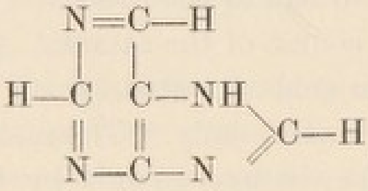
This synthetic origin of uric acid, which is so important in birds and snakes, does not, however, occur in mammals. In mammals uric acid is the chief end-product of the katabolism of cell nuclei or of nucleic acid, the principal constituent of the nuclei (see p. 64). This therefore leads us next to study:—

Purine Substances. — Emil Fischer has shown that among the decomposition products of nuclein are derivatives of a substance he has named purine. The empirical formulæ for purine, the purine bases, and uric acid are as follows:—

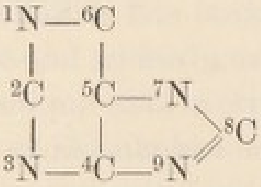
Purine . .	$C_5H_4N_4$		
Hypoxanthine .	$C_5H_4N_4O$	Monoxypurine	Purine bases.
Xanthine . .	$C_5H_4N_4O_2$	Dioxypurine	
Adenine . .	$C_5H_3N_4.NH_2$	Amino-purine	
Guanine . .	$C_5H_3N_4O.NH_2$	Amino-oxypurine	
Uric acid . .	$C_5H_4N_4O_3$	Trioxypurine	

There are a vast number of purine derivatives, but many of them have at present no physiological importance. Others in addition to those already enumerated are theophylline (dimethyl-xanthine), theobromine (also a dimethyl-xanthine), caffeine (trimethyl-xanthine); these are of interest, as they occur in tea, cocoa, and coffee. A few words more may be added in respect to those in our list.

Purine itself has never been discovered in the body. It has the following structural formula:—

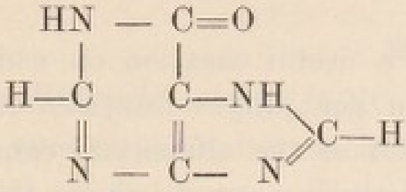


The purine nucleus is depicted in the next formula, and its atoms have been empirically numbered as shown, for convenience of description:—

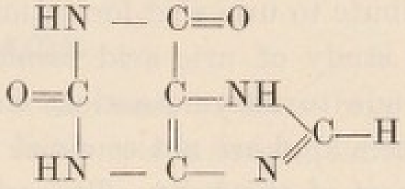


Hypoxanthine is found in the body tissues and fluids, and in the urine. It may be termed 6-oxypurine, as the oxygen is attached to the atom numbered 6 in the purine nucleus.

Xanthine is found with hypoxanthine in the body. It is 2, 6-dioxypurine, its oxygen atoms being attached to the atoms numbered 2 and 6 in the purine nucleus.



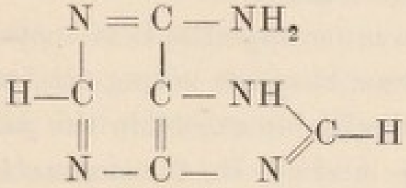
[hypoxanthine]



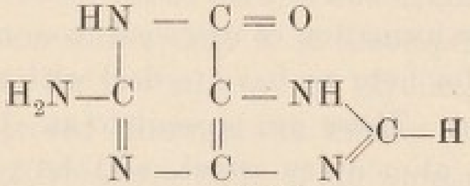
[xanthine]

Adenine is found in the tissues, blood, and urine. It is 6-amino-purine.

Guanine is also a decomposition product of nucleins. Combined with calcium it gives the brilliancy to the scales of fishes, and is also found in the bright tapetum of the eyes in these animals. It is a constituent of guano, and here is probably derived from the fish eaten by marine birds. It is 2-amino-6-oxypurine.

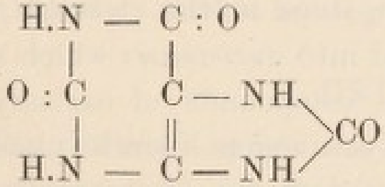


[adenine]

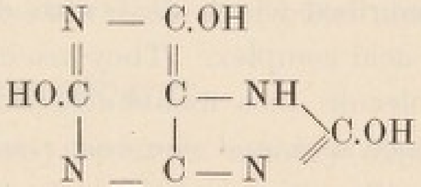


[guanine]

Uric acid (2, 6, 8-trioxypurine) offers an example of tautomerism (see p. 31). E. Fischer showed that it exists in two modifications according to the following formulæ :—



[lactam modification forming unstable α-urates]



[lactim modification forming stable β-urates]

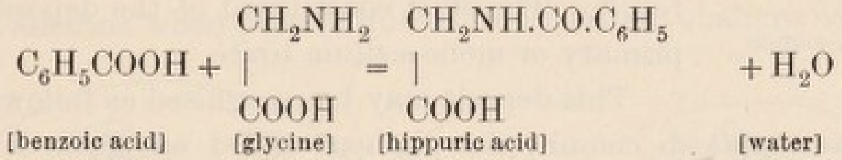
The close chemical relationship of uric acid to the purine bases is obvious from a study of the formulæ just given. Just as in the case of urea, uric acid, however, may be exogenously or endogenously formed. Certain kinds of food increase uric acid because they contain nuclein (for instance, sweetbreads) in abundance, or purine bases (for instance, hypoxanthine in meat); the uric acid which originates in this way is termed *exogenous*. Certain diets, on the other hand, increase uric acid formation by leading to an increase of leucocytes, and consequently to an increase in the metabolism of their nuclei; in other cases the leucocytes may increase from other causes, as in the disease named leucocythæmia. The uric acid that arises from nuclear katabolism is termed *endogenous*. Although special attention has been directed to the nuclei of leucocytes because they can be readily examined during life, it must be remembered that nuclein metabolism of all cells may contribute to uric acid formation.

A study of uric acid formation forms a useful occasion on which to allude to enzyme actions in metabolism generally. Enzymes of a digestive kind are not confined to the interior of the alimentary canal; but most of the body cells are provided with enzymes to assist them either in utilising the nutrient materials brought to them by the blood-stream, or in breaking them down previously to expelling them as waste substances. The enzyme which enables the liver cells to turn glycogen into sugar is the one which has been known longest. The enzyme called arginase (see p. 188), which leads to the hydrolysis of arginine into urea and ornithine, is one of the more recently discovered. Other examples which may be mentioned are proteolytic and peptolytic enzymes (tissue erepsin, etc.) found in many organs.

The formation of uric acid from nuclein is perhaps the best instance of all, for here we have to deal with numerous enzymes acting one after another. These are present to an almost negligible extent in the juices of the alimentary canal, and have been studied in the extracts of different organs; their distribution varies a good deal in different animals, and in the different organs of the same animal; speaking generally, they are most abundant in liver and spleen. The general term **nuclease** is given to the whole group, and a dozen or more have been described which deal with different steps in the cleavage of the nucleic acid complex. They are classified into *nucleinases* which resolve the molecule into mononucleotides, *i.e.*, compounds of carbohydrate, phosphoric acid, and one base; *nucleotidases* which liberate phosphoric acid, leaving the carbohydrate still united to the base; *nucleosidases* which hydrolytically cleave the base and carbohydrate apart; *deamidases* which remove the amino-group from the purine bases so set free; one of these, called *adenase*, converts adenine into hypoxanthine, and another, called *guanase*, converts guanïne into xanthine. Finally, *oxidases* step in, which convert hypoxanthine into xanthine, and xanthine into uric acid. But even that does not bring the list to a conclusion, for in some organs (especially the liver) there is a capacity to destroy uric acid after it is formed, and so animals are protected from a too great accumulation of this substance. What exactly happens to the uric acid is not certain, although it is clear that the products of its breakdown (probably allantoin and urea) are not so harmful as uric acid itself. The enzyme responsible for uric acid destruction is called the *uricolytic* enzyme. The uric acid which ultimately escapes as urates (normally) in the urine is the undestroyed residue. The uricolytic enzyme, however, is not present to any marked extent in the human subject.

HIPPURIC ACID

Hippuric acid ($C_9H_9NO_3$), combined with bases to form hippurates, is present in small quantities in human urine, but in large quantities in that of herbivora. This is due to the food of herbivora containing substances belonging to the aromatic group—the benzoic acid series. If benzoic acid is given to a man, it unites with glycine with the elimination of a molecule of water, and is excreted as hippuric acid :—



$$\underset{\text{[benzoic acid]}}{C_6H_5COOH} + \underset{\text{[glycine]}}{\begin{array}{l} CH_2NH_2 \\ | \\ COOH \end{array}} = \underset{\text{[hippuric acid]}}{\begin{array}{l} CH_2NH.CO.C_6H_5 \\ | \\ COOH \end{array}} + \underset{\text{[water]}}{H_2O}$$

This is a well-marked instance of synthesis carried out in the animal body, and experimental investigation shows that it is accomplished by the living cells of the kidney itself ; for if a mixture of glycine, benzoic acid, and defibrinated blood is injected through the kidney (or mixed with a minced kidney just removed from the body of an animal), their place is found to have been taken by hippuric acid.

It may be crystallised from horse's urine by evaporating to a syrup and saturating with hydrochloric acid. The crystals are dissolved in boiling water, filtered, and on cooling the acid again crystallises out. It melts at 186° C., and on further heating gives rise to the odour of bitter almond oil.

URINARY DEPOSITS

The different substances that may occur in urinary deposits are formed elements and chemical substances.

The **formed** or **histological elements** may consist of blood corpuscles, pus, mucus, epithelial cells, spermatozoa, casts of the urinary tubules, fungi, and entozoa. All of these, with the exception of a small quantity of mucus, which forms a flocculent cloud in the urine, are pathological, and the microscope is chiefly employed in their detection.

The **chemical substances** are uric acid, urates, calcium oxalate, calcium carbonate, and phosphates. Rarer forms are leucine, tyrosine, xanthine, and cystine. We shall, however, here only consider the commoner deposits, and for their identification the microscope and chemical tests must both be employed.

Deposit of Uric Acid.—This is a sandy reddish deposit resembling cayenne pepper. It may be recognised by its crystalline form (fig. 40, p. 199) and by the murexide reaction. The presence of these crystals generally indicates an increased formation of uric acid, and, if excessive, may lead to the formation of stones or calculi in the urinary tract.

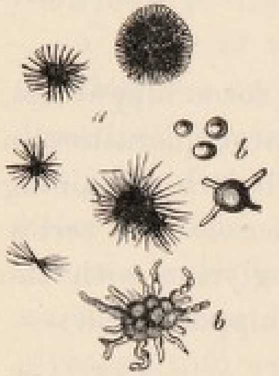


FIG. 41.—Mono-sodium urate.

Deposit of Urates.—This is much commoner, and may, if the urine is concentrated, occur in normal urine when it cools. It is generally found in the concentrated urine of fevers; and there appears to be a kind of fermentation, called the acid fermentation, which occurs in the urine after it has been passed, and which leads to the same result. The chief constituent of the deposit is the primary or mono-sodium urate.

This deposit may be recognised as follows :—

1. It has a pinkish colour; the pigment called *uro-erythrin* is one of the pigments of the urine, but its relationship to the other urinary pigments is not known (see further Lesson XXV.).

2. It dissolves upon warming the urine.

Microscopically it is usually amorphous, but crystalline forms similar to those depicted in figs. 41 and 42 may occur.

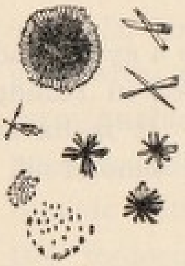


FIG. 42.—Mono-ammonium urate.

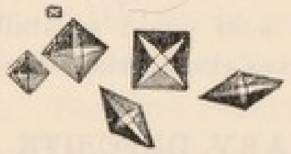


FIG. 43.—Envelope crystals of calcium oxalate.

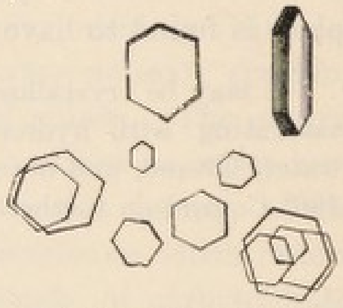


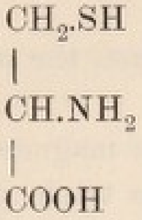
FIG. 44.—Cystine crystals.

Crystals of calcium oxalate may be mixed with this deposit (see fig. 43).

Deposit of Calcium Oxalate.—This occurs in envelope crystals (octahedra) or dumb-bells.

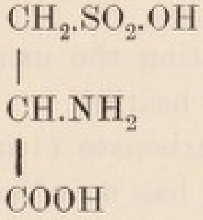
It is insoluble in ammonia, and in acetic acid. It is soluble with difficulty in hydrochloric acid.

Deposit of Cystine.—Cystine ($C_6H_{12}N_2S_2O_4$) is recognised by its colourless six-sided crystals (fig. 44). These are rare: they occur only in acid urine, and they may form concretions or calculi. The constitution of cystine is given on p. 49; by reduction a substance called *cysteine* (amino-thio-propionic acid) is obtained from it. Cysteine yields on oxidation cysteic acid, and this splits into taurine and carbon dioxide. There is a good deal of evidence that the taurine of the bile is the source of the cystine of the urine; this anomalous course of metabolism runs in families. The following formulæ indicate the relationship of cysteine, cysteic acid, and taurine:—



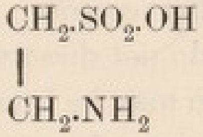
$$\begin{array}{l} CH_2.SH \\ | \\ CH.NH_2 \\ | \\ COOH \end{array}$$

[cysteine]



$$\begin{array}{l} CH_2.SO_2.OH \\ | \\ CH.NH_2 \\ | \\ COOH \end{array}$$

[cysteic acid]



$$\begin{array}{l} CH_2.SO_2.OH \\ | \\ CH_2.NH_2 \end{array}$$

[taurine]

Deposit of Phosphates.—These occur in alkaline urine. The urine may be alkaline when passed, due to fermentative changes occurring in the bladder. All urine, however, if exposed to the air (unless the air is perfectly pure, as on the top of a snow mountain), will in time become alkaline owing to an enzyme formed during the growth of the *micrococcus ureæ*. This forms ammonium carbonate from the urea.

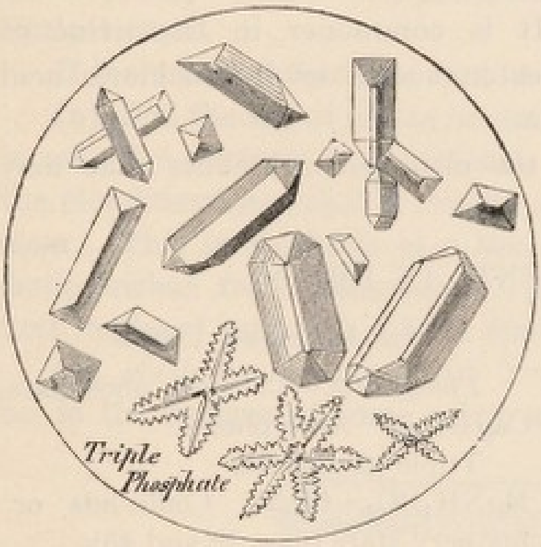


FIG. 45.—Triple phosphate crystals.

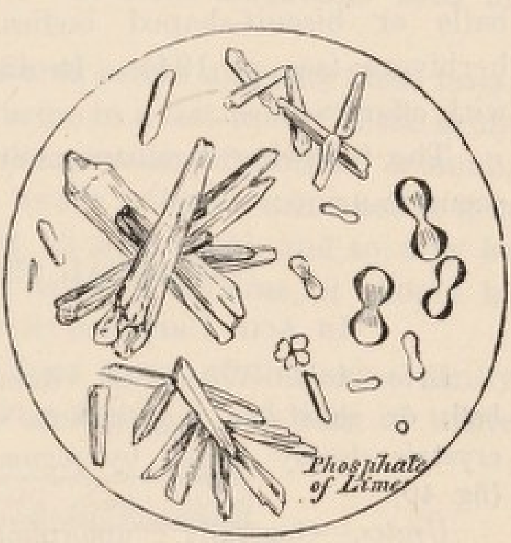


FIG. 46.—Crystals of phosphate of lime (stellar phosphate).

$$\underset{\text{[urea]}}{CON_2H_4} + \underset{\text{[water]}}{2H_2O} = \underset{\text{[ammonium carbonate]}}{(NH_4)_2CO_3}$$

The ammonia renders the urine alkaline, and precipitates the earthy phosphates. The chief forms of phosphates that occur in urinary deposits are:—

1. Calcium phosphate, $Ca_3(PO_4)_2$; amorphous.

2. Triple or ammonio-magnesium phosphate, $MgNH_4PO_4 + 6H_2O$; coffin-lids and feathery stars (fig. 45).

3. Crystalline phosphate of calcium, $CaHPO_4$, in rosettes of prisms, in spherules, or in dumb-bells (fig. 46).

4. Magnesium phosphate, $Mg_3(PO_4)_2 + 2H_2O$, occurs occasionally and crystallises in long plates.

All these phosphates are dissolved by acids, such as acetic acid, without effervescence.

They do not dissolve on heating the urine; in fact, the amount of precipitate may be increased by heating.

A solution of ammonium carbonate (1-in-5) eats magnesium phosphate away from the edges; it has no effect on the triple phosphate. A phosphate of calcium ($CaHPO_4 + 2H_2O$) may occasionally be deposited in acid urine. Pus in urine is apt to be mistaken for phosphates, but can be distinguished by the microscope.

Deposit of calcium carbonate, $CaCO_3$, appears but rarely as whitish balls or biscuit-shaped bodies. It is commoner in the urine of herbivora (see p. 194). It dissolves in acetic or hydrochloric acid with effervescence.

The following is a summary of the chemical sediments that may occur in urine:—

CHEMICAL SEDIMENTS IN URINE

IN ACID URINE

Uric Acid.—Whetstone, dumb-bell, or sheaf-like aggregations of crystals deeply tinged by pigment (fig. 40).

Urates.—Generally amorphous. The primary urate of sodium (fig. 41) and of ammonium (fig. 42) may sometimes occur in star-shaped clusters of needles of spheroidal clumps with projecting spines. Tinged brick-red. Soluble on warming.

Calcium Oxalate.— Octahedra, so-called envelope crystals (fig. 43). Insoluble in acetic acid.

Cystine.—Hexagonal plates (fig. 44). Rare.

Leucine and Tyrosine.—Rare.

Calcium Phosphate.
$CaHPO_4 + 2H_2O$.—Rare.

IN ALKALINE URINE

Phosphates.—Calcium phosphate, $Ca_3(PO_4)_2$. Amorphous.

Triple phosphate, $MgNH_4PO_4 + 6H_2O$. Coffin-lids or feathery stars (figs. 39 and 45).

Calcium hydrogen phosphate, $CaHPO_4$. Rosettes, spherules, or dumb-bells (fig. 46).

Magnesium phosphate, $Mg_3(PO_4)_2 + 2H_2O$. Long plates.

All soluble in acetic acid without effervescence.

Calcium Carbonate, $CaCO_3$.—Biscuit-shaped crystals. Soluble in acetic acid with effervescence.

Ammonium Urate. — "Thorn-apple" spherules.

Leucine and Tyrosine.—Very rare.

LESSON XII

PATHOLOGICAL URINE

1. **URINE A** is pathological urine containing albumin. It gives the usual protein tests. The following two are most frequently used in clinical work.

If the urine is cloudy, it should be filtered before applying these tests.

(a) Boil the top of a long column of urine in a test-tube. If the urine is acid, the albumin is coagulated. If the quantity of albumin is small, the cloudiness produced is readily seen, as the unboiled urine below it is clear. This is insoluble in a few drops of acetic acid, and so may be distinguished from phosphates. If the urine is alkaline, it should be first rendered acid with a little dilute acetic acid.

(b) *Heller's Nitric-Acid Test.*—Pour some of the urine gently on to the surface of some nitric acid in a test-tube. A ring of white precipitate occurs at the junction of the two liquids. This test is used for small quantities of albumin.

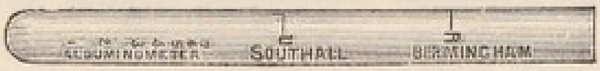


FIG. 47.—Albuminometer of Esbach.

2. **ESTIMATION OF ALBUMIN BY ESBACH'S ALBUMINOMETER.**—Esbach's reagent for precipitating the albumin is made by dissolving 10 grammes of picric acid and 20 grammes of citric acid in 800 or 900 c.c. of boiling water, and then adding sufficient water to make up to a litre (1000 c.c.).

Pour the urine into the tube up to the mark U; then the reagent up to the mark R. Close the tube with a cork, and to ensure complete mixture tilt it to and fro a dozen times without shaking. Allow the corked tube to stand upright twenty-four hours; then read off on the scale the height of the precipitate. The figures indicate grammes of dried albumin in a litre of urine. The percentage is obtained by dividing by 10. Thus, if the sediment stands at 3, the amount of albumin is 3 grammes per litre, or 0·3 gr. in 100 c.c. When the albumin is so abundant that the sediment is above 4, a more accurate result is obtained by first diluting the urine with one or two volumes of water, and then multiplying the resulting figure by 2 or 3, as the case may be. If the amount of albumin is less than 0·05 per cent., it cannot be accurately estimated by this method.

3. **URINE B** is diabetic urine. It has a high specific gravity. The presence of sugar is shown by the reduction (yellow precipitate of cuprous oxide) that occurs on boiling with Fehling's solution.

4. **ESTIMATION OF GLUCOSE.**—The following four methods are available for the quantitative determination of glucose: (a) The polarimetric method (see Appendix); (b) the fermentation method; (c) the gravimetric, and (d) volumetric methods by means of Fehling's or similar solutions.

The fermentation method is less accurate than the other methods. It is carried out in a fermentation saccharimeter, such as Einhorn's. This consists of a U-shaped tube, the longer limb of which is closed and carries an empirical graduation, indicating the percentage of glucose, corresponding to the amount of carbonic acid gas developed. Ten c.c. of the urine, mixed with some yeast, are taken and the apparatus is filled with this mixture, care being taken to remove all air-bubbles. After twenty-four hours' fermentation at room temperature, the percentage of glucose is read off.

Instead of fresh yeast it is more convenient to employ the commercial preparation Zymin, a dry powder consisting of yeast which has been killed by acetone treatment and which retains the active enzyme (zymase).

The gravimetric method is the most accurate of all, and of the many modifications of it the Kjeldahl-Allihn method is now considered the best. In this the reduction of Fehling's solution, taken in excess, is carried out in an atmosphere of hydrogen (or coal gas), the precipitate of cuprous oxide is filtered through a Soxhlet's asbestos tube and finally reduced to metallic copper by heating the tube in a current of hydrogen. From the amount of copper found by weighing the tube before and after the experiment, the quantity of glucose is calculated by means of a table compiled for this purpose.

Of the *volumetric methods* those of Ling and Rendle and of Benedict are given in this place; others will be found in Lesson XIII.

LING AND RENDLE'S VOLUMETRIC METHOD.—The sugar solution (diabetic urine) is allowed to run from a burette into a known volume of boiling Fehling's solution. The end point, *i.e.* the complete reduction of the cupric salt is recognised by means of a solution of ferrous thiocyanate. When a drop of this indicator is placed on a slab, and a drop of a solution containing a cupric salt brought in contact with it, oxidation of the ferrous salt occurs, with the immediate production of the well-known red colour of ferric thiocyanate.

Preparation of the Indicator.—One gramme of ferrous ammonium sulphate and 1·5 grammes of ammonium thiocyanate are dissolved in 10 c.c. of water at a moderate temperature and immediately cooled; 2·5 c.c. of concentrated hydrochloric acid are then added. The solution so obtained has invariably a brownish-red colour, due to the presence of ferric salt, which latter must therefore be reduced. This is effected by shaking with a small quantity of zinc dust.

Preparation of Fehling's Solution.—Solution No. 1.—69·278 grammes of crystallised copper sulphate are dissolved in water, and the solution made up to 1 litre.

Solution No. 2.—346 grammes of crystallised potassium-sodium tartrate (Rochelle salt) are dissolved in hot water, mixed with 142 grammes of caustic soda, also dissolved in water, and after cooling made up to 1 litre.

Equal volumes of these two solutions are accurately measured out and mixed in a dry flask before use. 10 c.c. of the mixed solution are equivalent to 0·05 gramme of glucose.

Analysis.—Freshly mixed Fehling's solution (10 c.c.) is accurately measured into a 200-c.c. boiling flask, diluted with about an equal quantity of water, and raised to boiling point. The urine is diluted with water and placed in a burette; the dilution should be adjusted so that 20 to 30 c.c. of the diluted urine are required to reduce the 10 c.c. of Fehling's solution. In the first experiment the urine should be diluted with nine times its volume of water. This diluted urine is then run into the boiling liquid in small amounts, commencing with 5 c.c. After each addition of sugar solution the mixture is boiled, the liquid being kept moving. About a dozen drops of the indicator are placed on a porcelain slab, and when it is judged that the precipitation of cuprous oxide is complete (that is, when the blue colour of the solution is disappearing), a drop of the liquid is withdrawn by a clean glass rod or by a capillary tube, and brought in contact with the middle of one of the drops of the indicator on the slab. The end point is reached when the mixture ceases to give a red colour with a drop of the indicator. It is essential to perform the titration as rapidly as possible, as an atmosphere of steam is then kept in the neck of the flask and the influence of atmospheric oxygen avoided. At the final point the liquid is boiled for about ten seconds. As in all volumetric methods the first titration may only give approximate results and a second will then be necessary to establish accurately the end point. Each titration should take from two to three minutes.

Example.—Suppose that the urine has been diluted tenfold with water, and that 20 c.c. of the diluted urine are found necessary to reduce the 10 c.c. of Fehling's solution; this will be equivalent to 2 c.c. of the original urine, and that amount will therefore contain 0·05 gramme of sugar; 1 c.c. will contain $\frac{0·05}{2}$, and 100 c.c. will contain $\frac{0·05}{2} \times 100 = 2·5$ grammes of sugar.

Pavy's modification of Fehling's solution is sometimes used. Here ammonia holds the cuprous oxide in solution, so that no precipitate forms in boiling Pavy's solution with a reducing sugar. The reduction is complete when the blue colour disappears. 10 c.c. of Pavy's solution = 1 c.c. of Fehling's solution = 0·005 gramme of glucose.

5. **BENEDICT'S METHOD.**—Benedict's reagent is an alkaline solution of copper sulphate containing potassium thiocyanate. This is kept boiling and the sugar solution is run into it from a burette until the blue colour disappears; the thiocyanate forms a white precipitate with the cuprous oxide formed, so that no red cuprous oxide obscures the blue tint.

Preparation of the Solution.—Sodium citrate, 200 grammes, sodium carbonate (crystalline), 200 grammes (or anhydrous sodium carbonate, 75 grammes), and potassium thiocyanate, 125 grammes, are dissolved in hot water; this when cool is made up with distilled water to 800 c.c. and filtered.

18 grammes of pure copper sulphate are then dissolved in 100 c.c. of water, and poured slowly with constant stirring into the first solution. 5 c.c. of a 5 per-cent. solution of potassium ferrocyanide are then added as an additional precaution to prevent any deposition of cuprous oxide; finally the total volume of the mixture is made up to 1000 c.c. with distilled water. 25 c.c. of this solution are reduced by 0·05 gramme of glucose.

Analysis.—3 or 4 grammes of anhydrous sodium carbonate are placed in a 4-oz. flask, then 25 c.c. of the above solution. This is kept boiling over a small flame, and the sugar solution run in from a burette until the last trace of blue colour disappears. The amount used for this purpose is then read off.

Calculation.—This may be illustrated by an example. If the sugar solution had been diluted 1-in-5 (that is, 10 c.c. with 40 c.c. of water), and the reading of the burette was 10 c.c., then

10 c.c. of the diluted solution = 2 c.c. of the original solution.
2 c.c. of the original solution contain 0·05 gramme of glucose.
1 c.c. „ „ contains $\frac{0·05}{2}$ „
and 100 c.c. „ „ contain $\frac{0·05 \times 100}{2}$ „
$= 2·5$ glucose per cent.

Estimation of other Reducing Sugars.—The same two methods may be used for the estimation of other reducing sugars; the only difference is in the final calculation:—

10 c.c. of Fehling's solution =0·05 gramme of glucose
(or 25 c.c. of Benedict's solution) =0·053 ,, fructose.
=0·0676 ,, lactose.
=0·074 ,, maltose.

Estimation of Sucrose.—Boil 40 c.c. of the sucrose solution with 30 c.c. of half-normal hydrochloric acid for one minute. Cool, neutralise with 30 c.c. of half-normal sodium hydroxide, cool and make up the total volume to 100 c.c. The reducing sugars so formed are then estimated as before, and the results calculated from the fact that 10 c.c. of Fehling's solution (or 25 c.c. of Benedict's solution)=0·0475 sucrose.

6. **ACETO-ACETIC ACID AND ACETONE** are frequently found in diabetic urine and may be detected as follows:—

(a) To 3 c.c. of the urine add a few drops of 10 per-cent. solution of ferric chloride as long as a precipitate (ferric phosphate) continues to be formed. Filter this off, and add to the filtrate a few more drops of the ferric chloride solution. A claret-like colour (which disappears on heating) is developed if *aceto-acetic acid* is present.[1]

(b) Acidulate the urine with sulphuric acid and shake up with ether; the ether on standing floats on the top; it contains the aceto-acetic acid in solution. Pour it off into another test-tube and shake with ferric chloride solution. A red colour is produced if aceto-acetic acid is present.

(c) Heat 250 c.c. of the urine with dilute acid or alkali; the aceto-acetic acid is converted into acetone. Distil the mixture, and collect the first 20 c.c. of the distillate, and examine this for acetone.

(d) *Acetone* in the urine itself or in the distillate just obtained may be detected by the following tests:—

i. Legal's test. Add a dilute freshly prepared solution of sodium nitro-prusside and a little 20 per-cent. caustic potash. A red colour is produced. Acidify with strong acetic acid; the colour disappears at once in the absence of acetone, but remains or is intensified into a purple in its presence. This test is also given by aceto-acetic acid.

ii. Lieben's iodoform test. Add a few c.c. of 20 per-cent. potash and then some iodine solution which contains also some potassium iodide. A whitish opalescence forms, and iodoform is detected by its characteristic odour.

iii. The iodoform test may be modified as follows: Add an alcoholic solution of iodine and a little strong ammonia; iodoform and a black precipitate of nitrogen iodide are formed; the latter gradually disappears, leaving the iodoform. (This is Gunning's test and serves to distinguish acetone from alcohol, Lieben's test being given by both alcohol and acetone.)

---

[1] Carbolic acid, salicylic acid, and phenaceturic acid, all of which may occur in urine after drug treatment, give a similar colour reaction in both fresh and previously boiled urine; whilst aceto-acetic acid does not give the reaction if the urine has been previously boiled.

iv. Acetone does not reduce Fehling's solution or ammoniacal silver solution as aldehyde does.

7. URINE C is from a case of jaundice and contains bile.

(a) *Bile pigment* may be detected by Gmelin's test (see p. 108), or by Huppert's test (Cole's modification), which is performed as follows:—Precipitate about 30 c.c. of the urine with baryta mixture; [1] heat and allow the precipitate to settle. The precipitate contains, among other things, a barium compound of bile pigment. Pour off the supernatant fluid, and then collect the precipitate on a filter. Scrape off the precipitate from the filter-paper and place it in a test-tube; add a few c.c. of alcohol, 2 drops of strong sulphuric acid, and 2 drops of a 5 per-cent. solution of potassium chlorate [2] and boil for a minute. Allow the precipitate of barium sulphate to settle, and the supernatant alcohol is seen to be coloured greenish-blue. On carefully adding fuming nitric acid after cooling, the solution becomes blue, violet, and red.

(b) *Bile salts* may be detected by Hay's sulphur test (p. 108). Pettenkofer's test may be performed in the following way: Warm a thin film of the urine and cane-sugar solution in a flat porcelain dish; then dip a glass rod in strong sulphuric acid and draw it across the film; its track is marked by a purplish line.

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[1] Made by mixing one volume of barium nitrate solution with two of barium hydrate solution, both saturated *in the cold*.

[2] Obermayer's reagent (hydrochloric acid containing 0·2 per cent. of ferric chloride, see under tests for indican, Lesson XXV.) may be used instead of potassium chlorate solution.

PROTEINS IN THE URINE

There is no protein matter in normal urine, and the most common cause of the appearance of albumin in the urine is disease of the kidney (Bright's disease). The best methods of testing for and estimating the albumin are given in the practical heading to this lesson. The term "albumin" is the one used by clinical observers. Properly speaking, it is a mixture of serum albumin and serum globulin.

A condition called "peptonuria," or peptone in the urine, is observed in certain pathological states, especially in diseases where there is a formation of pus, and particularly if the pus is decomposing owing to the action of a bacterial growth called staphylococcus; one of the products of disintegration of pus cells appears to be peptone; and this leaves the body by the urine. The term "peptone," however, is in the strict sense incorrect; the protein present is deutero-proteose. In certain diseases of bone, a proteose (Bence-Jones protein) may be found in the urine, which more nearly resembles hetero-proteose in its characters.

SUGAR IN THE URINE

Normal urine contains no sugar, or so little that for clinical purposes it may be considered absent. It occurs in the disease called diabetes mellitus, and can be artificially produced by the methods briefly referred to on p. 119.

The methods usually adopted for detecting and estimating the sugar are given at the head of this lesson. The sugar present is glucose. Lactose may occur in the urine of nursing mothers. The blood of diabetic persons often contains β-hydroxybutyric acid; some of this passes into the urine, but in the body it is largely converted into aceto-acetic acid and acetone, in which form it is passed in the urine (see p. 120).

β-hydroxybutyric acid may be detected by fermenting the urine completely with yeast, and then examining it with the polarimeter; the β-hydroxybutyric acid is not affected by yeast, and its presence is indicated by lævo-rotation.

Fehling's test is not absolutely trustworthy. Often a normal urine will decolorise Fehling's solution, although seldom a red precipitate is formed. This is due to excess of urates and creatinine. Another substance, called glycuronic acid ($C_6H_{10}O_7$), is also likely to be confused with sugar by Fehling's test; the cause of its appearance is sometimes the administration of drugs (chloral, camphor, etc.); but in rare cases it appears independently of drug treatment in normal urine. It is frequently found in diabetic urine. This acid is produced by the oxidation of glucose, the H_2 in the CH_2OH group being replaced by O.

Then, too, in the rare condition called alcaptonuria, confusion may similarly arise. Alcapton is a substance which originates from tyrosine by an unusual form of metabolism. It gives the urine a brown tint, which darkens on exposure to the air. It is an aromatic substance, and the researches of Baumann and Wolkow have identified it with homogentisic acid [$C_6H_3.(OH)_2CH_2.COOH$].

The best confirmatory tests for sugar are the *phenyl-hydrazine test* (see Lesson XIII.), and the *fermentation test.*

BILE IN THE URINE

This occurs in jaundice, the commonest cause of which is obstruction of the bile duct. The urine is dark brown, greenish, or in extreme cases almost black in colour. Excess of urobilin should not be mistaken for bile pigment.

BLOOD AND BLOOD PIGMENT IN THE URINE

When hæmorrhage occurs in any part of the urinary tract, blood appears in the urine. It is found in the acute stage of Bright's disease. If a large quantity is present, the urine is deep red. Microscopic examination then reveals the presence of blood corpuscles, and on spectroscopic examination the bands of oxyhæmoglobin are seen.

If only a small quantity of blood is present, the secretion—especially if acid—has a characteristic reddish-brown colour, which physicians term "smoky."

The blood pigment may, under certain conditions, appear in the urine without the presence of any blood corpuscles at all. This is produced by a disintegration of the corpuscles occurring in the circulation. The condition so produced is called *hæmoglobinuria*, and it occurs in several pathological states, as, for instance, in the tropical disease known as "Black-water fever." The pigment is in the condition of methæmoglobin mixed with more or less oxyhæmoglobin, and the spectroscope is the means used for identifying these substances.

PUS IN THE URINE

Pus occurs in the urine as the result of suppuration in any part of the urinary tract. It forms a white sediment resembling that of phosphates, and, indeed, is always mixed with phosphates. The pus corpuscles may be seen with the microscope; their nuclei are rendered evident by treatment with 1 per-cent. acetic acid, and the pus corpuscles are seen to resemble white blood corpuscles, which, in fact, they are in origin. Some of the protein constituents of the pus cells—and the same is true for blood—pass into solution, so that the urine pipetted off from the surface of the deposit gives the tests for albumin. On the addition of caustic potash to the deposit of pus cells a ropy gelatinous mass is obtained. This is distinctive. Mucus treated in the same way is dissolved.

AMINO-ACIDS IN URINE

Normal urine contains traces of glycine. Leucine, tyrosine, and other amino-acids may be present after extensive disintegration of tissue protein, such as occurs in acute atrophy of the liver (p. 186). Cystine may occur as a rare anomaly of metabolism. Associated with cystinuria one often finds diaminuria, that is, the passage of diamines into the urine; these are known as cadaverine and putrescine, and are the result of the removal of CO_2 from the diamino-acids lysine and ornithine respectively (see p. 116). Homogentisic acid, found in alcaptonuria (see preceding page), is another somewhat similar anomaly; it arises from tyrosine.

DETECTION OF SUBSTANCES OF PHYSIOLOGICAL IMPORTANCE

Subsequent lessons may be very usefully employed by the class in testing for the various substances the properties of which have been previously studied. If the substance is in solution, the following scheme will form a guide to the tests to be employed. If the substance under examination is solid, test its solubility in water; if it is soluble, the same scheme can then be applied to the solution. If the substance is insoluble in water, test its solubility in alkali and other reagents; insolubility in water will suggest such substances as uric acid. If the substance is a mixture part of which is soluble in water, filter off the solution and examine that; then test the solubility in alkali, etc., of the residue.

1. Note reaction, colour, clearness or opalescence, taste, smell. Coloured liquids suggest blood, bile, urine, etc. Opalescent liquids suggest starch, glycogen, or certain proteins.

2. Add iodine. A colour is produced:

If blue: **Starch.** Confirm by converting into a reducing sugar by saliva at 40° C., or by boiling with dilute sulphuric acid.

If reddish-brown: **Glycogen** or **dextrin,** the distinctions between which are given on p. 30.

3. Add copper sulphate and caustic potash.

(*a*) Blue solution: boil; yellow or red precipitate. **Glucose, fructose, maltose, lactose,** and other reducing sugars (for distinguishing tests see Lesson XIII.).

(*b*) Blue solution: no reduction on boiling; boil some of the original solution with 25 per-cent. sulphuric acid, and then boil with copper sulphate and *excess* of caustic potash; abundant yellow or red precipitate: **Sucrose.** Confirm by HCl test (see p. 21).

(*c*) Violet solution: **Proteins** (albumins, globulins, metaproteins). In presence of magnesium sulphate the potash causes also a white precipitate of magnesia.

(*d*) Pink solution; biuret reaction. **Peptones** or **proteoses.** In presence of ammonium sulphate very large excess of potash is necessary for this test. Only a trace of copper sulphate must be used.

4. When proteins are present proceed as follows: Boil the original solution (after adding a trace of 2 per-cent. acetic acid).

(*a*) Precipitate produced: **Albumins** or **globulins.**

(*b*) No precipitate: **Metaproteins, proteoses, or peptones.**

5. If albumin, or globulin, or both are present, saturate a fresh portion with magnesium sulphate or half saturate with ammonium sulphate; filter; the precipitate contains the **globulin,** the filtrate the **albumin.**

6. If proteins are present, but albumin or globulin absent:

(*a*) Neutralisation causes a precipitate soluble in excess of weak acid or alkali. **Acid** or **alkali metaprotein,** according as the reaction of the original liquid is acid or alkaline respectively. If the original liquid is neutral, metaproteins must be absent.

(*b*) Neutralisation produces no such precipitate: **Proteose** or **peptone.**

7. If proteose, or peptone, or both are present, saturate a fresh portion with ammonium sulphate:

(*a*) Precipitate: **Proteose.** (*b*) No precipitate: **Peptone.**

If both are present, the precipitate contains the proteose, and the filtrate the peptone.

8. To a fresh portion add nitric acid (proteins having been proved to be present).

(*a*) No precipitate, even though excess of sodium chloride be also added: **Peptone.**

(*b*) No precipitate, until excess of sodium chloride is added: **Deutero-proteose.**

(*c*) Precipitate which disappears on heating and reappears on cooling: **Proteoses.** This is a distinctive test for proteoses, and is given by all of them. For one of them, however (deutero-proteose), excess of sodium chloride must be added also.

(*d*) Precipitate little altered by heating: **Albumin** or **globulin.**

In all four cases nitric acid *plus* heat causes a yellow colour, turned orange by ammonia (xantho-proteic reaction).

9. Confirmatory tests for proteins:—

(*a*) Millon's test (see p. 41).

(*b*) Adamkiewicz's reaction or, better, the modification of this test introduced by Rosenheim (see p. 41).

(*c*) To test for fibrinogen :—

i. It coagulates by heat at 56° C.

ii. It is changed into fibrin by fibrin-ferment and calcium chloride.

(*d*) To test for caseinogen :—

i. It is not coagulated by heat.

ii. It is changed into casein by rennet and calcium chloride.

10. If **blood** is suspected :

(*a*) Examine spectroscopically, diluting if necessary.

i. Oxyhæmoglobin shows two bands between D and E.

ii. Add ammonium sulphide ; one band only appears.

iii. Carbonic oxide hæmoglobin shows two bands also, but will not reduce with ammonium sulphide.

iv. Methæmoglobin gives a typical band in the red between C and D.

v. Hæmatin, etc., show special spectra (see Advanced Course, Lesson XX.).

(*b*) Dry : boil with glacial acetic acid and a crystal of sodium chloride on a glass slide under a cover glass. When cold, hæmin crystals are seen.

(*c*) Try guaiacum test and Adler's test (p. 135).

(*d*) If the blood is old and dry, and its hæmoglobin converted into hæmatin :

i. Try hæmin test.

ii. Try guaiacum test and Adler's test.

iii. Dissolve it in potash ; add ammonium sulphide, and examine for spectrum of hæmochromogen.

11. If **bile** is suspected :

(*a*) Try Gmelin's test for bile pigments (see p. 108) ; also Huppert's test (p. 212).

(*b*) Try Pettenkofer's and also Hay's tests for bile salts (see p. 108).

12. Miscellaneous substances.

(*a*) **Mucin.** Precipitated by acetic acid or by alcohol. The precipitate is soluble in lime water. By collecting the precipitate and boiling it with 25 per-cent. sulphuric acid, a reducing sugar-like substance is obtained. Mucin gives the protein colour tests.

(*b*) **Nucleo-protein.** Precipitated by acetic acid or by alcohol. The precipitate is often viscous. It is soluble in dilute alkalis such as 1 per-cent. sodium carbonate. This solution causes intravascular clotting. If the precipitate is collected and subjected to gastric digestion, an insoluble deposit of nuclein is left, which is rich in phosphorus. Nucleo-protein gives the protein colour tests.

(*c*) **Gelatin.** This also gives some of the protein colour tests, but not those of Millon or Adamkiewicz. It is not coagulated, but dissolved in hot water. The solution gelatinises when cold.

(*d*) **Urea.** Very soluble in water. The solution effervesces when sodium hypobromite or fuming nitric acid is added. Concentrate a fresh portion, add nitric acid, and examine for crystals of urea nitrate. Solid urea heated in a dry test-tube gives off ammonia, and the residue is called biuret, which gives a rose-red colour with copper sulphate and caustic potash.

(*e*) **Uric acid.** Very insoluble in water ; soluble in potash, and precipitated from this solution in crystals by hydrochloric acid. Uric acid crystals from human urine are deeply pigmented red. Try murexide, Folin's and Schiff's tests (see p. 196).

(*f*) **Cholesterol.** Characteristic flat crystalline plates. For various colour tests see pp. 108, 109.

13. **Urine.** Normal constituents.

(*a*) Chlorides. Acidulate with nitric acid; add silver nitrate; white precipitate.

(*b*) Sulphates. Acidulate with nitric or hydrochloric acid; add barium chloride; white precipitate.

(*c*) Phosphates. Acidulate with nitric acid; add ammonium molybdate; boil; and a yellow crystalline precipitate forms. To another portion add ammonia; earthy (*i.e.* calcium and magnesium) phosphates are precipitated.

(*d*) Urea (see above).

(*e*) Uric acid. To 100 c.c. of urine add 5 c.c. of hydrochloric acid; leave for twenty-four hours, and pigmented crystals of uric acid are formed. For tests see above.

(*f*) Hippuric acid. Evaporate the urine with nitric acid, and heat the residue in a dry test-tube. A smell of oil of bitter almonds is given off.

(*g*) Creatinine. For colour tests see p. 181.

14. **Urine.** Abnormal constituents.

(*a*) *Blood.* Microscope (blood corpuscles). Spectroscope (for oxyhæmoglobin or methæmoglobin). Hæmin test.

(*b*) *Blood pigment* may be present without blood corpuscles. Spectroscope.

(*c*) *Bile.* Gmelin's, Huppert's, Hay's, and Pettenkofer's tests.

(*d*) *Pus.* White deposit. Microscope (pus cells). Add potash; it becomes stringy.

(*e*) *Albumin.* (i) Precipitated, if acid, by boiling; precipitate insoluble in acetic acid, so distinguishing it from phosphates. (ii) Precipitated by nitric acid in the cold. (iii) Precipitated by picric acid.

(*f*) *Sugar.* (i) Brown colour with potash and heat (Moore's test). (ii) Ferments with yeast. (iii) Reduces Fehling's solution. (iv) Urine has a high specific gravity. (v) Add picric acid, potash, and boil; the urine becomes a dark opaque red; the similar slight coloration in normal urine is due to creatinine.

(*g*) *Aceto-acetic acid and acetone.* For tests see p. 211.

(*h*) *Mucus.* Flocculent cloud; may be increased by acetic acid; soluble in alkalis. A little mucus in urine is not abnormal.

(*i*) *Deposits.*

i. Examine microscopically for blood corpuscles, pus cells, crystals, etc.

ii. Phosphates. White deposit often mixed with mucus or pus. Insoluble on heating; soluble in acetic acid. Urine generally alkaline. Examine microscopically for coffin-lids of triple phosphate and star-like clusters of stellar (calcium) phosphate.

iii. Urates. Pink deposit, usually amorphous; may be mixed with envelope crystals of calcium oxalate. Deposit soluble on heating urine. Murexide test.

iv. Uric acid. Deposit like cayenne pepper. Microscope. Tests as above.

ADVANCED COURSE

INTRODUCTION

It will be presupposed that students who take the following lessons have already been through the elementary course. The order in which the subjects are treated is the same as that already adopted. The instructions given will be mainly practical; theoretical matter on which they depend, or to which they lead, is, as a rule, too lengthy to be discussed in a short manual like the present volume. The Appendix contains a description of various instruments which are not generally contained in sufficient numbers in a physiological laboratory to admit of each student being able to use them in a class. It also contains a description of certain methods of research which should always be shown in demonstrations, though there may be practical difficulties in allowing each member of the class to perform the experiment. The few experiments in which living animals are employed will also necessarily be of the nature of demonstrations.

LESSON XIII

CARBOHYDRATES

1. **Glycogen.**—A rabbit which has been fed five or six hours previously on carrots is killed by bleeding. The chest and abdomen are opened quickly and a cannula inserted into the portal vein, and another into the vena cava inferior. A stream of salt solution is then allowed to pass through the liver until it is uniformly pale. The washings are collected in three beakers labelled *a*, *b*, and *c*.

The liver is cut out quickly, chopped into small pieces, and thrown into boiling water acidulated with acetic acid. The acidulated water extracts a small quantity of glycogen. The pieces of scalded liver are then ground up in a mortar with hot water, and thoroughly extracted with boiling water. Filter. A strong solution of glycogen is thus obtained ; but hot water will not extract glycogen thoroughly.

Test the solution when cold with iodine.

To separate the glycogen evaporate the solution to a small bulk on the water-bath and then add excess of alcohol ; the glycogen is precipitated as a flocculent powder, which is collected on a filter and dried in an oven at the temperature of 100°.

2. Examine the washings of the liver in the beakers *a*, *b*, and *c*, for sugar. This may be done in a rough quantitative manner as follows :—Take equal quantities of *a*, *b*, and *c* in three test-tubes ; to each add an equal amount of Fehling's solution, and boil : *a* will give a heavy precipitate of cuprous oxide, *b* one not so heavy, and *c* least of all, or none at all.

3. **Pflüger's Method of estimating Glycogen.**—20 to 100 grammes of the finely chopped liver are boiled for two to three hours with 100 c.c. of 60 per-cent potash. After cooling wash the contents of the flask into a beaker, and add 200 c.c. of water, and then 400 c.c. of 94 per-cent. alcohol, and the mixture is allowed to stand overnight. The glycogen is thus precipitated free from protein. Collect the precipitate on a filter, and wash once with 1 vol. 15 per-cent. potash and 2 vols. of alcohol ; then wash with 66 per-cent. alcohol. After this transfer the precipitate and filter-paper to a large beaker and boil thoroughly with water. Neutralise the solution and filter. Dilute the filtrate to 500 c.c. and add 25 c.c. of hydrochloric acid of specific gravity 1·19. Heat for three hours on the boiling water-bath ; this converts the glycogen into glucose. After cooling, neutralise with 20 per-cent. potash and filter ; the filtrate is brought up to 250 c.c. ; estimate the glucose in this either polarimetrically or by a good volumetric method. The percentage of glucose multiplied by 0·927 gives the amount of glycogen.

4. **Microchemical Detection of Glycogen.**—A thin piece of the liver is hardened in 90 per-cent. alcohol. Sections are cut by the free hand, or after embedding in paraffin. If paraffin is used, this is got rid of by means of turpentine ; and the sections prepared by either method are treated with chloroform in which iodine is dissolved, and mounted in chloroform balsam containing some iodine. The glycogen is stained brown, and is most abundant in the cells around the radicals of the hepatic vein.

5. **Phenyl-Hydrazine Test for Sugars.**—To 5 c.c. of the suspected fluid (*e.g.* diabetic urine) add 1 decigramme of phenyl-hydrazine hydrochloride, 2 decigrammes of sodium acetate, and heat on the water-bath at 100° C. for thirty to sixty minutes. On cooling, if not before, a crystalline or amorphous precipitate separates out. If amorphous, dissolve it in hot alcohol ; dilute the solution with water, and boil to expel the alcohol, whereupon the osazone separates out in yellow crystals. Examine the crystals with the microscope (see accompanying plate).

Glucose gives a precipitate of phenyl-glucosazone $C_6H_{10}O_4(N_2H.C_6H_5)_2$, which crystallises in yellow needles (melting point 205° C.).

Fructose yields an osazone identical with this.

Galactose yields a very similar osazone (phenyl-galactosazone). It differs from phenyl-glucosazone by melting at 190–193°, and in being optically inactive when dissolved in glacial acetic acid.

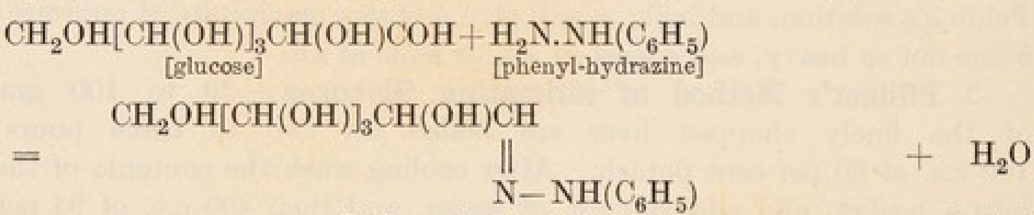
Sucrose does not form a compound with phenyl-hydrazine.

Lactose yields phenyl-lactosazone $C_{12}H_{20}O_9(N_2H.C_6H_5)_2$. It crystallises in needles, usually in clusters (melting point 200° C.). It is soluble in 80–90 parts of boiling water. Lactose in urine does not give this test readily.

Maltose yields phenyl-maltosazone ($C_{24}H_{32}N_4O_9$). It crystallises in yellow needles much wider than those yielded by glucose or lactose (melting point 206° C.). Unlike phenyl-glucosazone, it dissolves in 75 parts of boiling water, and is still more soluble in hot alcohol.

The chemistry of the phenyl-hydrazine reaction is represented in the following equations, glucose being taken as an example of the sugar used :—

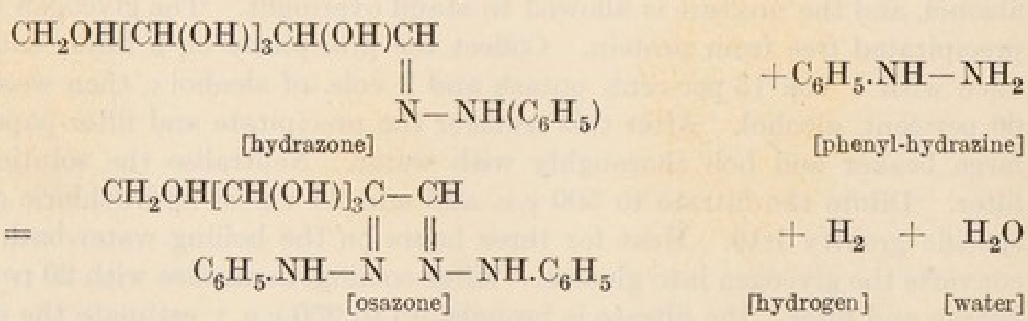
I.



$$\underset{\text{[glucose]}}{CH_2OH[CH(OH)]_3CH(OH)COH} + \underset{\text{[phenyl-hydrazine]}}{H_2N.NH(C_6H_5)}$$

$$= \underset{\text{[hydrazone]}}{\begin{array}{rl} CH_2OH[CH(OH)]_3CH(OH) & CH \\ & \| \\ & N-NH(C_6H_5) \end{array}} + \underset{\text{[water]}}{H_2O}$$

II.



$$\underset{\text{[hydrazone]}}{\begin{array}{rl} CH_2OH[CH(OH)]_3CH(OH) & CH \\ & \| \\ & N-NH(C_6H_5) \end{array}} + \underset{\text{[phenyl-hydrazine]}}{C_6H_5.NH-NH_2}$$

$$= \underset{\text{[osazone]}}{\begin{array}{rll} CH_2OH[CH(OH)]_3 & C- & CH \\ & \| & \| \\ C_6H_5.NH- & N & N-NH.C_6H_5 \end{array}} + \underset{\text{[hydrogen]}}{H_2} + \underset{\text{[water]}}{H_2O}$$

6. To determine the *melting point* of the osazones (or other organic substances) place a small quantity of the powder in a capillary thin-walled tube,

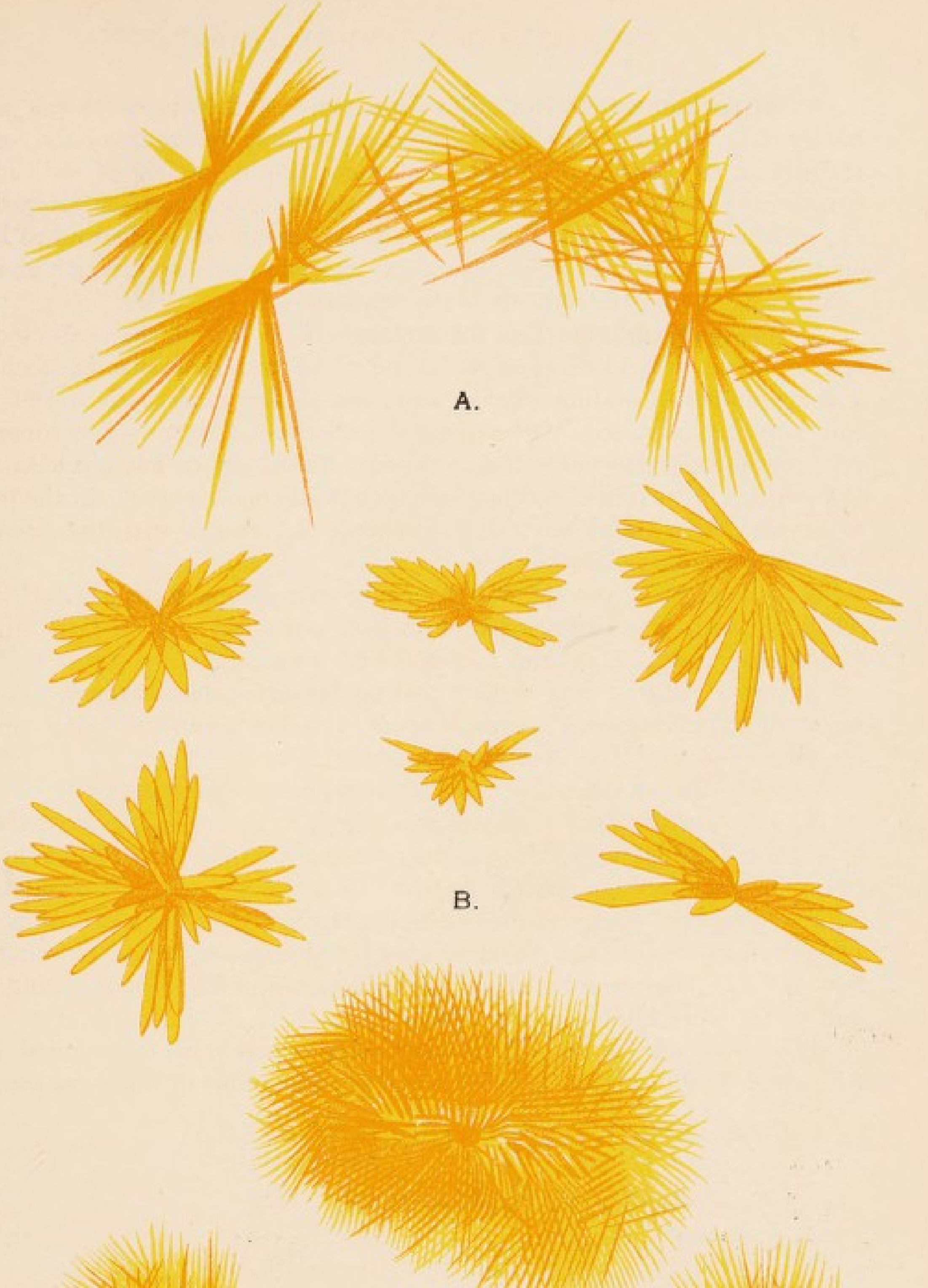


Fig. 48. —Plate of osazone crystals highly magnified.

A, phenyl-glucosazone. B, phenyl-maltosazone. C, phenyl-lactosazone.

strapped on to a thermometer by an indiarubber band. Place this in a sulphuric acid bath which is gradually heated, and note the temperature at which the powder melts.

7. **The Polarimeter.**—Estimate the strength of a solution of glucose by means of the polarimeter (see Appendix). The polarimetric method is a rapid one. When used for the estimation of glucose in urine, 50 c.c. of the urine are mixed with 5 c.c. of a 10 per-cent. solution of lead acetate in order to precipitate pigments, etc. Filter through a dry filter, and fill the tube of the polarimeter with the clear filtrate. The dilution due to the lead acetate solution must be taken into account when calculating the percentage of glucose from the rotation observed.

8. **Formation of Mucic Acid.**—Take 1 gramme of lactose and heat it in a porcelain capsule with 12 c.c. of nitric acid on a water-bath until the fluid is reduced to one-third of its original volume. Cool overnight, and a crystalline precipitate of mucic acid separates out. Cane sugar, maltose, dextrose, dextrin, and starch, treated in the same way, yield an isomeric acid called saccharic acid, which, being soluble, does not separate out. Lactose yields both acids ; galactose, mucic acid only.

9. **Pentoses** give the ordinary reduction tests for sugar and yield osazones, but do not ferment with yeast. They give the two following characteristic tests ; they may be performed with gum arabic (which contains arabinose) or pine-wood shavings (which contain xylose).

(*a*) Phloroglucin reaction. Warm some distilled water with an equal volume of concentrated hydrochloric acid in a test-tube and add phloroglucin until a little remains undissolved. Add a small quantity of gum arabic, and keep the mixture warm in the water-bath at 100° C. The solution becomes cherry-red, and a precipitate settles out, which is soluble in amyl alcohol. This solution gives an absorption band between the D and E lines.

(*b*) Orcin reaction. Substitute orcin for phloroglucin in the foregoing experiment. The solution becomes violet on warming, then blue, red, and finally green. A bluish-green precipitate settles out, soluble in amyl alcohol. This solution gives an absorption band between C and D.

10. **Glycuronic acid** (see p. 213) gives all the above reactions ; it may be distinguished as follows :—

(*a*) Take 50 c.c. of glycuronic acid solution in a capsule ; add 1 gramme of *p*-bromphenyl-hydrazine and rather more than the same amount of sodium acetate. Keep the mixture in the water-bath at 100° C. for a quarter of an hour, when yellow crystals of *p*-bromphenyl-hydrazone of glycuronic acid separate out. After cooling filter off the crystals and wash them with absolute alcohol, in which they are insoluble. Under the same conditions carbohydrates yield *p*-bromphenyl-osazones, but these are soluble in absolute alcohol. The *p*-bromphenyl-hydrazone is soluble in absolute alcohol to which pyridine has been added ; the rotatory power of this solution is greater than that of any of the osazones.

(*b*) *Tollens' Test.*—To 5 c.c. of urine add 0·5 c.c. of a 1 per-cent. naphtho-resorcin solution in alcohol, and 5 c.c. of hydrochloric acid (sp. gr. 1·19). Raise the mixture to boiling point and boil for one minute over a small flame. Let it stand for four minutes, and then cool under the tap. Shake it with an equal volume of ether. If glycuronic acid is present, the ether becomes blue or violet, and shows an absorption band near the D line. From 60 normal urines, 40 gave the reaction ; it is especially strong after the administration of camphor, chloral, salicylic acid, creosote, etc. The reaction is not absolutely distinctive for glycuronic acid, since it is also given by glyoxylic acid and by all acids which contain both a carbonyl and a carboxyl group ; but none of these substances are likely to occur in urine.

Micro

Bang's Volumetric Method for the Estimation of Glucose

Principle of the Method.—A copper solution, containing carbonates and sulphocyanide of potassium, is boiled with a quantity of the sugar solution which is not sufficient to reduce all the cupric salts. Under these circumstances the cupro-thiocyanate, formed by the reduction, is kept in solution. The excess of the cupric salt is then titrated back in the cold by means of a standard hydroxylamine solution, the end point being a change from a blue to a colourless solution.

Hydroxylamine Solution used in c.c.	Glucose in Milli-grammes.	Hydroxylamine Solution used in c.c.	Glucose in Milli-grammes.	Hydroxylamine Solution used in c.c.	Glucose in Milli-grammes.
0·75	60·0	16	35·1	33	15·4
1·00	59·4	17	33·9	34	14·4
1·50	58·4	18	32·6	35	13·4
2	57·3	19	31·4	36	12·4
3	55·0	20	30·2	37	11·4
4	53·4	21	29·0	38	10·4
5	51·6	22	27·7	39	9·4
6	49·8	23	26·5	40	8·5
7	48·0	24	25·2	41	7·6
8	46·3	25	24·1	42	6·7
9	44·7	26	22·9	43	5·8
10	43·3	27	21·8	44	4·9
11	41·8	28	20·7	45	4·1
12	40·4	29	19·6	46	3·3
13	39·0	30	18·6	47	2·5
14	37·7	31	17·5	48	1·7
15	36·4	32	16·5	49	0·9

Preparation of the Copper Solution.—500 grammes of potassium carbonate, 100 grammes of potassium bicarbonate, and 400 grammes of potassium thiocyanate are dissolved in a 2-litre measuring flask in about 1500 c.c. distilled water at 60° C., and afterwards cooled to about 30° C. A cold solution of 25 grammes of crystallised copper sulphate in about 150 c.c. distilled water is then poured slowly into the carbonate solution, taking care that no development of carbonic acid takes place. The solution is made up to 2 litres and filtered after twenty-four hours. The solution keeps indefinitely.

Preparation of the Hydroxylamine Solution.—200 grammes of potassium thiocyanate are dissolved in a 2-litre measuring flask in about 1500 c.c. of distilled water. 6·55 grammes of hydroxylamine sulphate are dissolved in distilled water and added to the other solution, which is then made up with distilled water to the mark. This solution also keeps well in a dark bottle.

Analysis.—Measure 50 c.c. of the copper solution into a 200-c.c. flask and add 10 c.c. of the sugar solution. If this contains more than 0·6 per cent. sugar, only 5 c.c. (or less) and 5 c.c. of water ought to be taken. Heat the mixture to boiling on a wire gauze and boil exactly for three minutes. Cool rapidly to room temperature and titrate with the hydroxylamine solution until colourless. From the number of c.c. of the hydroxylamine solution used the quantity of sugar is calculated by means of the table on the preceding page :—

Bertrand's Volumetric Method for the Estimation of Glucose

This method has recently come into vogue for the estimation of glucose in blood etc. It is not so suitable for urine as Bang's method, owing to the fact that in many urines cuprous oxide does not easily settle, but remains partially in colloidal suspension.

Principle of the Method.—The precipitate of cuprous oxide formed on boiling an excess of Fehling's solution with a glucose solution is filtered off, washed and dissolved in a solution of ferric sulphate in sulphuric acid. The ferrous salt formed is titrated with a standard permanganate solution.

Solutions required.—(1) A solution which contains 40 grammes of pure copper sulphate in one litre. (2) A solution which contains 200 grammes of Rochelle salt and 150 grammes sodium hydroxide in one litre. (3) A solution which contains 50 grammes ferric sulphate (free from ferrous sulphate), and 200 c.c. concentrated sulphuric acid in one litre. (4) A solution which contains 5 grammes potassium permanganate in one litre. This solution is standardised in the following way :—Weigh out 250 milligrammes ammonium oxalate, dissolve it in 50 c.c. water, add 2 c.c. concentrated sulphuric acid, warm to 60–80° C., and titrate with the permanganate solution, until a pink colour persists.

Analysis.—Measure 20 c.c. of the sugar solution [1] into a flask of 150 c.c. capacity, and add 20 c.c. each of the copper sulphate and Rochelle salt solutions. Heat to boiling and keep on the boil for three minutes. Filter through an asbestos filter, leaving most of the precipitate in the flask. Wash the precipitate in the flask with a little distilled water and decant through the same filter. Dissolve the precipitate in the flask in about 20 c.c. of the ferric sulphate solution and pour the solution through the filter. A green solution results which is titrated at once with permanganate until the green colour changes sharply into pink.

Calculation.—The amount of copper, precipitated as cuprous oxide, is calculated first from the following equations taking place during the reaction :—

$$\begin{gathered}
Cu_2O + Fe_2(SO_4)_3 + H_2SO_4 = 2CuSO_4 + 2FeSO_4 + H_2O \\
10FeSO_4 + 2KMnO_4 + 8H_2SO_4 = 5Fe_2(SO_4)_3 + K_2SO_4 + 2MnSO_4 + 8H_2O \\
5C_2H_2O_4 + 2KMnO_4 + 3H_2SO_4 = 10CO_2 + 2MnSO_4 + K_2SO_4 + 8H_2O.
\end{gathered}$$

One molecule of ammonium oxalate corresponds to 2Fe and therefore to 2Cu. The amount of ammonium oxalate taken multiplied with 0·8951 $\left(=\frac{63{\cdot}6\times 2}{142{\cdot}1}\right)$ gives therefore the amount of copper corresponding to the number of c.c. of permanganate used. From the amount of copper found the quantity of sugar is calculated by means of the following table :—

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[1] The solution must not contain more than 100 milligrammes of glucose.

Glucose in mg.	Cu in mg.	Glucose in mg.	Cu in mg.	Glucose in mg.	Cu in mg.	Glucose in mg.	Cu in mg.	Glucose in mg.	Cu in mg.
10	20·4	29	57·2	47	90·0	65	121·3	83	150·9
11	22·4	30	59·1	48	91·8	66	123·0	84	152·5
12	24·3	31	60·9	49	93·6	67	124·7	85	154·0
13	26·3	32	62·8	50	95·4	68	126·4	86	155·6
14	28·3	33	64·6	51	97·1	69	128·1	87	157·2
15	30·2	34	66·5	52	98·9	70	129·8	88	158·8
16	32·2	35	68·3	53	100·6	71	131·4	89	160·4
17	34·2	36	70·1	54	102·3	72	133·1	90	162·0
18	36·2	37	72·0	55	104·1	73	134·7	91	163·6
19	38·1	38	73·8	56	105·8	74	136·3	92	165·2
20	40·1	39	75·7	57	107·6	75	137·9	93	166·7
21	42·0	40	77·5	58	109·3	76	139·6	94	168·3
22	43·9	41	79·3	59	111·1	77	141·2	95	169·8
23	45·8	42	81·1	60	112·8	78	142·8	96	171·4
24	47·7	43	82·9	61	114·5	79	144·5	97	173·1
25	49·6	44	84·7	62	116·2	80	146·1	98	174·6
26	51·5	45	86·4	63	117·9	81	147·7	99	176·2
27	53·4	46	88·2	64	119·6	82	149·3	100	177·8
28	55·3								

LESSON XIV

CARBOHYDRATES : ACTION OF DIASTASE UPON STARCH

1. Prepare a 0·5 per-cent. solution of starch.

2. Prepare some malt extract by digesting 10 grammes of powdered malt with 50 c.c. of water at 50° C. for three hours, and subsequently straining. This extract contains the diastatic or malting enzyme.

Solutions 1 and 2 may be conveniently prepared beforehand by the demonstrator.

3. To the starch solution add one-tenth of its volume of malt extract, and place the mixture in a water-bath at 40° C. From time to time test portions of the liquid by mixing a drop with a drop of iodine solution on a testing slab. The blue colour at first seen is soon replaced by violet (mixture of blue and red), and then by a red reaction (due to *erythro-dextrin*), which gradually vanishes (*achromic point*). Alcohol added to the liquid when all starch and erythro-dextrin have gone still causes a precipitate of a dextrin, which, as it gives no colour with iodine, is called *achroō-dextrin*. The liquid also contains a reducing sugar, maltose.

4. Take 50 c.c. of a solution of maltose and determine how much of it is necessary to reduce 10 c.c. of Fehling's solution.

5. Take another 50 c.c. and boil it with 1 c.c. of strong sulphuric acid for half an hour in a flask. This converts it into glucose. After cooling bring the liquid to its original volume (50 c.c.) by adding water, and again determine its increased reducing power with Fehling's solution. If x=c.c. of maltose solution are necessary to reduce 10 c.c. of Fehling's solution, then $\frac{2x}{3}$=c.c. of glucose solution are approximately necessary for the same purpose. The strength of the maltose solution can be calculated from the fact that 10 c.c. of Fehling's solution correspond to 0·05 gramme of dextrose.

6. *Wohlgemuth's* method for the quantitative determination of diastatic enzymes. A series of test-tubes of equal size, each containing 5 c.c. of a 1 per-cent. starch solution, are kept in a vessel filled with ice water, and decreasing quantities of the enzyme solution to be examined are added. By the use of ice water the action of the enzyme is prevented until all the tubes are prepared. They are now transferred into a water-bath kept at 40°, by which means the action of the enzyme begins in all the tubes simultaneously. They are kept at this temperature for thirty to sixty minutes and then again transferred into ice water, in order to stop the action.

All the tubes are then filled with distilled water and one drop of decinormal iodine solution is added to each. After shaking one observes various colours, such as dark blue, blue violet, reddish brown and yellow, according to the quantity and activity of the enzyme. Those tubes which show a yellow to a reddish colour contain achroö-dextrin or erythro-dextrin, those with a blue violet a mixture of erythro-dextrin and starch. The last tube, in which a violet colour is produced, is taken as the limit of activity. In the one immediately preceding this, all the starch has been converted into dextrin and from it the strength of the enzyme solution is calculated by estimating the number of c.c. of a 1 per-cent. starch solution, which has been converted into dextrin by 1 c.c. of the enzyme solution during the time of the experiment. An example will make this clear. The tube immediately preceding the one which shows a violet colour contained 0·02 c.c. of saliva. The time of experiment was thirty minutes. Therefore, 0·02 c.c. saliva was able to convert 5 c.c. of a 1 per-cent. starch solution into dextrin within thirty minutes and therefore 1 c.c. of saliva would have produced the same change in $\frac{5}{0{\cdot}02}=250$ c.c. of the starch solution. If D designates the diastatic strength, we obtain therefore $D\,\frac{40^\circ}{30'}=250$. In another set of experiments with a different enzyme, the index tube contained 0·0125 c.c. saliva. We find, therefore, that

0·0125 c.c. in 30′ digests 5 c.c. 1 per cent. of a starch solution
∴ 1·0 c.c. „ „ 400 c.c. of the starch solution.

Therefore, $D\,\frac{40^\circ}{30'}=400$. The diastatic power of this saliva was therefore nearly twice that of the former. The same method and mode of notation may be used for any diastatic enzyme.

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LESSON XV

CRYSTALLISATION OF PROTEINS

1. **Egg-albumin.**—Fresh egg-white is mixed with an equal bulk of fully saturated, filtered, neutral ammonium sulphate solution. 100 c.c. of the former are measured into a porcelain basin or strong beaker, and 100 c.c. of ammonium sulphate solution are added in successive quantities of 10 or 15 c.c., the mixture being thoroughly churned with an egg whisk after each addition. The whole should be finally so thoroughly beaten up as to form a large proportion of light froth. After the greater part of the froth has broken down, the mixture is thrown on a folded filter-paper, moderately rapid filtration being obtained without the use of a filter-pump. The filtrate is strongly alkaline to litmus, and smells of ammonia. To the filtrate in a flask, or to as much of it as can be obtained in a convenient time of filtration, further ammonium sulphate solution is very cautiously added (best, drop by drop from a burette) until a slight permanent precipitate remains, and this precipitate is afterwards just redissolved by the equally cautious addition of water. Dilute acetic acid (10 per cent.) from a burette is now added drop by drop until such a stage of reaction is reached that a precipitate forms and only just redissolves. Finally one or two drops (not more) of acid are added in excess of this, whereupon a bulky white precipitate falls. The flask is now corked and allowed to stand. In twenty-four hours or less the precipitate, which will have increased in quantity, will be found to consist entirely of acicular crystals. Small portions should be examined under a ⅛th objective, avoiding pressure on the cover slip. (F. G. Hopkins.)

2. **Serum Albumin.**—Crystals of this protein may be obtained by the same method. Horse's serum is the best to use.

3. **Edestin.**—This may be taken as a type of the crystallisable vegetable globulins. One kilogramme of hemp seed is ground, or pressed in an oil press. The remainder of the fat is then removed by extracting with light petroleum. When free from this solvent, the seeds are digested at 60° with 1 litre of 5 per-cent. solution of sodium chloride. The liquid is then filtered off from the residue through calico and allowed to cool. A precipitate forms and settles at the bottom of the vessel. The supernatant liquid is then decanted off and the precipitate washed by decantation with distilled water. It is then redissolved in 500 c.c. of 5 per-cent. salt solution, and the solution filtered through a warm filter. On cooling, beautiful crystals of the regular system separate. These are washed with cold 5 per-cent. salt solution, distilled water, alcohol, and ether. The yield is about 100 grammes from a kilogramme of ground hemp seed.

LESSON XVI

MILK

1. Caseinogen in milk exists in the form of a salt (calcium caseinogenate). Add acetic acid to milk, and this salt is decomposed, and free caseinogen (with entangled fat) is precipitated. Collect the precipitate so produced from about 500 c.c. of milk on a filter, and wash thoroughly with distilled water: grind it up with calcium carbonate in a mortar, and add about 500 c.c. of distilled water; allow the mixture to stand for about an hour. The fat rises to the top: the excess of calcium carbonate falls to the bottom. The intermediate fluid contains the caseinogen in solution; it is usually very opalescent. Take some of this solution and divide it into three parts, A, B, and C.

To A add rennet.

To B add a few drops of 2 per-cent. solution of calcium chloride.

To C add both rennet and calcium chloride.

Put all three in the water-bath at 40° C. A clot of casein forms in C, but not in A if all calcium salts have been successfully washed away, nor in B.

2. The formation of casein from caseinogen is a double process; the first action is that of the enzyme, which converts the caseinogen into what may be called soluble casein; the second action is that of the calcium salt, which precipitates the casein in an insoluble form, or curd. This may be shown by taking some of the caseinogen solution and adding rennet. Warm to 40° C.; no visible change occurs, but nevertheless soluble casein and not caseinogen is now present. Then boil this mixture to destroy the enzyme, cool, and add calcium chloride. A formation of insoluble curd now occurs.

3. Caseinogen may be precipitated as a salt from milk by the addition of alcohol. This reagent also precipitates the other milk proteins.

4. The method of salting out described in Lesson VI., Exercise 10 (p. 69), may also be used. Add to some milk an equal volume of saturated solution of ammonium sulphate. Caseinogen as a salt is thus precipitated, and entangles the fat with it. Filter off the precipitate and examine the filtrate as follows:—Saturate it with sodium chloride; a small amount of precipitate comes down. This is the so-called lacto-globulin. This contains only a trace of true globulin; it is mostly caseinogen previously left in solution together with calcium sulphate. Filter it off; acidify the filtrate with a few drops of 2 per-cent. acetic acid and heat it in a water-bath gradually. About 77° C. the remaining protein (lactalbumin) is coagulated.

5. **Fat Estimation in Milk** (*Gerber's Acido-butyrometric Method*).

Principle of the Method.—The proteins of milk and the other solids not fat are dissolved in concentrated sulphuric acid, and the fat is subsequently separated by centrifugal force. The separation is helped by the addition of amylic alcohol. The whole process is carried out in a special simple centrifuge, holding two or more acido-butyrometer tubes, which allow the direct reading off of the fat percentage on the graduated narrow stem of the tube.

Analysis.—By means of the special pipettes supplied with the apparatus measure into the tubes 10 c.c. of concentrated sulphuric acid, then 11 c.c.

of the sample of milk, and finally 1 c.c. of amylic alcohol. Insert the rubber cork and shake the tube with an up-and-down motion until the curd is dissolved. Push up the cork, if necessary, so that the graduated neck is full, and place the tubes into the cups of the centrifuge, screw on the cover, and spin the centrifuge for two or three minutes. If the fat is not in a clear limpid layer in the neck, or if the upper portion is frothy, the rotation has not been sufficient and must be repeated. Read off the percentage of fat by adjusting, by slight pressure on the cork, the bottom layer to one of the larger lines on the scale and count up the number of divisions between this and the lowest curved line at the top. Each of the larger divisions is equal to 1 per cent. of fat, and the smaller 0·1 per cent. of fat.

LESSON XVII

THE PROTEOSES

1. Witte's peptone contains very little true peptone, but consists chiefly of proteoses, which are soluble, like peptone, in neutral saline solutions.

2. Make a solution of this substance in 10 per-cent. sodium chloride solution, and filter. Very little residue is left on the filter. This consists of dysalbumose, an insoluble form of hetero-albumose, formed during the process of preparing the substance. If hot saline solution is used instead of cold as a solvent, this amount of insoluble residue is increased, hetero-albumose being to a slight extent precipitated by heat.

3. The solution gives the following tests :—

(*a*) It does not coagulate on heating.

(*b*) Biuret reaction (due both to peptone and proteoses).

(*c*) A drop of nitric acid, best added by a glass rod, gives a precipitate which dissolves upon heating and reappears on cooling. (This is due to the proteoses present.)

(*d*) The precipitate produced by the addition of acetic acid and a drop of potassium ferrocyanide is also soluble on heating, and reappears on cooling.

4. For the separation of the proteoses and peptone proceed as follows :—

(*a*) Saturate the solution with ammonium sulphate, and filter. The filtrate contains the peptone, and the precipitate the proteoses. The peptone is not precipitated by nitric acid, nor by many of the reagents that precipitate other proteins. It is precipitated completely by alcohol, tannin, and potassio-mercuric iodide; imperfectly by phosphotungstic and phosphomolybdic acid.

It gives the biuret reaction, *but in the presence of ammonium sulphate a large excess of caustic potash is necessary.*

(*b*) Dialyse another portion of the solution; hetero-proteose is precipitated.

(*c*) Saturate another portion of the solution with sodium chloride (or half saturate with ammonium sulphate) after faintly acidulating with acetic acid. Proto-proteose and hetero-proteose are precipitated. Filter. The filtrate contains the deutero-proteose and peptone.

The proto- and hetero-proteose may be redissolved by adding distilled water, and may be separated from each other by dialysis (see *b*).

Deutero-proteose may be separated from the peptone by saturation with ammonium sulphate, or by the addition of a crystal of phosphoric acid. These reagents precipitate the deutero-proteose, but not the peptone.

Deutero-proteose gives the nitric acid reaction (see 3, *c*) characteristic of the proteoses only in the presence of excess of salt. If the salt is removed by dialysis, nitric acid then causes no precipitate.

5. Another delicate test introduced by McWilliam may here be mentioned. Salicyl-sulphonic acid precipitates albumins and globulins: on heating, the precipitate is coagulated. The same reagent precipitates proteoses. On heating, the precipitate dissolves and reappears on cooling. It does not precipitate peptones.

6. The use of trichloracetic acid for the separation of various proteins may be illustrated by the following experiment:—Take some blood and add to it some solution of Witte's peptone (*i.e.* proteoses and peptone). Add to this mixture an equal volume of a 10 per-cent. solution of trichloracetic acid. There is an abundant precipitate. Boil rapidly and filter hot. The filtrate contains the proteoses and peptone, all the other proteins being contained in the precipitate. On cooling, the filtrate deposits some of the proteose. The proteose and peptone in the filtrate may be detected in the usual way.

LESSON XVIII

DIGESTION

Numerous methods have been devised for the purpose of comparing the proteoclastic activity of digestive enzymes, and for determining their rate of action. These methods may be conveniently grouped into two classes:—

(*a*) Methods in which the rate of solution of a solid protein is used as the index of the action of the enzyme (Grützner's, Roaf's, and Mett's methods).

(*b*) Methods in which the rate of formation of the products (amino-acids) serves as the index (Sörensen's and Van Slyke's methods).

1. **Roaf's Method.**—This is a modification of Grützner's method. Grützner used fibrin stained with carmine, and when the fibrin is dissolved the carmine is set free, and from the depth of colour the amount of fibrin digested can be estimated. The disadvantage of the method is that it can only be used for gastric juice, for when alkali is present, as in pancreatic fluid, the carmine is dissolved out by the alkali before digestion sets in. This was overcome by Roaf by using congo-red instead of carmine.

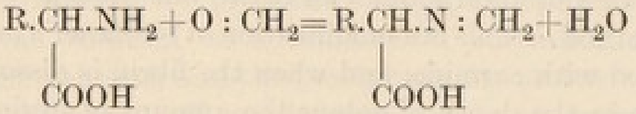
Preparation of the Stained Fibrin.—Clean fibrin is minced, and placed in a 0·5 per-cent. solution of congo-red solution for twenty-four hours (50 grammes of moist fibrin per 100 c.c. of staining solution). This is then poured into excess of water and heated to 80° C. for five minutes. The fibrin is then collected on a cloth and washed under the tap. It is squeezed as dry as possible and kept in equal parts of glycerin and water, a little toluol being added to prevent the growth of moulds. As instances of the way in which experiments may be performed, the following may be taken :—

(*a*) Put an equal weighed quantity of the stained fibrin into two test-tubes ; add to each an equal volume of two artificial pancreatic fluids. At the end of a given time (say fifteen minutes) remove the tubes, and filter ; the fluid will be more deeply coloured which contained the more active enzyme ; dilute this until it has the same tint as the lighter fluid, and the amount of dilution necessary will measure the relative efficiency of the two preparations.

(*b*) Repeat the experiment, using two specimens of artificial gastric juice. Their relative efficiency is determined in the same way, except that as the acid of the juice has turned the red into a bluish colour, the reaction should be rendered just alkaline by the addition of a few crystals of sodium carbonate. It is easier to determine the relative depth of tint in red than in blue fluids. When comparing the depth of colour of an acid digest with that resulting from digestion in an alkaline medium, the neutralisation of the former is carried out in the same way, and the depth of the two red solutions can then be directly compared.

2. **Mett's Method.**—A method which is now very generally employed for estimating the proteolytic activity of a digestive juice is one originally introduced by Mett. Pieces of capillary glass tubing of known length are filled with white of egg. This is set into a solid by heating to 95° C. They are then placed in the digestive fluid at 36° C., and the coagulated egg-white is digested. After a given time the tubes are removed ; and if the digestive process has not gone too far, only a part of the little column of coagulated protein will have disappeared ; the length of the remaining column is easily measured, and the length that has been digested is a measure of the digestive strength of the fluid.[1] This forms a very convenient method to use in experiments on velocity of reaction. Schütz's Law states that the amount of action psn is proportional to the square root of the amount of ~~enzyme~~. In most other cases of enzyme activity the rapidity of action is directly proportional to the amount of enzyme present (see pp. 92–95).

3. **Sörensen's Method.**—This very simple method for the estimation of amino-acids depends on the action of formaldehyde on these substances. Amino-acids combine with formaldehyde to form methylene compounds :—



$$\underset{\text{COOH}}{\text{R.}\underset{|}{\text{CH}}\text{.NH}_2} + \text{O} : \text{CH}_2 = \underset{\text{COOH}}{\text{R.}\underset{|}{\text{CH}}\text{.N}} : \text{CH}_2 + \text{H}_2\text{O}$$

The basic character of the amino-acid thus being destroyed, the carboxyl (COOH) or acid group may be titrated in the usual way. The method is carried out by adding an excess of a neutral formaldehyde solution to the digested fluid, and titrating the acid set free with decinormal alkali, as described under estimation of ammonia in urine (Lesson XXII.).

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[1] Hamburger has used the same method in investigating the digestive action of juices on gelatin. The tubes are filled with warm gelatin solution, and this jellies on cooling. They are placed as before in the digestive mixture, and the length of the column that disappears can be easily measured. These experiments must, however, be performed at room temperature, for the usual temperature (36°–40° C.) at which artificial digestion is usually carried out would melt the gelatin. He has also used the same method for estimating amylolytic activity, by filling the tubes with thick starch paste.

4. **The Method of Gross and Fuld.**—In this method a solution of caseinogen is used as the substrate. As caseinogen is soluble in dilute hydrochloric acid as well as in alkali, the method can be used for observations in both peptic and tryptic enzymes. In the case of peptic digestion, the caseinogen which is still undigested is precipitated by sodium acetate, while the cleavage products remain in solution. In tryptic digestion the end point is estimated by precipitating the undigested caseinogen with alcohol and acetic acid.

(*a*) *Caseinogen Method for Pepsin Estimations.*—The caseinogen solution is prepared by dissolving 1 gramme of caseinogen (Hammarsten's casein) in 1 litre of dilute hydrochloric acid (16 c.c. of HCl, specific gravity 1·124, and 986 c.c. of water). A series of test-tubes are charged with 10 c.c. of the caseinogen solution, and decreasing amounts of the gastric juice. The tubes are incubated for fifteen minutes at body temperature, and then a few drops of a concentrated solution of sodium acetate are added to each. Those tubes in which all the caseinogen is digested will show no precipitate ; those tubes in which much caseinogen remains undigested will show a heavy precipitate ; the first tube in which a mere cloud is observed is taken as containing the amount of enzyme just sufficient for digestion, and this amount is taken as the unit. Normal gastric juice by this method shows 33 units.

(*b*) *Caseinogen Method for Trypsin Estimations.*—One gramme of caseinogen is dissolved in 10 c.c. of decinormal soda, neutralised with decinormal hydrochloric acid, and made up to a litre with distilled water. Again a series of test-tubes are charged with 2 c.c. of the solution and decreasing amounts of the pancreatic fluid. These are incubated for an hour at body temperature, and then a few drops of acid alcohol (1 c.c. acetic acid, 50 c.c. alcohol, 49 c.c. water) added to each. The tube which shows only the faintest cloud is taken as before as the unit. Human pancreatic juice shows by this method on the average 250 units, that of the dog 125–250 units.

5. **Estimation of Amino-Nitrogen by Van Slyke's Method.**—The principle of this method is based on the well-known reaction of nitrous acid on aliphatic substances which contain an amino-group. The reaction proceeds according to the formula—

$$R.NH_2 + HNO_2 = R.OH + H_2O + N_2$$

R being the fatty radical. It will be seen that the amount of nitrogen evolved is double that contained in the amino-compound. Therefore the final result must be divided by 2.

Fig. 49 shows the apparatus designed by Van Slyke for obtaining and measuring the nitrogen evolved.

The apparatus is first filled with nitric oxide in order to displace the air ; this gas is also used to wash the evolved nitrogen into the eudiometer (F), which is filled with 1 per cent. sulphuric acid. The excess of nitric oxide is removed by permanganate solution contained in a Hempel pipette (H). The tube A serves for the supply of nitrous acid (sodium nitrite and glacial acetic acid). The reaction between the amino-substance and nitrous acid is carried out in D. The amino-substance in solution is run into D from the graduated tube B. The description of the determination may be divided into three stages:—

(1) *The Displacement of Air by Nitric Oxide.*—The acidulated water in F fills the capillary tube leading to the Hempel pipette (H), and also the capillary tube as far as *c*. Glacial acetic acid is poured into A up to the mark; this is run into D, the stopcock *c* being turned so as to let the air escape from D. Through A one next pours sodium nitrite solution (30 grammes of sodium nitrite per 100 c.c. of water) until D is full of solution. The gas exit from D is now closed by the stopcock *c*, and *a* being open, D is shaken for a few seconds. The nitric oxide which instantly collects is let out at *c*, and the shaking repeated. The second amount of nitric oxide then evolved, which washes out the last portions of air, is also let out through *c*. D is now shaken until all but 20 c.c. of the solution have been displaced by nitric oxide, and driven back into A. A mark on D indicates the 20 c.c. point. One then closes *a*, and turns *c* and *f* so that D and F are connected.

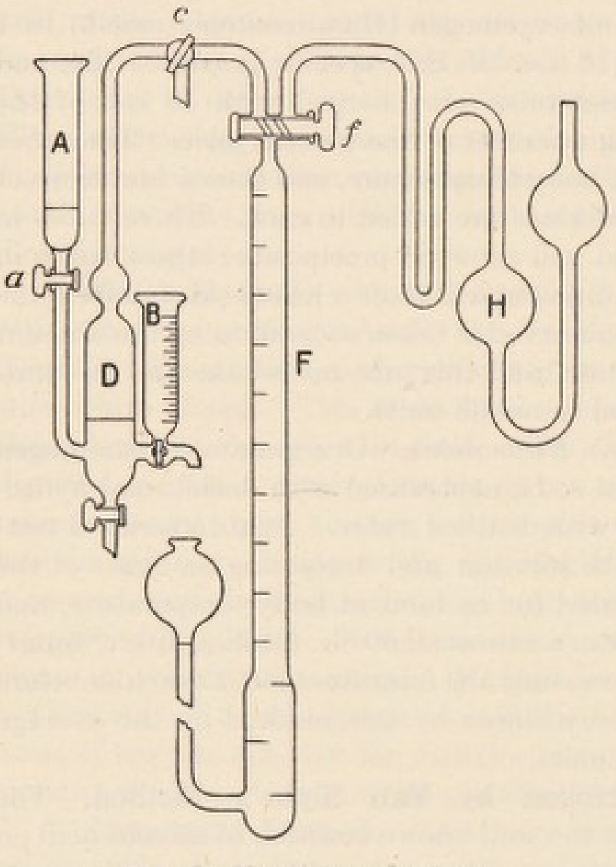

FIG. 49.—Van Slyke's apparatus.

(2) *Decomposition of the Amino-substance.*—Ten c.c. (or less) are measured off in B, and a known amount of this is run into D, and D is shaken for three to five minutes. With α-amino-acids, proteins, or partially or completely hydrolysed proteins, five minutes' vigorous shaking is sufficient. (A small motor may be arranged for the purpose of shaking D and H.) In cases where the solution is viscid, and the liquid threatens to froth over into F, B is rinsed out and a little caprylic alcohol added through it.

(3) *Absorption of Nitric Oxide and Measurement of Nitrogen.*—The reaction being completed, all the gas in D is displaced into F by liquid from A, and this gaseous mixture of nitrogen and nitric oxide is drawn from F into the absorption pipette H. The latter, which is filled with permanganate solution (50 grammes potassium permanganate and 25 grammes caustic potash per litre) is then shaken for a minute, and thus the nitric oxide is absorbed. The remaining gas (which is pure nitrogen) is then returned to F and measured. This amount divided by 2 (see equation) gives the amount of amino-nitrogen, from which the amount of the amino-substance analysed is calculated in the usual way.

6. **The Acid of Gastric Juice.**—The digestive powers of the acids are

proportional to their dissociation and the number of H ions liberated. The anions, however, modify this by having different powers of retarding the action. The greater suitability of hydrochloric over lactic acid, for instance, in gastric digestion is due to the fact that the former acid more readily undergoes dissociation.

Hydrochloric acid is absent or diminished in some diseases of the stomach, especially in cancer ; this is true for cancer in general even when the stomach is not involved ; the best colour tests for it are the following :—

(*a*) Günzburg's reagent consists of 2 parts of phloroglucinol, 1 part of vanillin, and 30 parts of rectified spirit. A drop of filtered gastric juice is evaporated with an equal quantity of the reagent. Red crystals form, or, if much peptone is present, there will be a red paste. The residue is of a bright red colour, even when only 1 part of hydrochloric acid in 10,000 is present. The organic acids do not give the reaction.

(*b*) Tropæolin test. Drops of a saturated solution of tropæolin-OO in 94 per-cent. methylated spirits are allowed to dry on a porcelain slab at 40° C. A drop of the fluid to be tested is placed on the tropæolin drop, still at 40° C. ; and if hydrochloric acid is present a violet spot is left when the fluid has evaporated. A drop of 0·006 per-cent. hydrochloric acid leaves a distinct mark.

(*c*) Töpfer's test. A drop of dimethyl-amino-azo-benzene is spread in a thin film on a white plate. A drop of dilute hydrochloric acid (up to 1 in 10,000) strikes with this in the cold a bright red colour.

Tropæolin and Töpfer's reagent are two of many aniline dyes which can be used for the purpose.

Lactic acid is sometimes present in the gastric contents, being derived by fermentative processes from the food. It is soluble in ether, and is generally detected by making an ethereal extract of the stomach contents, and evaporating the ether. If lactic acid is present in the residue it may be identified by Uffelmann's reaction in the following way :—

A solution of dilute ferric chloride and carbolic acid is made as follows :—

10 c.c. of a 4 per-cent. solution of carbolic acid.
20 c.c. of distilled water.
1 drop of ferric chloride solution.

On mixing a solution containing a mere trace (up to 1 part in 10,000) of lactic acid with this violet solution, it is instantly turned yellow. Larger percentages of other acids—for instance, more than 0·2 per cent. of hydrochloric acid—are necessary to decolorise the solution, but the yellow colour produced by lactic acid is not obtained.

Hopkins's Reaction for Lactic Acid.—Place 3 drops of a 1 per-cent. alcoholic solution of lactic acid in a clean, *dry* test-tube, add 5 c.c. of concentrated sulphuric acid and 3 drops of a saturated solution of copper sulphate. Mix thoroughly and place the test-tube in a beaker of boiling water for five minutes. Then cool thoroughly under the tap, and add 2 drops of a 0·2 per-cent. alcoholic solution of thiophene and shake. Replace the tube in the boiling water ; as the mixture gets warm a cherry-red colour develops.

7. **Demonstration of Pancreatic Secretion.**—In an anæsthetised dog insert a cannula into the main pancreatic duct, and collect the juice in a suitable

vessel. Inject some 0·4 per-cent. hydrochloric acid into the duodenum, and note after some minutes the abundant flow of pancreatic juice. Next ligature off and remove two or three feet of the upper part of the small intestine, wash out the contents and slit it open; scrape off the mucous membrane with the back of a scalpel; preserve a small quantity of the scrapings for future use and label this A. Grind up the remainder in a mortar with clean sand or powdered glass, and add 0·4 per-cent. hydrochloric acid. Transfer the mixture to a flask, boil, and when cool neutralise with a little caustic soda solution. Filter; the filtrate contains *secretin*, which has been formed by the acid from the *pro-secretin* of the intestinal epithelium. Inject some of this solution through a cannula into the external jugular vein of the dog, and an abundant flow of pancreatic juice is an almost immediate result.

Characters of the juice so obtained:—

(*a*) It is a clear, colourless fluid, and very strongly alkaline.

(*b*) Mixed with starch solution and kept at 40° C. dextrin and maltose are rapidly formed.

(*c*) Mixed with milk there may be some curdling produced, but the most marked effect is that the milk rapidly becomes acid, and a smell of fatty acids is noticeable.

(*d*) Added to fibrin and kept at 40° protein digestion occurs very slowly; next day, however, the fibrin will be in some measure digested.

(*e*) Mix some of the pancreatic juice with the scraping of the intestine which was preserved and labelled A. Then add fibrin. The fluid is now strongly proteolytic, and at 40° C. the fibrin rapidly dissolves; trypsin has been liberated from the trypsinogen of the juice by the intestinal *entero-kinase.*

8. **Products of Pancreatic Digestion of Proteins.**—A pancreatic digest should be prepared beforehand by the demonstrator. This may be done by digesting a quantity of protein with artificial pancreatic juice, if the natural juice prepared by the action of secretin is not available; in the latter case the addition of intestinal epithelium (entero-kinase) should not be forgotten. Unless an antiseptic has been added putrefaction will also occur, and its odour will be very perceptible after the mixture has been placed in the warm chamber for some time.

A very good mixture for the purpose will be found to be the following:—

100 grammes of plasmon (caseinogen).
10 grammes of sodium carbonate.
1 litre of water.
25 c.c. of Benger's liquor pancreaticus.
0·5 gramme sodium fluoride.
3 c.c. chloroform.

The last two items on the list are added to prevent putrefaction.

After digestion has progressed for one to two days another 10 c.c. of liquor pancreaticus may be added.

The products of digestion in one case should be examined, say, after six hours' digestion, and in another case after thirty-six hours' or more. The digestive products should then be searched for; the early products of digestion (alkali-albumin, deutero-proteose, etc.) will become less abundant with the length of time that digestion has been allowed to progress, and the later products (peptone, leucine, tyrosine, tryptophane, etc.) will become more abundant.

(*a*) *Tryptophane.*—Add a few drops of bromine water; a violet colour is produced. Add 2 or 3 c.c. of amyl alcohol and shake. The alcohol rises on standing and contains the pigment in solution.

(*b*) *Leucine and Tyrosine.*—i. Examine microscopical specimens of these. The deposit generally found in rather old specimens of Benger's liquor pancreaticus will be a convenient source of these crystals.

ii. To some of the pancreatic digest add Millon's reagent and filter off the precipitated protein. Boil the filtrate, and the presence of tyrosine is indicated by a red colour. If tyrosine is abundant the red colour appears without boiling. Leucine does not give this test.

iii. Faintly acidify another portion of the filtered digest with acetic acid and boil; if any protein matter is still undigested it will be thus coagulated and can be filtered off. Reduce the filtrate to a small bulk until it begins to become syrupy. Leave overnight in a cool place, and crystals mainly of tyrosine will separate out. Filter these off through fine muslin, and evaporate down the filtrate to the consistency of a thick syrup; leave this overnight again, and a second crop of crystals, forming a scum on the surface and consisting mainly of leucine, will have separated out.

iv. *Mörner's Test for Tyrosine.*—The following reagent is used:—1 c.c. formalin, 45 c.c. distilled water, 55 c.c. concentrated sulphuric acid. If a portion of this solution is boiled with a little tyrosine (in the solid form or in solution), an emerald-green colour appears. This test often fails in the presence of organic impurities.

9. In a digest in which putrefaction has occurred, test for *indole* as follows:—Add a little sulphuric acid and a few drops of a dilute solution of sodium nitrite; a bright red colour is produced (Cholera-red reaction).

10. **Zymogen Granules.**—Examine microscopically, mounting in aqueous humour or serum (or in glycerol after treatment with osmic acid vapour), small pieces of the pancreas, parotid, and submaxillary glands in a normal guinea-pig,[1] and also in one in which profuse secretion had been produced by the administration of pilocarpine.

Note that zymogen granules are abundant in the former, and scarce in the latter, being situated chiefly at the free border of the cells.

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[1] The guinea-pigs should be killed by bleeding, and the blood collected and defibrinated, and utilised for the preparation of oxyhæmoglobin crystals. This will give students an opportunity of seeing the exceptional form (tetrahedra) in which the blood-pigment of this animal crystallises.

The three methods of obtaining crystals described on p. 145 all give good results. If amyl nitrite is used instead of ether in the third method, crystals of methæmoglobin are obtained.

LESSON XIX

THE BLOOD

1. **Effect of Decalcifying Agents in hindering Coagulation.**—From an anæsthetised dog collect samples of blood from the carotid artery, into which a suitable cannula should have been previously inserted.

(*a*) Collect the first sample in an equal volume of 0·4 per-cent. solution of potassium oxalate made with physiological salt solution.

(*b*) Collect the second sample in a tenth of its volume of a 3 per-cent. solution of sodium fluoride.

(*c*) Collect the third sample in a quarter of its volume of 10 per-cent. solution of sodium citrate.

In all three cases mix thoroughly, and coagulation is hindered owing to decalcification, as explained on p. 139.

The separation of the plasma from the corpuscles may be most readily carried out by a centrifugal machine; the corpuscles settle and the supernatant plasma can be then pipetted off. Sedimentation is specially rapid in the case of citrate blood, and a well-marked layer of colourless corpuscles and platelets may usually be seen on the top of the mass of red corpuscles.

Oxalate plasma and citrate plasma coagulate on the restoration of the calcium by adding a few drops of calcium chloride solution, as we have already seen in the elementary course (p. 133). Fluoride plasma does not coagulate unless fibrin ferment (or some fluid such as serum which contains fibrin-ferment or thrombin) is added as well as the calcium salt. Fluoride plasma thus forms a convenient test-fluid for fibrin ferment.

If in either case the plasma is previously heated to 60° C. and filtered, coagulation—that is to say, fibrin formation—can never be produced, because its mother substance, fibrinogen, which is coagulated by heat at 56° C., has been coagulated and removed.

2. **Influence of Leech Extract on Coagulation.**—The same dog still under the anæsthetic may be next used for the following experiments:—

(*a*) Draw off a sample of blood into a clean test-tube, and note the time it takes to clot.

(*b*) Draw off a second sample into about half its volume of leech extract made by grinding up the heads of about twenty leeches in 20 c.c. of salt solution, and filtering. This remains unclotted for hours or days.

(*c*) Inject 10 c.c. of the extract into the jugular vein of the animal, and draw off samples of blood from time to time, comparing the coagulation time (which gradually lengthens) with that of specimen *a*.

(*d*) Having obtained a specimen which does not clot at all, dilute it with salt solution and pass a stream of carbon dioxide through it. Clotting is not produced as it is in "peptone" blood (see 3 *g*, below). In order to produce clotting, excess of serum, or some fluid containing thrombin, must be added. The action of leech extract is mainly due to the fact that it contains antithrombin.

(*e*) The experiments described under *d* may be repeated with leech extract plasma, obtained from the blood by centrifugalising.

(*f*) Instead of leech extract, a solution of its active principle (hirudin) may be used. This produces little or no fall of blood pressure, and so contrasts with what occurs in "peptone" injection. Leech extract produces a very small fall of arterial pressure.

3. **Influence of Commercial Peptones (Proteoses) on Coagulation.**—For the purpose of the following experiments another dog must be employed.

The animal having been anæsthetised a cannula is placed in the external jugular vein for the injection of the "peptone."

The carotid artery is connected to a mercurial manometer for the registration of arterial pressure.

Another convenient artery must be exposed and a cannula inserted into it for collection of samples of blood.

(*a*) First draw off a sample of blood and note its coagulation time.

(*b*) Draw a second sample into a strong solution of commercial peptone. The coagulation time is somewhat longer than in *a*.

(*c*) Draw off a small sample and make a blood film, staining it with methylene blue; count the colourless corpuscles.

(*d*) Then inject the peptone quickly, so that the animal receives 0·3 gramme per kilo. of body-weight. Note during and for some time after the injection a great fall in arterial blood-pressure. This has been shown by the oncometer to be due to vascular dilatation.

(*e*) After the injection draw off successive samples, and note the great prolongation of the coagulation time which is soon produced.

(*f*) Make a stained blood film from one sample as before, and note the great scarcity of colourless corpuscles.

(*g*) Dilute some of the blood which does not clot with twice its volume of salt solution, and pass a stream of carbonic acid through the mixture; coagulation soon occurs.

(*h*) The same experiment may be repeated with the same result, if "peptone" plasma obtained by centrifugalising is used instead of the whole blood.

(*i*) Finally bleed the animal to death, collecting the blood in three successive glass cylinders. Place them in the ice chest, and examine them a few days or a week later.

The first lot of blood collected will show sedimentation of corpuscles, and a slight clot at the junction of the corpuscles and supernatant plasma—that is, at the place where the white corpuscles and platelets lie.

The last lot of blood collected shows less sedimentation, and will probably have clotted throughout. This is because the blood removed last has been diluted by tissue lymph, which has passed into the blood-stream in an attempt to increase the volume of the blood, which has been lessened by the previous bleeding; the clot produced is probably due to the action of thrombokinase.

The middle sample will show something intermediate between the two extremes, the usual state of things being clot through the sediment, and the plasma above it still fluid.

4. **Intravascular Coagulation.**—A solution of nucleo-protein from the thymus, testis, lymphatic glands, or kidney has been prepared beforehand by the demonstrator. It may be prepared in one of two ways.

(*a*) *Wooldridge's Method.*—The gland is cut up small and extracted with water for twenty-four hours. Weak acetic acid (0·5 c.c. of the acetic acid of the " Pharmacopœia " diluted with twice its volume of water for every 100 c.c. of extract) is then added to the decanted liquid. After some hours the precipitated nucleo-pretein (called tissue-fibrinogen by Wooldridge) falls to the bottom of the vessel. This is collected and dissolved in 1 per-cent. sodium carbonate solution.

(*b*) *The Sodium Chloride Method.*—The finely divided gland is ground up in a mortar with about an equal quantity of solid sodium chloride and a small quantity of water. The resulting viscous mass is poured into excess of distilled water. The nucleo-protein rises to the surface of the water, where it may be collected and dissolved as before.

A rabbit is anæsthetised, and a cannula inserted into the external jugular vein. The solution is injected into the circulation through this. The animal soon dies from cessation of respiration ; the eyeballs protrude and the pupils are widely dilated. On opening the animal the heart will be found still beating, and its cavities (especially on the right side) distended with clotted blood. The vessels, especially the veins, also are full of clot. The blood of the portal vein is usually clotted most. If a dog is employed instead of a rabbit in this experiment, coagulation is usually confined to the portal area. This is related to the greater venosity of the blood in this situation. If venosity is increased in any other area, as by tetanising the muscles of one leg, clotting will be found also in the veins of this region.

5. **The Estimation of Glucose in Blood and Blood Serum.**—The estimation of glucose necessitates the complete removal of proteins. Of the many methods suggested for this purpose that of Michaelis and Rona is the best. It depends on the property which proteins possess of being absorbed by other colloids, and they are thus carried down with them when they come into contact with electrolytes. Colloidal iron (ferric hydrate) is generally used for this purpose.

(*a*) *In Serum.*—50 c.c serum are diluted to 500 c.c. with distilled water, and 40 c.c. of the colloidal iron solution are added slowly with constant shaking. On filtering, a water-clear filtrate is obtained, free from iron and protein. After exactly measuring its volume, it is slightly acidified with acetic acid and concentrated *in vacuo* or on a water-bath, and made up to 50 c.c. in a measuring flask. Glucose is estimated polarimetrically, and by any of the reduction methods described (see Lessons XII. and XIII.).

(*b*) *In the whole Blood.*—A measured or a weighed quantity of blood (30–40 c.c. or 30–40 grammes) is diluted to 1 litre with distilled water and the iron solution added as before. For 1 gramme of blood about 3 c.c. iron solution are required. The mixture is left standing, with frequent shaking, for about ten minutes. Now one adds 1–1·5 grammes finely powdered magnesium sulphate and shakes for one to two minutes. Filter and proceed as before. Although the addition of magnesium sulphate is necessary in the whole blood, such an addition can be dispensed with in the case of serum, the salts of which act as the electrolytes in the reaction. The reason why the addition of a salt to the whole blood should be necessary is not clear.

(*c*) A microchemical method for the estimation of glucose in blood has recently been described by Bang, which makes it possible to estimate it accurately in two or three drops of blood (100 mg.).

LESSON XX

HÆMOGLOBIN AND ITS DERIVATIVES

Defibrinated ox-blood suitably diluted may be used in the following experiments as in those described in Lesson IX.

1. Place some in a vessel with flat sides in front of the large spectroscope. Note the position of the two characteristic bands of **oxyhæmoglobin;** these are replaced by the single band of **hæmoglobin** after reduction by the addition of Stokes's reagent or of ammonium sulphide. By means of a small rectangular prism a comparison spectrum showing the bright sodium line (in the position of the dark line named D in the solar spectrum) may be obtained, and focussed with the absorption spectrum.

2. Obtain similar comparison spectra by the use of the microspectroscope. For this purpose a cell containing a small quantity of oxyhæmoglobin solution may be placed on the microscope stage, and a test-tube containing **carbonic oxide hæmoglobin** in front of the slit in the side of the instrument. Notice that the two bands of carbonic oxide hæmoglobin are very like those of oxyhæmoglobin, but are a little nearer to the violet end of the spectrum.

Carbonic oxide hæmoglobin may be readily prepared by passing a stream of coal gas through the diluted blood. It has a cherry-red colour, and is not reduced by the addition of ammonium sulphide (fig. 50, spectrum 4).

3. **Methæmoglobin.**—Add a few drops of ferricyanide of potassium to dilute blood, and warm gently. The colour changes to mahogany-brown. Place the test-tube in front of the small direct-vision spectroscope. Note the characteristic band in the red (fig. 50, spectrum 5). On dilution, other bands appear (fig. 50, spectrum 6). Treat with ammonium sulphide, and the band of hæmoglobin appears.

4. **Acid Hæmatin.**—(*a*) Prepare the following mixture:—150 c.c. of 90 per-cent. alcohol and 6 c.c. of concentrated sulphuric acid; take about 5 c.c. of this mixture and boil it in a test-tube. While still hot, drop into it a few drops of undiluted defibrinated blood, and filter. Note the brown colour of the fibrate. Compare the position of the absorption band in the red with that of methæmoglobin; that of acid hæmatin is further from the D line (fig. 50, spectrum 7).

(*b*) Add some glacial acetic acid to undiluted defibrinated blood. Extract this with ether by gently agitating it with that fluid. The ethereal extract should then be poured off and examined spectroscopically. The band in the red is seen, and on further diluting with ether, three additional bands appear.

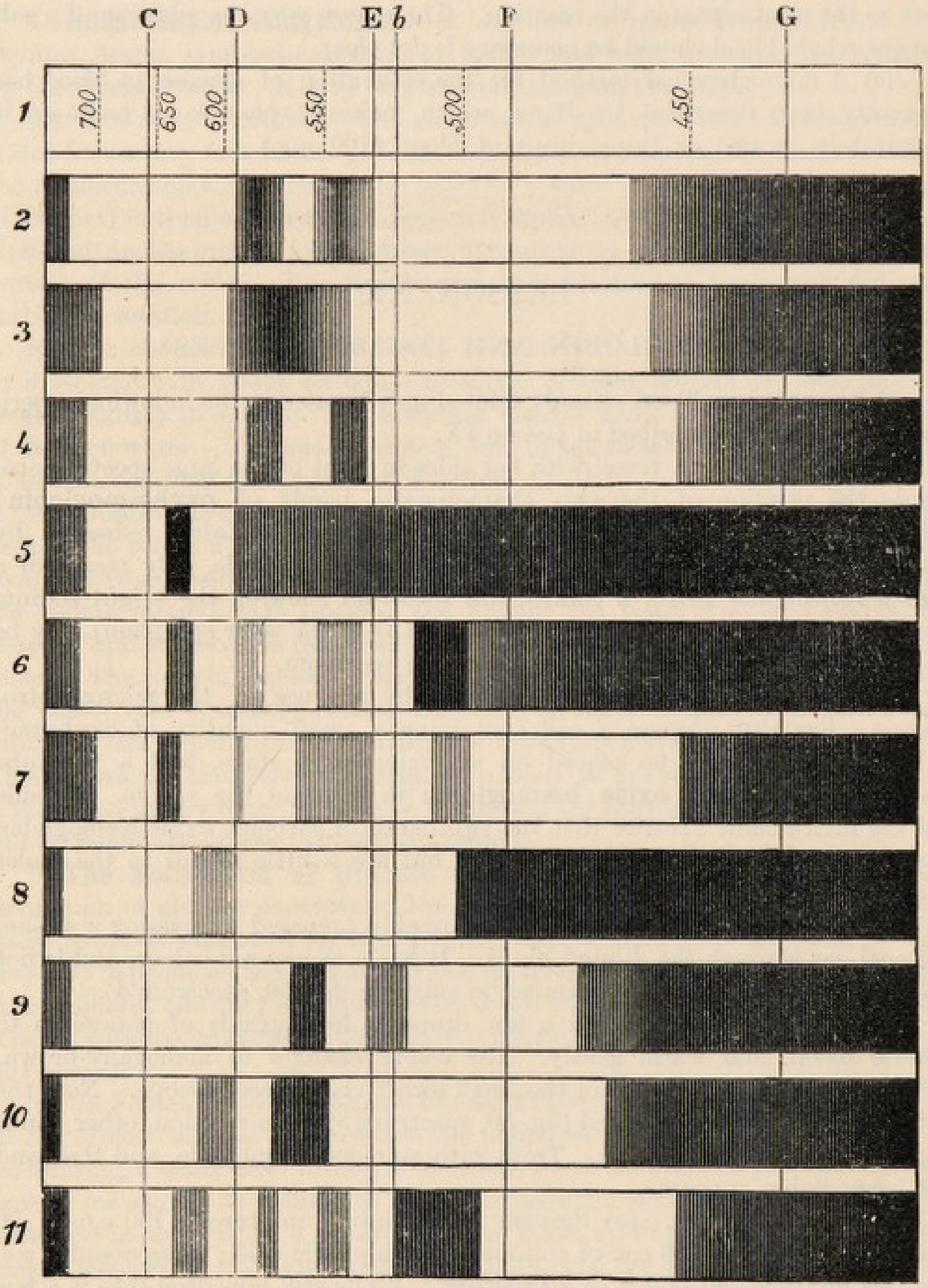

FIG. 50.—1, Solar spectrum. 2, Spectrum of oxyhæmoglobin (0·37 p.c. solution). First band, λ 589–564; second band, λ 555–517. 3, Spectrum of hæmoglobin. Band, λ 597–535. 4, Spectrum of CO-hæmoglobin. First band, λ 583–564; second band, λ 547–521. 5, Spectrum of methæmoglobin (concentrated solution). 6, Spectrum of methæmoglobin (dilute solution). First band, λ 647–622; second band, λ 587–571; third band, λ 552–532; fourth band, λ 514–490. 7, Spectrum of acid hæmatin (ethereal solution). First band, λ 656–615; second band, λ 597–577; third band, λ 557–529; fourth band, λ 517–488. 8, Spectrum of alkaline hæmatin. Band from λ 630–581. 9, Spectrum of hæmochromogen (reduced hæmatin). First band, λ 569–542; second band, λ 535–504. 10, Spectrum of acid hæmatoporphyrin. First band, λ 607–593; second band, λ 585–536. 11, Spectrum of alkaline hæmatoporphyrin. First band, λ 633–612; second band, λ 589–564; third band, λ 549–529; fourth band, λ 518–488. The above measurements (after MacMunn) are in millionths of a millimetre. The liquid was examined in a layer 1 centimetre thick. The edges of ill-defined bands vary a great deal with the concentration of the solutions.

5. **Alkaline Hæmatin.**—(*a*) Add to diluted blood a small quantity of strong caustic potash, and boil. The colour changes to brown, and with the spectroscope a faint shading on the left side of the D line is seen (fig. 50, spectrum 8).

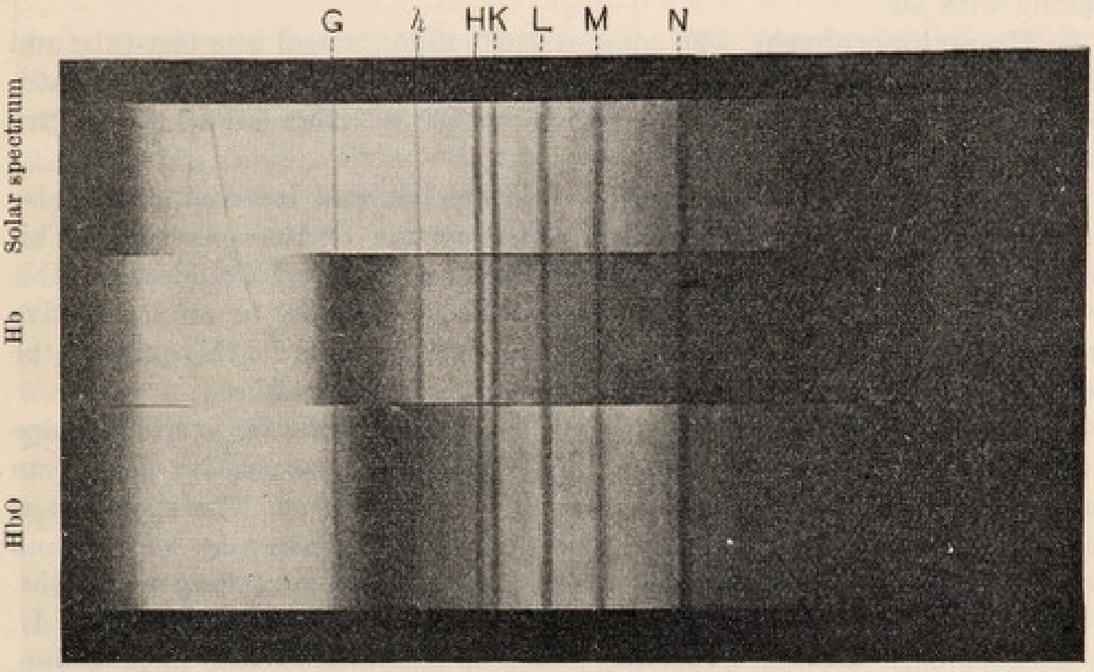

FIG. 51.—The photographic spectrum of hæmoglobin and oxyhæmoglobin. (Gamgee.)

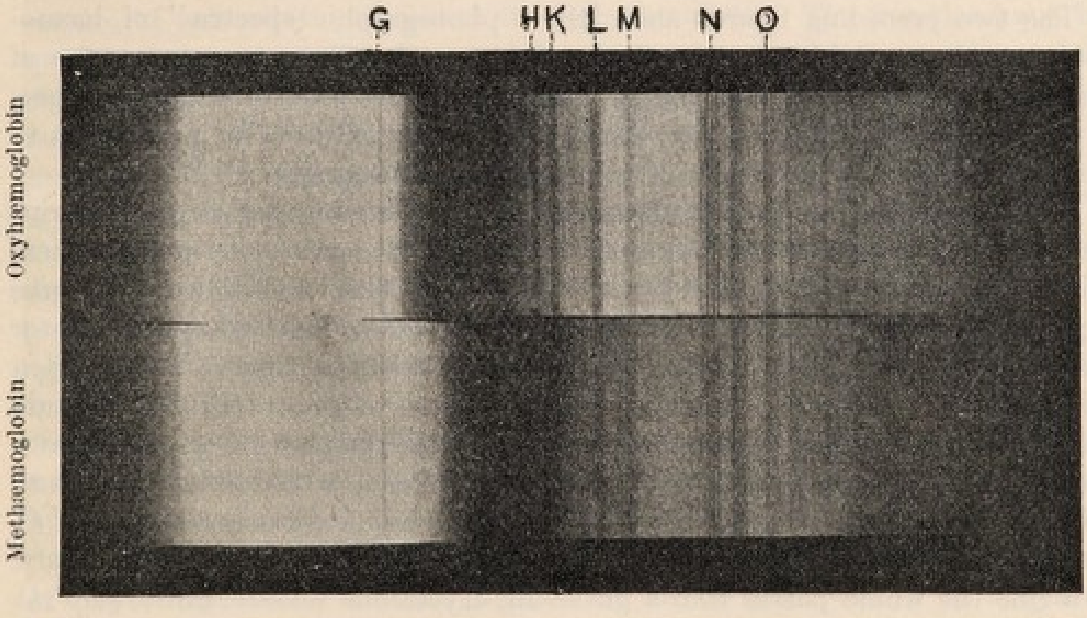

FIG. 52.—The photographic spectrum of oxyhæmoglobin and methæmoglobin. (Gamgee.)

(*b*) The band is much better seen in an alcoholic solution. Prepare the following mixture:—150 c.c. of 90 per-cent. alcohol, and 18 c.c. of 50 per-cent. potash. Take about 5 c.c. of this mixture in a test-tube and boil it. While still hot, drop into it a few drops of undiluted defibrinated blood. The fluid shows the spectrum of alkaline hæmatin. This may then be used for the next experiment.

6. **Hæmochromogen.**—Add ammonium sulphide to the solution of alkaline hæmatin; the colour changes to red, and two bands are seen, one between D and E, and the other nearly coinciding with E and *b* (fig. 50, spectrum 9). The spectrum of alkaline hæmatin reappears for a short time after vigorous shaking with air.

7. **Hæmatoporphyrin.**—To some strong sulphuric acid in a test-tube add a few drops of undiluted blood, and observe the spectrum of acid hæmatoporphyrin (iron-free hæmatin) (fig. 50, spectrum 10). Map out all the spectra you see on a chart.

8. **The Photographic Spectrum.**—Hæmoglobin and its compounds also show absorption bands in the ultra-violet portion of the spectrum. This portion of the spectrum is not visible to the eye, but can be rendered visible by allowing the spectrum to fall on a fluorescent screen, or on a sensitive photographic plate. In order to show absorption bands in this part of the spectrum very dilute solutions of the pigment must be used.

In order to demonstrate these bands, the telescope of a large spectroscope is removed, and a beam of sunlight or of light from the positive pole of an arc lamp is allowed to fall on the slit of the collimator. The spectrum is focussed on a fluorescent screen.[1] The slit is then opened very widely, and the coloured solution is interposed on the path of the beam falling on the slit.

Oxyhæmoglobin shows a band (Soret's band) between the lines G and H. In hæmoglobin, carbonic oxide hæmoglobin, and nitric oxide hæmoglobin, this band is rather nearer G. Methæmoglobin and hæmatoporphyrin show similar bands.

The two preceding figures show the "photographic spectra" of hæmoglobin, oxyhæmoglobin, and methæmoglobin, and will serve as examples of the results obtained. I am greatly indebted to the late Professor Gamgee, to whom we owe most of our knowledge on this subject, for permission to reproduce these two specimens of his numerous photographs.

9. **Preparation of Pure Oxyhæmoglobin.**—Centrifugalise dog's defibrinated blood, and pour off the serum. Centrifugalise again with physiological saline solution repeatedly until the supernatant fluid contains only traces of protein. Mix the magma of corpuscles with two or three volumes of water saturated with acid-free ether; the solution becomes clear. Then add a few drops of 1 per-cent. solution of acid sodium sulphate till the mixture looks tinted like fresh blood, owing to the precipitation of the stromata. These can be separated by filtration. Pour off the clear red fluid: cool it to 0° C., add one-fourth of its volume of absolute alcohol previously cooled to 0° C. Shake well, and then let the mixture stand at 5°–15° C. for twenty-four hours. As a rule the whole passes into a glittering crystalline mass. Filter at 0° C. and wash with ice-cold 25 per-cent. alcohol. Redissolve the crystals in a small quantity of water, and recrystallise as before. The crystals may then be spread on plates of porous porcelain, and dried in a vacuum over sulphuric acid.

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[1] Fluorescent screens, similar to those in common use in observations made with Röntgen rays, may be made by coating white cardboard with barium platinocyanide.

LESSON XXI

MUSCLE AND NERVOUS TISSUES

1. **Hopkins's Lactic Acid Test** (see p. 235) may be applied as follows. Remove one hind limb of a pithed frog. Stimulate the sacral plexus of the other side for ten minutes with a strong Faradic current. Then amputate the other hind limb. Skin both legs, and chop up the muscles of the two sides separately. Pound each in a mortar with clean sand and then with 15 c.c. of 95 per-cent. alcohol. Transfer the mixture to a beaker, and warm in the water-bath for a few minutes. Filter, and evaporate the filtrate to dryness in a water-bath. Extract the residue with about 5 c.c. of cold water, rubbing it up thoroughly with a glass rod. Filter and boil the filtrate in a test-tube for about a minute with as much animal charcoal as will lie on a threepenny-piece. Filter again and evaporate the filtrate to dryness in a water-bath. Allow the residue to cool, and dissolve it by shaking in 5 c.c. of concentrated sulphuric acid. Transfer this to a dry test-tube; add 3 drops of saturated solution of copper sulphate, and place the tube in boiling water for five minutes. Cool and add 2 drops of 0·2 per-cent. solution of thiophene in alcohol; replace the tube in the boiling water. A cherry-red colour develops in the tube containing the extract from tetanised muscle, but not in the other.

2. A rabbit has been killed and its muscles washed free from blood by a stream of salt solution injected through the aorta. The muscles have been quickly removed, chopped up small, and extracted with 5 per-cent. solution of magnesium sulphate. This extract is given out. It will probably be faintly acid. The acid is sarco-lactic acid. It may be identified by Uffelmann's or Hopkins's reaction (p. 235).

3. The coagulation of muscle somewhat resembles that of blood. This may be shown with the salted muscle plasma (the extract given out) as follows: Dilute some of it with four times its volume of water; divide it into two parts; keep one at 40° C. and the other at the ordinary temperature. Coagulation—that is, formation of a clot of myosin—occurs in both, but earliest in that at 40° C.

[illegible]

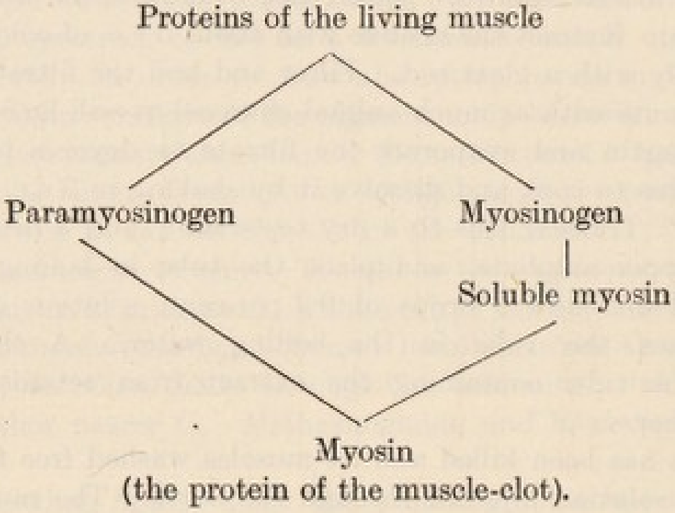
4. Remove the clot of myosin from 3; observe it is soluble in 10 per-cent. sodium chloride, and also in 0·2 per-cent. hydrochloric acid, forming acid meta-protein.

5. Make an extract of muscle in the same way, using a small quantity of physiological salt solution instead of the strong solution of magnesium sulphate employed in the foregoing experiments. Such an extract contains the two principal proteins, viz. paramyosinogen (v. Fürth's myosin) and myosinogen (v. Fürth's myogen), the two precursors of the muscle-clot or myosin (v. Fürth's muscle-fibrin). Small quantities of other proteins also present are mainly due to unavoidable mixture with small amounts of blood and lymph.

These two proteins differ in temperature of heat coagulation. Take the extract and heat it in a test-tube within a water-bath; at 47° C. paramyosinogen is coagulated ; filter this off, and heat the filtrate ; at 56° C. flocculi of the coagulated myosinogen separate out.

6. Paramyosinogen is precipitable by dialysis, and is a true globulin. Myosinogen is what is called an atypical globulin, and corresponds to the pseudo-globulin of blood serum and egg-white. Though readily salted out of solution like paramyosinogen it is not precipitable by dialysis.

7. In the process of clotting, such as occurs in *rigor mortis*, paramyosinogen is directly converted into myosin ; whereas myosinogen first passes into a soluble modification (coagulable by heat at the remarkably low temperature of 40° C.) before myosin is formed. This is shown in a diagrammatic way in the following scheme :—


Proteins of the living muscle
Paramyosinogen
Myosinogen
Soluble myosin
Myosin
(the protein of the muscle-clot).

8. When a muscle is gradually heated, at a certain temperature it contracts permanently and loses its irritability. This phenomenon is known as *heat-rigor*, and is due to the coagulation of the proteins in the muscle. If a tracing is taken of the shortening, it is found that the first shortening occurs at the coagulation temperature of paramyosinogen (47°–50° C.), and if the heating is continued a second shortening occurs at 56° C., the coagulation temperature of myosinogen. If frog's muscles are used there are three shortenings—namely, at 40°, 47°, and 56° C. ; frog's muscle thus contains an additional protein which coagulates at 40° C. This additional protein may be the soluble myosin alluded to above, some of which, in the muscle of cold-blooded animals, is present before *rigor mortis* occurs ; at any rate, it has the same coagulation temperature.

In addition to the proteins mentioned, there is a small quantity of nucleo-protein.

9. **Involuntary Muscle.**—The main facts just described for voluntary are true also for involuntary muscle. The chief distinction lies in the quantity of nucleo-protein, which is more abundant in those forms of muscle the fibres of which are least different from the mesoblastic cells from which all ultimately originate. This may be readily shown by the following simple experiment.

Take equal parts of voluntary muscle, heart muscle, and plain muscle (say from the stomach wall), and extract each for the same time with equal amounts of 0·15 per-cent. solution of sodium carbonate. Filter and add to each filtrate acetic acid, drop by drop. The extract of voluntary muscle gives an opalescence only; in the case of the plain muscle there is an abundant precipitate; the heart muscle gives a result intermediate between the other two.

b(a)/hre

10. **Pigments of Muscle:**—

(*a*) Notice the difference between the red and pale muscles of the rabbit.

(*b*) Examine a piece of red muscle (*e.g.* the diaphragm) spectroscopically for oxyhæmoglobin (or it may be more convenient to make an aqueous extract of the muscle, and examine that).

(*c*) A piece of the pectoral muscle of a pigeon has been soaked in glycerol. Press a small piece between two glass slides, and place it in front of the spectroscope. Observe and map out the bands of myohæmatin. This pigment is doubtless a derivative of hæmoglobin.

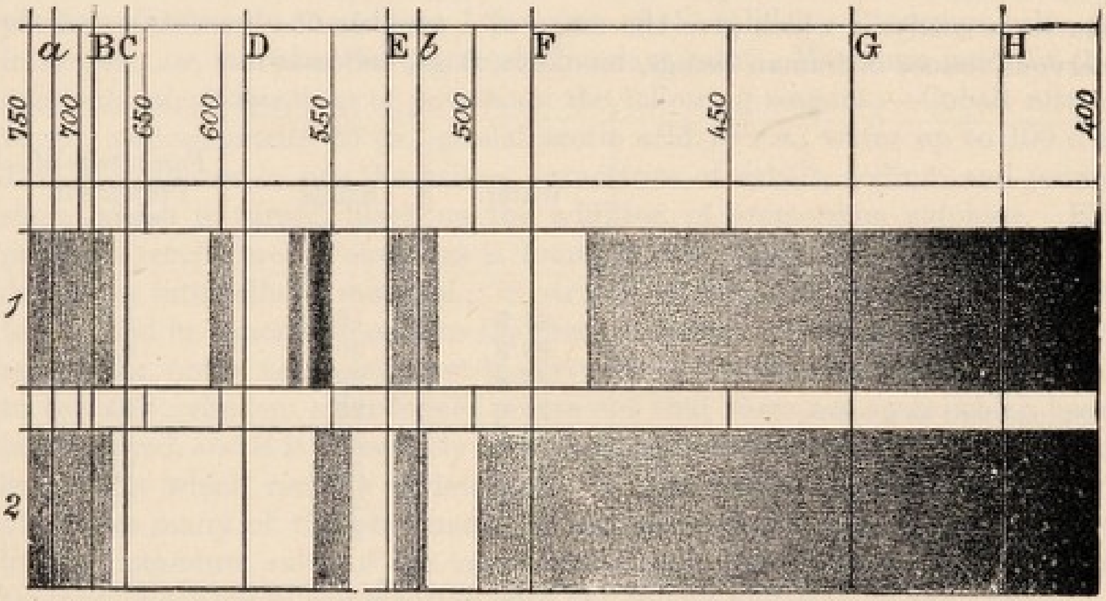


FIG. 53.—1, Absorption spectrum of myohæmatin, as seen in muscle rendered transparent by glycerin. 2, Absorption spectrum of modified myohæmatin.

(*d*) Pieces of the same muscles have been placed in ether for twenty-four hours. The ether dissolves out a yellow lipochrome from the adherent fat. A watery fluid below contains modified myohæmatin. Filter it; compare its spectrum with that of hæmochromogen. The myohæmatin bands are rather nearer the violet end of the spectrum (fig. 53, spectrum 2) than those of hæmochromogen (fig. 50, spectrum 9).

11. **Creatine:**—

(*a*) Take some of the red fluid described in 10 *d*, and let it evaporate to dryness in a desiccator over sulphuric acid.

In a day or two, crystals of creatine tinged with myohæmatin separate out.

(*b*) Take an aqueous extract of muscle, such as Liebig's extract or beef-tea; add baryta water to precipitate the phosphates, and filter. Remove excess of baryta by a stream of carbonic acid; filter off the barium carbonate and evaporate the filtrate on the water-bath to a thick syrup. Set it aside to cool, and in a few days a crystalline deposit of creatine will be found at the bottom of the vessel. This is washed with alcohol and dissolved in hot water. On concentrating the aqueous solution, crystals once more separate out, which may be still further purified by recrystallisation.

NERVOUS TISSUES

The chemical investigation of nervous tissues is not well adapted to class exercises ; still it may not be uninteresting to state briefly the principal known facts in relation to this subject. The most important points which any table of analyses will show are : (1) the large percentage of water, especially in the grey matter ; (2) the large percentage of protein. In grey matter, where the cells are prominent structures, this is most marked, and of the solids, protein material here comprises more than half of the total. The following are some analyses which give the mean of a number of observations on the nervous tissues of human beings, monkeys, dogs, and cats :—

	Water.	Solids.	Percentage of Proteins in Solids.
Cerebral grey matter	83·5	16·5	51
,, white ,,	69·9	30·1	33
Cerebellum	79·8	20·2	42
Spinal cord as a whole	71·6	28·4	31
Cervical cord	72·5	27·5	31
Dorsal cord	69·8	30·2	28
Lumbar cord	72·6	27·4	33
Sciatic nerves	65·1	34·9	29

The most important protein is *nucleo-protein* ; there is also a certain amount of *globulin*, which, like the paramyosinogen of muscle, is coagulated by heat at the low temperature of 47° C. A certain small amount of neurokeratin (especially abundant in white matter) is included in the foregoing table with the proteins. The granules in nerve cells (Nissl's bodies) are nucleo-protein in nature.

Heat Contraction in Nerve.—A nerve, when heated, shortens ; this shortening occurs in a series of steps, which, as in the case of muscle, take place at the coagulation temperatures of the proteins present. The first step in the shortening occurs in the frog at about 40°, in the mammal at about 47°, and in the bird at about 52° C. The nerve is killed at the same temperatures.

Lipoids.—After the proteins, the next most abundant substances present are the *lipoids*. A fuller consideration of these substances is given in Lesson IV. (see pp. 37 to 40). They comprise :—

1. *Phosphatides* ; of these, lecithin is the best known ; kephalin and sphingomyelin are others.
2. *Galactosides* ; these are nitrogenous glucosides free from phosphorus ; they yield on hydrolysis the reducing sugar galactose.

3. *Cholesterol*; a crystalline monohydric alcohol free from both nitrogen and phosphorus.

The following are some recent analyses of nerve by Falk, the numbers given are percentages of the total solids :—

	Medullated Nerve.	Non-medullated Nerve.
Cholesterol	25·0	47·0
Lecithin	2·9	9·8
Kephalin	12·4	23·7
Galactosides	18·2	6·0

Fresh nervous tissues are alkaline, but, like most other living structures, they turn acid after death. The change is particularly rapid in grey matter. The acidity is due to sarco-lactic acid.

Finally, there are smaller quantities of other extractives and a small proportion of mineral salts (about 1 per cent. of the solids). Potassium salts, as in muscle, are stated to be the most abundant salts. Macallum used for the micro-chemical detection of potassium the following reagent :—Cobalt nitrite 20 gr., sodium nitritè 35 gr., glacial acetic acid 10 c.c., water up to 100 c.c. This precipitates *in situ* the yellow hexanitrite of cobalt, sodium and potassium, which is turned black on the addition of ammonium sulphide. His principal results are : Potassium is found in cell protoplasm, but more abundantly in intercellular material ; in striped muscle it is limited to the dark bands, and in pancreatic cells to the granular zone. It is not discoverable in any nuclei, nor in nerve cells, but in nerve-fibres it is found in patches external to the axis cylinder. Macdonald points out that these are spots which have been injured, and it is apparently only on injury that the potassium is liberated in a form which renders it detectable by Macallum's reagent. Macdonald attributes many of the phenomena of nervous action to electrolytic changes in the potassium salts of the nerve-fibres, and which are present in large amounts, possibly in combination with the colloid materials of the axon.

Very little is known of the chemical changes nervous tissues undergo during activity. We know that oxygen is very essential, especially for the activity of grey matter ; cerebral anæmia is rapidly followed by loss of consciousness and death. Similar respiratory exchanges, though less in amount, occur in peripheral nerves. It can hardly be doubted that the lipoids, and especially the phosphatides, which are extremely labile substances, participate in metabolism.

Cerebro-spinal Fluid.—This is secreted by the epithelium which covers the choroid plexuses (choroid gland), and plays the part of the lymph of the central nervous system. It is a very watery fluid, containing, besides some inorganic salts similar to those of the blood, a trace of protein matter (globulin) and a small amount of sugar. It contains no choline or cholesterol normally.

Chemistry of Nerve Degeneration.—In Wallerian degeneration of nerve, several investigators have attempted to discover how the degenerated nerve differs from a healthy nerve. Little or no change in the peripheral end can be detected up to about three days after a nerve has been divided, and the nerve-fibres remain excitable up to that time. They then show a progressive increase in the quantity of water, and a corresponding decrease in the proportion of solids. The percentage of phosphorus also decreases, and it entirely disappears in a little more than three weeks after the nerve is cut. When regeneration occurs, the nerves return approximately to their previous composition.

It has also been shown that in spinal cords in which a unilateral degeneration of the pyramidal tract has been produced by a lesion in the opposite cerebral hemisphere, there is a similar increase of water and diminution of phosphorus on the degenerated side. Further, in a divided nerve Noll has shown that the phosphorised material also diminishes somewhat in the central end, due to "disuse atrophy."

This disappearance of phosphorus must be due to the break-up of phosphatides, and the liberation of phosphoric acid, which is carried away as phosphates by the lymph and blood.

The staining reactions of a degenerated nerve also indicate that the appearances are not only due to a breakdown in an anatomical sense, but in a chemical sense also. Of these staining reactions the one most often employed is that which is associated with the name of Marchi. This is the black staining which the medullary sheaths of degenerated nerve-fibres show when after being hardened in Müller's fluid they are treated with Marchi's reagent, a mixture of Müller's fluid, and osmic acid. Healthy nerve-fibres are not blackened by this reagent, because the more rapidly penetrating chromic acid of the Müller's fluid has already supplied the unsaturated oleic acid radical in the lecithin and other phosphatides with all the oxygen they can take up. But when the nerve is degenerated, the oleic acid is either increased in amount, or so liberated from its previous combination in the lecithin molecule, that it is then able also to take oxygen from osmic acid and reduce it to a lower black oxide. In the later stages of degeneration the Marchi reaction is not obtained, because the fat globules have then been absorbed.

In certain diseases of the central nervous system, such as General Paralysis of the insane, degeneration occurs on a large scale, and the products of the chemical disintegration of the cerebral tissue have been sought for in the blood, but with more profitable results in the cerebro-spinal fluid. This fluid under those circumstances shows an excess of protein which is mainly nucleo-protein; cholesterol can also be usually detected in the fluid, and so also can choline or similar bases which originate from the decomposition of phosphatides. Although many physiologists have taken up the choline question and the methods for identifying this base, it must be admitted that the tests hitherto devised are not absolutely conclusive, for sufficient of the base cannot be collected for a complete analysis. The base which is present if not choline is a nearly related substance, perhaps a derivative of choline, and according to the latest view the questionable material is trimethylamine, which we have already seen is a cleavage product of choline.

The tests employed to detect choline are mainly three :—(1) The fluid is diluted with about five times its volume of absolute alcohol and the precipitated proteins are filtered off. The filtrate is evaporated to dryness at 40° C., and the residue dissolved in absolute alcohol and filtered ; the filtrate from this is again evaporated to dryness, and again dissolved in absolute alcohol, and this should be again repeated. To the final alcoholic solution, an alcoholic solution of platinum chloride is added, and the precipitate so formed is allowed to settle and is washed with absolute alcohol by decantation ; the precipitate is then dissolved in 15 per-cent. alcohol, filtered, and the filtrate is allowed to slowly evaporate in a watch-glass at 40° C. The crystals can then be seen with the microscope. They are recognised not only by their yellow colour and octahedral form, and by their solubility in water and 15 per-cent. alcohol, but also by the fact that on incineration they yield 31 per cent. of platinum and give off the odour of trimethylamine. There is a danger of mistaking such crystals for those obtained from the chlorides of potassium and ammonium ; but the presence of such contaminations may be minimised by the use of alcohol as water-free as possible. The crystals of choline platinum chloride are doubly refracting, whereas the platinum chlorides of ammonium and potassium are not. (2) The following test is distinctive of choline and leads to no risk of confusion with other substances. The final alcoholic solution prepared as above is evaporated to dryness, and the residue taken up with water, to this is added a strong solution of iodine (2 grammes of iodine and 6 grammes of potassium iodide in 100 c.c. of water). In a few minutes dark-brown prisms of choline periodide are formed. These look very like hæmin crystals. If the slide is allowed to stand so that the liquid gradually evaporates, the crystals slowly disappear, and their place is taken by brown oily droplets, but if a fresh drop of the iodine solution is added the crystals slowly form once more. (3) A physiological test, namely, the lowering of arterial blood-pressure (partly cardiac in origin, and partly due to dilatation of peripheral vessels) which a saline solution of the residue of the alcoholic extract produces : this fall is abolished, or even replaced by a rise of arterial pressure, if the animal has been atropinised. Such tests have already been shown to be of diagnostic value in the distinction between organic and so-called functional diseases of the nervous system.

LESSON XXII

THE URINE

TOTAL NITROGEN, UREA, AND AMMONIA

Kjeldahl's Method of Estimating Total Nitrogen.—This simple method can be used in connection with most substances of physiological importance. Briefly, it consists in converting all the nitrogen present into ammonium sulphate by means of sulphuric acid ; then rendering alkaline with soda, and distilling over the ammonia into standard acid, the diminution in acidity of which measures the amount of ammonia present.

The following modification of the original method is used in this laboratory.

About 1 gramme of the substance under investigation (or in the case of urine when one wishes to make an estimation of total nitrogen, 5 or 10 c.c.

of that fluid) is placed in a round-bottomed Jena flask of about 250 c.c. capacity, and 20 c.c. of pure sulphuric acid are added. Six grammes of potassium sulphate and about half a gramme of copper sulphate are also added. The flask should be provided with a loose balloon stopper, and arranged in a sloping direction over a small flame. The mixture is heated slowly until it boils. In about twenty minutes the fluid becomes nearly colourless; boiling is continued for another forty-five minutes. By this time all the nitrogen will be in combination as ammonia.

After cooling, the fluid is washed into a litre flask of Jena glass (fig. 54, A) and water added until the total volume of the fluid is about 400 c.c. Add then an excess of 40 per-cent. caustic soda solution, a few pieces of granulated zinc to avoid bumping in the subsequent distillation, and immediately fit the glass tube B into the neck of the flask by means of a well-fitting rubber stopper. The other end of B leads into the flask C which contains a measured amount (50 or 100 c.c.) of standard sulphuric acid; $\frac{1}{5}$-normal acid is a convenient strength to use. The bulb D shown in the figure guards against regurgitation, and the end of the tube should dip just below the surface of the acid in C. The mixture in the flask is now boiled for about half an hour, when all the ammonia will have distilled over; the use of a condenser around the tube B is unnecessary. The acidity of the standard acid is then determined by titrating with standard alkali, a few drops of lacmoid being added to act as the indicator of the end of the reaction. This gives a pink colour with acid, blue with alkali.

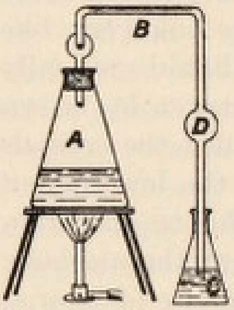


FIG. 54.—Kjeldahl's method: distilling apparatus.

Example.—Suppose 1 gramme of a nitrogenous substance is taken, and the ammonia distilled over into 100 c.c. of $\frac{1}{5}$-normal sulphuric acid. This is then titrated with a corresponding solution of sodium hydrate, and it is found that the neutral point is reached when 60 c.c. of the soda solution have been added. The other 40 c.c. must therefore have been neutralised by the ammonia derived from the substance under investigation. This 40 c.c. of acid $=$ 8 c.c. of normal acid $=$ 8 c.c. of normal ammonia $= 8 \times 0{\cdot}017 = 0{\cdot}136$ gramme of ammonia. One gramme of the substance analysed, therefore, yields 0·136 gramme of ammonia, and this contains 0·112 gramme of nitrogen; 100 grammes will therefore contain 11·2 grammes of nitrogen. If the strength of the acid is that just recommended each c.c. corresponds to 0·0028 gramme of nitrogen.

ESTIMATION OF AMMONIA IN URINE

In all the exact methods the ammonia is set free by alkali, and absorbed by standard acid. As boiling with strong alkalis splits off ammonia from other urinary constituents, *Schlössing* originally used lime-water as the alkali and allowed the ammonia to be absorbed by decinormal acid under a bell-jar. This method occupies three to four days for its completion, but may be carried out more rapidly by distilling off the ammonia *in vacuo* (Wurster's, Nencki's, and other methods). Folin's method avoids these drawbacks by driving off the ammonia by means of an air current.

1. **Folin's Method.**—Twenty-five c.c. of urine are measured into a tall cylinder; 1 gramme of anhydrous sodium carbonate and 10 c.c. kerosene oil (to prevent frothing) are added, and air is drawn through the apparatus for two hours. The air current is previously passed through acid in order to remove ammonia from it. The ammonia set free from the urine is carried away and absorbed in a specially designed absorption apparatus, which contains 20 c.c. of decinormal sulphuric acid. After two hours the ammonia is estimated by titrating the contents of the absorption vessel with decinormal alkali. The number of c.c. decinormal alkali subtracted from 20 gives the number of c.c. of decinormal acid neutralised by ammonia. 1 c.c. of decinormal acid=0·0017 gramme of ammonia. Even with this method at least two hours are necessary. In Folin's most recent method *the time is reduced to fifteen minutes* by only taking 2 c.c. of urine, and estimating the ammonia colorimetrically by means of Nessler's reagent.[1]

2. **The Formalin Method.**—This method is an adaptation to urine of Sörensen's method for the estimation of amino-acids (see p. 232). When neutral solutions of ammonium salts are treated with an excess of formaldehyde, the compound hexamethylene tetramine (urotropine) is formed, and a corresponding amount of acid is set free from the ammonium salt ($4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 6H_2O + 4HCl$), which can be titrated in the usual way.

This method, when applied to urine, does not give exact results, because the small amounts of amino-acids present in the urine also enter into the reaction. It is, however, of value as a rapid clinical method for the estimation of ammonia.

Twenty-five c.c. of urine are measured into a flask; 15 grammes finely powdered potassium oxalate[2] and a few drops of phenolphthalein solution (as an indicator) are added. Shake for a few minutes and titrate with decinormal alkali. (The number of c.c. required furnishes at the same time data for calculating the acidity of the urine.) Now add 10 c.c. of formalin[3] diluted with 10 c.c. water and neutralised with decinormal alkali (using phenolphthalein as an indicator). The pink colour disappears on mixing the urine and the formalin solution. Run in as much of the decinormal alkali as is necessary to bring back the same tint as before.

$$1 \text{ c.c. } \frac{N}{10}\text{NaOH} = 0{\cdot}0017 \text{ grammes } NH_3.$$

PREPARATION OF UREA FROM URINE

Evaporate about 50 c.c. of urine to a small bulk and finally to complete dryness on the water-bath at boiling temperature. Turn out the flame and extract the residue with about 10 c.c. of acetone. The dish may be replaced on the water-bath, which will still be quite hot enough to boil the acetone. Pour off the hot acetone extract into a dry watch glass or evaporating basin.

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[1] Nessler's reagent is an alkaline solution of mercuric iodide, which gives a characteristic yellow colour with traces of ammonia.

[2] Potassium oxalate is added to precipitate calcium salts, which interfere with the end point.

[3] Formalin is a commercial name for a solution containing 40 per cent. of formaldehyde.

On cooling, urea crystallises out in silky needles. The acetone quickly evaporates spontaneously, and a crystalline mass of urea is left. Dissolve some of this in water; place a drop of the aqueous solution on a slide, and allow it to crystallise; examine the crystals with the microscope. Place another drop on another slide, and add a drop of nitric acid; examine microscopically the crystals of urea nitrate which separate out.

ESTIMATION OF UREA

A great many methods have been proposed for the purpose of urea estimation, which all had to be modified repeatedly, and it remains still to be seen if the methods now in use stand the test of further investigation. All the methods are indirect, *i.e.* the urea is decomposed in some way, and the quantity of one of the decomposition products is estimated. According to the nature of this decomposition the methods may be divided into two main groups:—

1. **Methods which are based on the Decomposition of Urea into Nitrogen, Carbonic Acid, and Water.**—The hypobromite method (see p. 180) is a type of these methods, which have been abandoned for accurate work owing to the fact that (1) even pure urea solutions yield variable amounts of nitrogen, and (2) other urinary constituents (ammonia, uric acid, creatinine, allantoin, etc.) also yield nitrogen under the conditions of the experiment.

2. **Methods which are based on the Decomposition of Urea into Ammonia and Carbonic Acid.**—This decomposition is brought about by heating with either phosphoric or hydrochloric acid, or with salts like magnesium chloride, lithium chloride, and hydrochloric acid, potassium acetate and acetic acid, potassium bisulphate and zinc sulphate. As the urine contains preformed ammonia, this has to be estimated previously.

Of the methods now in use that of Benedict and Folin seem to be the most satisfactory.

(*a*) *Benedict's Method.*—In this method the urine is heated with potassium bisulphate and zinc sulphate to 165° C. for one hour. The fluid is diluted, made alkaline, and the ammonia distilled off as in Kjeldahl's method. Five c.c. of urine are introduced into a rather wide Jena test-tube, and about 3 grammes of potassium bisulphate and 1–2 grammes zinc sulphate are added. A bit of paraffin and a little powdered pumice are introduced to prevent frothing and the mixture boiled practically to dryness, either over a free flame or in a sulphuric acid bath kept at about 130° C. The tube is then immersed into a sulphuric acid bath maintained at 162° and left there for one hour. The contents are transferred with distilled water into a distilling apparatus, made alkaline with sodium carbonate and distilled as described under Kjeldahl's method. When the distillation is completed, the standard acid in the receiver should be boiled to remove CO_2 before titration.

$$1 \text{ c.c. } \frac{N}{5} H_2SO_4 = 0{\cdot}0028 \text{ grammes N} = 0{\cdot}006 \text{ grammes urea.}$$

(*b*) *Folin's Method.*—In this method only a very small quantity of urine is used (1 c.c. of the ten times diluted urine, containing from 0·75–1·5 milligrammes of urea nitrogen). The decomposition into ammonia and CO_2 is brought about by heating with potassium acetate and acetic acid to 155° C. for ten minutes.[1] Caustic alkali is added and the ammonia is aspirated into $\frac{N}{10}$ acid. As the quantity of ammonia is too small to be titrated accurately, it is estimated colorimetrically, as described previously (see Ammonia estimation, p. 253).

LESSON XXIII

THE URINE (*continued*)

URIC ACID AND CREATININE

Preparation of Pure Uric Acid.—This may best be done by taking the solid urine of a bird or a snake (which consists principally of the acid ammonium urate); one has not then to separate any pigment.

The material is boiled with 10 per-cent. caustic soda or ammonia, diluted, and then allowed to stand. The clear fluid is decanted and poured into a large excess of water to which 10 per cent. of hydrochloric acid has been added; the crystals of uric acid which form are filtered off after standing twenty-four hours. These may be purified by washing, re-solution in soda, and re-precipitation by acid.

ESTIMATION OF URIC ACID

In human urine, the normal amount of uric acid (in the form of urates) excreted in the day varies from 0·4 to 0·8 gramme. If the daily volume of urine is taken as 1500 c.c., the percentage of uric acid will therefore be from 0·026 to 0·052.

The Folin-Shaffer Method.—Hopkins's method referred to in the text (p. 199) is now generally replaced by the modification of it introduced by Folin and Shaffer. The chief alteration introduced is the substitution of ammonium sulphate for ammonium chloride as a precipitant. This is first added in an acid solution; this precipitates a mucoid from the urine which interferes with the subsequent titration; the urine is then rendered alkaline, and the uric acid comes down as ammonium urate. The Folin-Shaffer reagent also contains uranium acetate; this precipitates the phosphates, and thus facilitates filtration and removal of the mucoid.

Analysis.—To 200 c.c. of urine add 50 c.c. of the Folin-Shaffer reagent (500 grammes ammonium sulphate, 5 grammes uranium acetate, 60 c.c. of 10 per-cent. acetic acid in 650 c.c. of water; the volume of the solution is then almost exactly 1 litre). Allow the mixture to stand for some minutes and then filter through a double dry filter into a dry flask. Measure 125 c.c.

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[1] A temperature indicator is used in this process, which consists of a sealed tube containing powdered chloride-iodide of mercury, a salt which is bright red and melts to a clear dark red fluid at 155° C. The sealed tube is heated together with the urine and the potassium acetate mixture.

of the filtrate (corresponding to 100 c.c. urine) into a beaker, and add 5 c.c. of concentrated ammonia; allow this to stand for twenty-four hours. Decant the fluid from the precipitate through a small filter; wash the precipitate on to the filter with 10 per-cent. solution of ammonium sulphate, and repeat the washing twice more to remove the chlorides. Rinse the precipitate back into the beaker with almost 100 c.c. of hot water; cool, and then add 15 c.c. of concentrated sulphuric acid, and titrate at once without cooling with $\frac{1}{20}$ normal potassium permanganate solution (1·581 gramme potassium permanganate to 1 litre of water) until a permanent rose colour is obtained. The number of c.c. of the permanganate solution used from the burette, multiplied by 3·75, gives the quantity of uric acid in milligrammes per 100 c.c. of urine. Three milligrammes should be added to the result to allow for the solubility of ammonium urate in the reagents used.

Folin's and Macallum's Colorimetric Method.—In this method the blue colour which is produced by the action of phosphotungstic acid on uric acid is made use of for the quantitative estimation of the latter by comparing it in a colorimeter with a standard solution of uric acid similarly treated. Since the colour reaction is very delicate, only a very small volume of urine is required for analysis. The method is only applicable to normal urines. For pathological urines (diabetic, albuminous) a modified method has been worked out by Folin and Denis. With some modifications it can also be applied for the estimation of uric acid in blood.

From 2 to 5 c.c. of urine (depending on the specific gravity), say 3 c.c., are measured into a 100 c.c. beaker, and after adding a drop of saturated oxalic acid solution the whole is evaporated to dryness on the water-bath. To the dry, cool residue are added 10–15 c.c. of a mixture of 2 parts of dry ether and 1 part of methyl alcohol. After standing for a few minutes, the solution is decanted and the residue extracted once more in the same way. By these means certain substances (polyphenols) are removed, which also give a blue colour with phosphotungstic acid. (For ordinary class work 90 per cent. alcohol can be substituted for the ether-alcohol mixture, although the slight solubility of uric acid in it introduces a small source of error.)

To the washed residue in the bottom of the beaker is next added water (5–10 c.c.) and a drop of saturated sodium carbonate solution, and the mixture is shaken so as to secure complete solution. Add 2 c.c. of the phosphotungstic acid solution,[1] and 20 c.c. of a saturated solution of sodium carbonate. The resulting blue solution is transferred to a 100-c.c. measuring flask, diluted with water up to the mark, and poured into one of the tubes of the colorimeter (see next page). The second tube is filled with the standard solution for comparison, which is obtained by treating 1 milligramme of uric acid in 0·4 per-cent. lithium carbonate solution with phosphotungstic acid in exactly the same proportions.[2]

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[1] The solution is prepared by boiling 100 grammes of sodium tungstate with 80 c.c. of 85 per-cent. phosphoric acid and 750 c.c. of water for a couple of hours and then diluting to 1 litre.

[2] This standard solution must be freshly prepared, and in order to avoid this inconvenience, Folin and Denis have more recently introduced a uric acid formaldehyde solution which keeps. One gramme of uric acid in a litre flask is dissolved in 200 c.c. of a 0·4 per-cent. solution of lithium carbonate. To this 40 c.c. of 40 per

Calculation.—Set the standard at a depth of x mm. Equality of tint is obtained, when the depth of the unknown solution is y. Therefore 3 c.c. of urine originally taken for analysis contain $\frac{x}{y}$ milligrammes uric acid, and 100 c.c. contain $\frac{100x}{3y}$.

ESTIMATION OF CREATININE

The following colorimetric method (Folin's) is now generally employed for the estimation of creatinine; and with a slight modification it may also be used for the estimation of creatine. It is based on the red colour which has been shown by Jaffé to develop when an alkaline solution of picric acid is added to a solution of creatinine; this is compared with the colour of a standard solution of potassium bichromate, the tint of the two fluids being almost identical. If creatine has to be estimated, this is first transformed into creatinine by boiling with hydrochloric acid. The apparatus and reagents necessary are :—

1. A colorimeter consisting of two tubes, the height of the column in which can be read by graduations in tenths of a millimetre.
2. A half-normal solution of potassium bichromate (24·5 grammes per litre).
3. A saturated solution of picric acid.
4. 10 per-cent. caustic soda.

To perform the analysis one tube of the colorimeter is filled with the bichromate solution up to the height of 8 mm. 10 c.c. of urine are measured into a ½-litre flask, 15 c.c. of the picric acid solution and 5 c.c. of the caustic soda solution added; after standing five minutes water is added until the total volume of the mixture is 500 c.c. This solution is poured into the second tube of the colorimeter to such a height (which is read off) that, on looking down through it the intensity of the colour is the same as that in the standard tube by its side. Folin found that a layer 8 mm. deep of the standard solution has the same colour as a layer 8·1 mm. deep of a solution prepared from 10 milligrammes of pure creatinine, picric acid, and caustic soda. The calculation for x, the number of milligrammes of creatinine in the urine, is therefore $x = 10 \times \frac{8{\cdot}1}{y}$, where y is the millimetre depth of the unknown solution which matches 8 mm. of the standard bichromate solution.

cent. formaldehyde are added, and the mixture shaken and allowed to stand for a few minutes. It is then acidified with 20 c.c. of normal acetic acid, and the whole diluted with water to 1 litre. Next day it is standardised against a freshly prepared lithium carbonate solution of uric acid. The colour produced by 5 c.c. of the solution corresponds very nearly with the colour obtained from 1 milligramme of uric acid. The colorimetric reading obtained for the solution when thus compared against 1 milligramme of pure uric acid is, of course, thereafter to be used as the standard value corresponding to 1 milligramme of uric acid.

LESSON XXIV

THE URINE (*continued*)

THE INORGANIC SALTS

The principal inorganic salts in urine are chlorides, sulphates, and phosphates.

The chlorides consist chiefly of those of sodium and potassium, the latter being present in quite small quantities. The amount of sodium chloride in human urine is 10–15 gramme *per diem* ; taking the total volume of the daily urine at 1500 c.c., this corresponds to 0·6 to 1 per cent.

The phosphoric acid in the urine is combined with sodium, potassium, calcium, and magnesium. The total P_2O_5 in the twenty-four hours is about 3·5 grammes, or 0·24 per cent. In addition to the inorganic phosphates, there are small amounts of organic compounds of phosphorus, such as glycerophosphates. The following exercises therefore contain one for the estimation of total phosphorus.

The sulphates in the urine are of two kinds: the inorganic sulphates, namely, those of sodium and potassium, and the ethereal sulphates (see p. 193). In human urine about 2 grammes of total sulphuric acid are passed *per diem*, or 0·13 per cent.

Although the major portion of the sulphur in the urine is present as sulphate, there is in addition a small quantity combined in organic compounds. It is therefore necessary to add an exercise for the estimation of total sulphur.

ESTIMATION OF CHLORIDES

Volhard's method adopted for the determination of the total chlorides consists in their precipitation by excess of a standard solution of silver nitrate in the presence of nitric acid. The excess of silver is then estimated in an aliquot part of the filtrate with a solution of potassium or ammonium thiocyanate, which has been previously standardised against the silver solution, a ferric salt being used as indicator.

The following solutions are necessary :—

i. A standard solution of silver nitrate of such a strength that 1 c.c. corresponds to 0·01 gramme of sodium chloride (29·075 grammes of fused silver nitrate in a litre of distilled water).

ii. Solution of potassium thiocyanate (8 grammes to the litre).

iii. Pure nitric acid free from chlorides.

iv. A saturated solution of iron alum.

The potassium thiocyanate solution is first standardised in the following way :—

Place 10 c.c. of the silver solution in a beaker, add 5 c.c. of the nitric acid, 5 c.c. of the solution of iron alum, and 80 c.c. of water. Run into this from a burette the thiocyanate solution until a permanent red tinge is obtained. Note the number of c.c. necessary for this purpose, and call this number x.

Analysis.—Place 10 c.c. of the urine in a 100-c.c. measuring flask with a pipette; add about 4 c.c. of pure nitric acid, and 20 c.c. of the standard solution of silver nitrate. Fill up the flask to the 100-c.c. mark with distilled water, mix thoroughly, and filter through a dry filter-paper into a dry vessel.

Measure out 50 c.c. (that is exactly half) of the filtrate, add 5 c.c. of the iron alum solution, and titrate with the thiocyanate solution until a permanent red colour is obtained. Call the number of c.c. so used a. This must be doubled to represent what the total fluid (100 c.c.) would have required.

In the previous standardisation of the thiocyanate solution we found that x c.c. of the thiocyanate solution is equivalent to 10 c.c. of the silver solution; therefore $2a$ c.c. are equivalent to $\frac{10 \times 2a}{x}$ of the silver solution, and this represents the amount of silver solution not used in precipitating the chlorides. Therefore $20 - \frac{10 \times 2a}{x}$ is the number of c.c of the silver nitrate solution utilised in the precipitation of the chlorides.

Ten c.c. of urine (the amount taken for analysis) therefore contains the amount of chlorides which require $20 - \frac{20a}{x}$ c.c. of the standard silver nitrate for their precipitation, and as each c.c. of the standard solution is equivalent to 0·01 gramme of sodium chloride, the total chlorides in the 10 c.c. of urine (expressed as sodium chloride) is $\left(20 - \frac{20a}{x}\right) \times 0{\cdot}01 = 0{\cdot}2 - \frac{0{\cdot}2a}{x}$ grammes. If one multiplies this by 10, we obtain $2 - \frac{2a}{x}$ grammes, which is the amount per 100 c.c. of urine. If the total urine passed in the day is 1500 c.c., we have further to multiply this by 15 to obtain the amount excreted in the twenty-four hours.

ESTIMATION OF PHOSPHATES

(*a*) *Estimation of the total phosphates.*

For this purpose the following reagents are necessary:—

i. A standard solution of uranium nitrate. The uranium nitrate solution contains 35·5 grammes in a litre of water; 1 c.c. corresponds to 0·005 gramme of phosphoric acid (P_2O_5).

ii. Acid solution of sodium acetate. Dissolve 100 grammes of sodium acetate in 900 c.c. of water; add to this 100 c.c. of glacial acetic acid.

iii. Solution of potassium ferrocyanide.

Method.—Take 50 c.c. of urine. Add 5 c.c. of the acid solution of sodium acetate.[1] Heat the mixture to 80° C.

Run into it while hot the standard uranium nitrate solution from a burette until a drop of the mixture gives a distinct brown colour with a drop of potassium ferrocyanide placed on a porcelain slab. Read off the quantity of solution used and calculate therefrom the percentage amount of phosphoric acid in the urine.

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[1] In using uranium nitrate it is imperative that sodium acetate should accompany the titration in order to avoid the possible occurrence of free nitric acid in the solution. If uranium acetate is used, it may be omitted.

Another indicator which may be used is cochineal tincture, a few drops of which may be added to the mixture. A change of colour from red to green is the sign of the end of the reaction.

(*b*) *Estimation of the phosphoric acid combined with calcium and magnesium* (alkaline earths).

Take 200 c.c. of urine. Render it alkaline with ammonia. Lay the mixture aside for twelve hours. Collect the precipitated earthy phosphates on a filter; wash with dilute ammonia (1 in 3). Wash the precipitate off the filter with water acidified by a few drops of acetic acid. Dissolve with the aid of heat, adding a little more acetic acid if necessary. Add 5 c.c. of the acid solution of sodium acetate. Bring the volume up to 50 c.c., and estimate the phosphates in this volumetrically by the standard uranium nitrate as before. Subtract the phosphoric acid combined with the alkaline earths thus obtained from the total quantity of phosphoric acid, and the difference is the amount of acid combined with the alkaline metals, sodium and potassium.

ESTIMATION OF TOTAL PHOSPHORUS

The phosphorus of the urine is mainly present in the form of inorganic phosphates; but there are in addition certain organic compounds of phosphorus such as glycero-phosphates. In order to estimate the total phosphorus, the following method is the best to employ.

Estimation of total phosphorus by Neumann's method :—

This method is now in general use for the estimation of phosphorus in organic substances, on account of the ease with which the destruction of the organic matter is carried out. The solution obtained after the first stage of the process serves equally well for the estimation of iron, calcium, magnesium, sodium, and potassium. An estimation of hydrochloric acid may be carried out by a slight modification of the method.

Principle of the Method.—The organic matter is oxidised with a mixture of equal parts of nitric and sulphuric acids. The phosphoric acid so formed is then precipitated as ammonium phosphomolybdate, and this precipitate, after washing it free from acid, is dissolved in excess of half-normal alkali and titrated with half-normal acid. The difference multiplied by 0·553 gives the amount of phosphorus in milligrammes. If multiplied by 1·268, the result is expressed in terms of milligrammes of P_2O_5.

Analysis.—Measure 5 c.c. of the urine into a Kjeldahl flask, and add 20 c.c. of the acid mixture (equal parts of nitric and sulphuric acids). Heat in a fume cupboard over a small flame until the evolution of brown fumes ceases. Allow to cool, and add a small quantity of fuming nitric acid; heat strongly until a clear solution is obtained. After cooling add 100 c.c. of distilled water, heat on the water-bath, and add 100 c.c. of a molybdic acid solution.[1] The yellow precipitate of ammonium phosphomolybdate is filtered and rapidly washed free from acid,[1] transferred into a flask, and a measured quantity of half-normal sodium hydrate added until a colourless fluid results. A slight excess (5 to 6 c.c.) of the half-normal sodium hydrate is added, and the solution boiled for about fifteen minutes, until all the ammonia has been removed. After cooling and adding a few drops of phenolphthalein as an indicator, the pink solution is titrated with half-normal sulphuric acid until it is colourless. If $a =$ the c.c. of the sodium hydrate solution actually taken, and $b =$ the c.c. of the sodium hydrate solution left when the reaction is over ($=$ the c.c. of half-normal acid added), then $x = a - b$, x being the number of c.c. of sodium hydrate used up in the reaction with the phosphomolybdate. Further, $x \times 0{\cdot}553 =$ milligrammes of phosphorus present; and $x \times 1{\cdot}268 = P_2O_5$.

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[1] The solution is prepared as follows: a solution of 75 grammes of ammonium molybdate, in 500 c.c. of water, is poured into 500 c.c. nitric acid (250 c.c. of concentrated nitric acid and 250 c.c. of water), and 1 litre of ammonium nitrate solution (500 grammes dissolved in 1 litre of distilled water) is added to the mixture. Various formulæ for the making of this mixture are given, but the above is the one used in this laboratory.

ESTIMATION OF SULPHATES

Benzidin

(*a*) *Estimation of total* (*inorganic and ethereal*) *sulphates* (Folin's modification):—

Twenty-five c.c. of urine and 20 c.c. of dilute hydrochloric acid (1 part of hydrochloric acid to 4 parts of water by volume) are gently boiled in a flask, covered with a small watch-glass, for twenty to thirty minutes. This effects the splitting of the ethereal sulphates and the liberation of sulphuric acid. The flask is cooled for two or three minutes in running water, and the contents are diluted with cold water to about 150 c.c. To this cold solution is then added 10 c.c. of a 5 per-cent. solution of barium chloride without any shaking or stirring during the addition. The barium chloride should be added drop by drop, preferably by means of an automatic dropper. At the end of an hour or later the mixture is shaken up and filtered through a weighed Gooch crucible (a porcelain crucible with a perforated bottom containing a layer of asbestos). The precipitate is washed with water, ignited, and weighed. The increase in weight is the amount of barium sulphate obtained.

(*b*) *Estimation of inorganic sulphate* (Folin's modification):—

About 100 c.c. of water, 10 c.c. of the dilute hydrochloric acid, and 25 c.c. of urine are measured into a flask. Ten c.c. of a 5 per-cent. solution of barium chloride are added as described above, and the filtration, washing, and weighing carried out as before.

(*c*) The *ethereal sulphates* are represented by the difference between the total and the inorganic sulphates. They may, however, also be estimated directly by the following method:—125 c.c. of urine are diluted with 75 c.c. of water and 30 c.c. of the dilute hydrochloric acid. The solution is precipitated in the cold by the addition of 20 c.c. of 5 per-cent. solution of barium chloride as before, and the mixture is filtered, after one hour's standing, through a dry filter. Inorganic sulphates are thus removed. Half of the filtrate (that is 125 c.c.) is then quietly boiled for less than thirty minutes. By this means sulphuric acid is liberated from the ethereal sulphates, and this is precipitated by the barium chloride present as barium sulphate, which is collected, washed, ignited, and weighed as before. Double the total thus obtained will give in terms of barium sulphate the amount of ethereal sulphate present in the 125 c.c. of urine originally taken, and from this the percentage and amount passed in the twenty-four hours can be calculated.

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[1] It is essential that the filtration and washing should be effected rapidly. In Neumann's original method of filtering through filter-paper several slight modifications have been introduced by different authors. In this laboratory the following method is used, which allows the filtration and washing to be carried out within five minutes: Filter-pulp is prepared by shaking up 30 grammes of a pure filtering paper with 1 litre of water and 50 c.c. of concentrated hydrochloric acid. The pulp is filtered under pressure, well washed with boiling water and then kept, suspended in 2 litres of water, ready for use. About 30 to 40 c.c. of filter-paper pulp are poured into a funnel containing a small perforated porcelain filter-plate, and the filtration and washing of the molybdate precipitate is carried out by the help of the filter-pump.

Calculation.—233 parts of barium sulphate correspond to 80 parts of SO_3, or 32 parts of S. To calculate the SO_3, multiply the weight of barium sulphate by $\frac{80}{233}=0{\cdot}343$; to calculate the S, multiply it by $\frac{32}{233}=0{\cdot}137$. Example: 100 c.c. of urine gave 0·5 gramme of total barium sulphate; this multiplied by $0{\cdot}343=0{\cdot}171$ of total SO_3. Another 100 c.c. of the same urine gave 0·45 gramme of barium sulphate from inorganic sulphates; this multiplied by $0{\cdot}343=0{\cdot}154$ of SO_3 in inorganic combination. If this is subtracted from the total SO_3 ($0{\cdot}171-0{\cdot}154$) we see that 0·017 gramme of SO_3 was in combination as ethereal sulphate, or about one-tenth of the total, which is the average proportion in normal urine.

ESTIMATION OF TOTAL SULPHUR

Benedict's Method (Wolf and Oesterberg's modification).—In this method the organic matter is destroyed by boiling the substance with fuming nitric acid, and the oxidation of sulphur to sulphuric acid is completed by heating with copper nitrate and potassium chlorate. In the solution finally obtained, sulphuric acid is estimated gravimetrically as barium sulphate.

Analysis.—Measure 10 c.c. of urine into a Kjeldahl flask of 300 c.c. capacity; add 20 c.c. fuming nitric acid and heat over a small flame. Continue to boil until the fluid is free from solid particles. Transfer the solution to a porcelain dish and add 20 c.c. of Benedict's solution (200 grammes copper nitrate, and 50 grammes potassium chlorate dissolved in 1 litre of water). Evaporate to dryness on a sand-bath; then heat over the free flame until the residue is blackened by the formation of copper oxide. Raise the flame and heat to redness for ten minutes. After cooling add 25 c.c. of 10 per-cent. hydrochloric acid and dissolve the black residue by warming gently. The solution is washed into a flask with about 150 c.c. of water, and the sulphuric acid estimated by means of barium chloride in the way described above (*a*). The filtrate from the barium sulphate precipitate may be used for the estimation of phosphorus by Neumann's method.

LESSON XXV

THE URINE (*continued*)

THE PIGMENTS

The urinary pigments are numerous, and have from time to time been described under different names by various observers.

1. **Urochrome.**—This is the essential yellow pigment of the urine. The word was originally introduced by Thudichum, whose investigations have in the main been confirmed and supplemented by the recent work of Dombrowski.

Preparation of Urochrome (Dombrowski).—After removal of sulphates, phosphates, and urates by a mixture of barium and calcium acetates and ammonia (86 grammes of calcium acetate, 53 grammes of barium acetate, and 43 c.c. of 21 per-cent. ammonia to 10 litres of urine), the filtered urine is neutralised with acetic acid, and urochrome is precipitated by adding copper acetate. The greenish precipitate is filtered off, well washed, and then suspended in water, and the copper is removed by sulphuretted hydrogen at 50° C. Baryta water is added to the filtrate, and excess of barium removed by a stream of carbon dioxide ; the barium salt is then concentrated *in vacuo* and precipitated by alcohol. The precipitate is dissolved in water and freed from chlorine by silver nitrate. The soluble silver salt so formed is precipitated by alcohol and ether, washed free from silver nitrate, dried, and analysed. The free urochrome can be obtained by removing the silver with sulphuretted hydrogen ; it is amorphous and easily soluble in 90 per-cent. alcohol, in which it keeps well.

Estimation.—The copper salt obtained as above is dissolved in ammonia, and the purine bases precipitated with ammoniacal silver solution. Nitrogen is estimated in the copper and silver precipitates ; the difference gives the nitrogen of urochrome. Urochrome contains 11·1 per cent. of nitrogen.

It shows no absorption bands, but cuts off the violet part of the spectrum which urobilin allows to pass. It does not fluoresce with zinc salts as urobilin does. It yields a pyrrol derivative which is not identical with hæmopyrrol, and so probably urochrome is not related to urobilin. It contains 5 per cent. of sulphur, most of which is easily split off as sulphide by cold alkali. Cystine-sulphur is not present. It is an acid, and reddens litmus. It is probably derived from protein.

2. **Urobilin.**—Urobilin is a derivative of the blood pigment, and is identical with stercobilin (see pp. 124, 182). Probably both reduction and hydration occur in its formation. It is very like the substance named hydrobilirubin by Maly, which he obtained by the action of sodium amalgam on bilirubin. The following formulæ show the relationship between these allied pigments :—

Hæmatin	$C_{31}H_{33 \text{ or } 35}.O_5N_4Fe$
Bilirubin	$C_{32}H_{36}N_4O_6$
Hydrobilirubin	$C_{32}H_{40}N_4O_7$

Normal urine contains but little urobilin; what is present is chiefly in the form of a colourless chromogen, which by oxidation is converted into urobilin. In numerous pathological conditions urobilin is abundant.

The following are the two methods introduced by Garrod and Hopkins for its separation from the urine:—

(*a*) The urine is first saturated with ammonium chloride, and the urate so precipitated is filtered off. The filtrate is then acidified with sulphuric acid and saturated with ammonium sulphate. This causes a precipitate of urobilin, which may be collected and dissolved in water. The aqueous solution is again saturated with ammonium sulphate, and the pigment is thus precipitated in a state of purity.

(*b*) The urates are first removed, then the urine is acidified and saturated with ammonium sulphate as before. The urobilin is then extracted from the mixture by shaking it with a mixture of chloroform and ether (1 : 2) in a large separating funnel. The ether-chloroform extract is then rendered faintly alkaline and shaken with distilled water, and the urobilin passes into solution in the water. The aqueous solution is now once more saturated with ammonium sulphate and slightly acidified; it then once more yields its pigment to ether-chloroform.

By means of either of these methods urobilin is obtained in a pure condition; even normal urine will give some, for the chromogen is partly converted into the pigment by the acid employed.

Urobilin dissolved in alcohol exhibits a green fluorescence, which is greatly increased by the addition of zinc chloride and ammonia. It shows a well-marked absorption band between *b* and F, slightly overlapping the latter (fig. 55, spectrum 4).

Urobilin, like most animal pigments, shows acidic tendencies and forms compounds with bases; it is liberated from such combinations by the addition of an acid.

If urobilin is dissolved in caustic potash or soda, and sufficient sulphuric or hydrochloric acid is added to render the liquid faintly acid, a turbidity is produced. This turbid liquid shows an additional band in the region of the E line (fig. 55, spectrum 6), which is probably due to the special light absorption exercised by fine particles of urobilin in suspension. It wholly disappears when the precipitate is filtered off, and when it is redissolved the ordinary band alone is visible.

3. **Uroerythrin.**—This is the colouring matter of pink urate sediments. It may be separated from the sediment as follows:—The deposit is washed with ice-cold water, dried, and placed in absolute alcohol. The alcohol, though a solvent for uroerythrin, does not extract it from the urates. The alcohol is poured off, and the deposit dissolved in warm water. From this solution the pigment is easily extracted by amylic alcohol.

Uroerythrin has a great affinity for urates, with which it appears to form a loose compound. Its solutions are rapidly decolorised by light. Spectroscopically it shows two rather ill-defined bands (fig. 55, spectrum 7). It gives a green colour with caustic potash, and red or pink with mineral acids. Uroerythrin appears to be a small but constant constituent of urine. Its origin and relationship to the other pigments are unknown.

4. **Hæmatoporphyrin.**—This also occurs in small quantities in normal urine. In some pathological conditions, especially after the administration of certain drugs (*e.g.* sulphonal), its amount is increased. Its amount is stated to increase when the urine stands; this points to the existence of a colourless chromogen. It may be separated from the urine as follows:—Caustic alkali is added to the urine; this causes a precipitate of phosphates, which carries down the pigment with it: the pigment may be dissolved out with chloroform. The chloroform is evaporated, the residue washed with

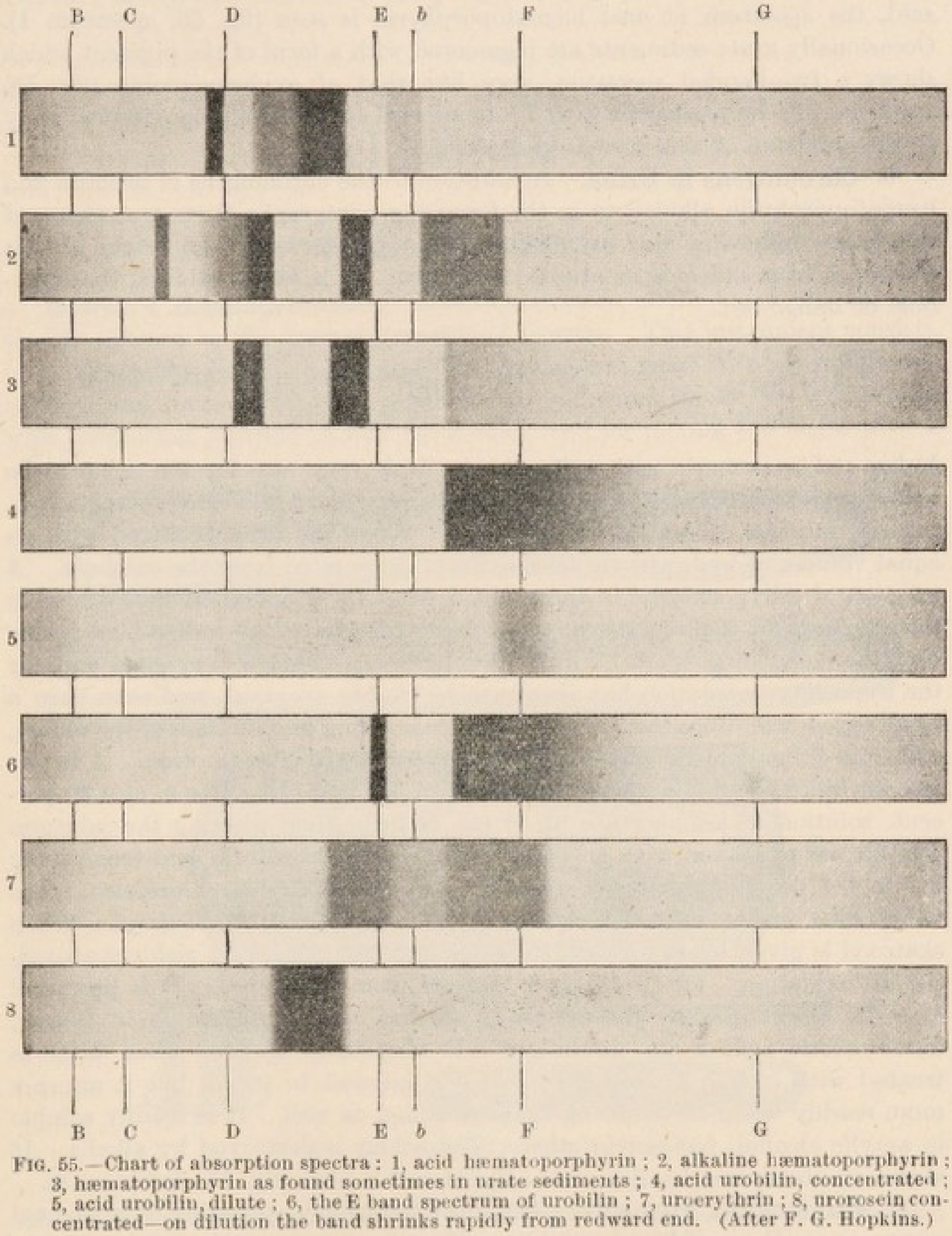
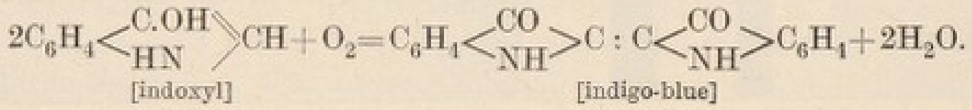


FIG. 55.—Chart of absorption spectra: 1, acid hæmatoporphyrin; 2, alkaline hæmatoporphyrin; 3, hæmatoporphyrin as found sometimes in urate sediments; 4, acid urobilin, concentrated; 5, acid urobilin, dilute; 6, the E band spectrum of urobilin; 7, uroerythrin; 8, urorosein concentrated—on dilution the band shrinks rapidly from redward end. (After F. G. Hopkins.)

alcohol, and finally dissolved in acidified alcohol. Urines rich in the pigment yield it easily to acetic ether or to amylic alcohol.

When the urine is sufficiently rich in the pigment, the bands shown are those of alkaline hæmatoporphyrin (fig. 55, spectrum 2). On adding sulphuric acid, the spectrum of acid hæmatoporphyrin is seen (fig. 55, spectrum 1). Occasionally urate sediments are pigmented with a form of the pigment which shows a two-banded spectrum, very like that of oxyhæmoglobin (fig. 55, spectrum 3); by treatment with dilute mineral acids this changes immediately to the spectrum of acid hæmatoporphyrin.

5. **Chromogens in Urine.**—In addition to the chromogens of urobilin and hæmatoporphyrin alluded to in the foregoing paragraphs there are others, of which the following may be mentioned:—(*a*) *Indoxyl.*—The origin of this substance from indole is mentioned on p. 193. It is easily oxidised to indigo-blue or indigo-red.



$$\underset{\text{[indoxyl]}}{2C_6H_4{<}\begin{matrix}C.OH\\HN\end{matrix}{>}CH} + O_2 = \underset{\text{[indigo-blue]}}{C_6H_4{<}\begin{matrix}CO\\NH\end{matrix}{>}C : C{<}\begin{matrix}CO\\NH\end{matrix}{>}C_6H_4} + 2H_2O.$$

Indigo-red is isomeric with indigo-blue. It is very rare for the urine to be actually pigmented with indigo, for the urinary indoxyl is excreted as a conjugated sulphate which resists oxidation. When the urine is mixed with an equal volume of hydrochloric acid, indoxyl is liberated from the sulphate. A solution of a hypochlorite is then added drop by drop, when indigo-blue is formed, and on shaking the mixture with chloroform the indigo-blue passes into the chloroform (Jaffé). This test, however, is not a very good one, for the hypochlorite solution has always to be freshly prepared, and even then a small excess will cause the colour to disappear owing to oxidation of the indigo, and it is difficult to hit off the exact amount to give the reaction. A better test for indoxyl-sulphuric acid (indican) consists in adding 10 c.c. of a 20 per-cent. solution of lead acetate to 50 c.c. of urine, and filtering the mixture. The filtrate is shaken with an equal volume of hydrochloric acid (containing 0·2 to 0·4 per cent. of ferric chloride) and a few c.c. of chloroform. The indigo-blue passes into the chloroform (Obermayer). (*b*) *Skatoxyl.*—When skatoxyl is given by the mouth it passes into the urine, and yields skatoxyl-red on oxidation. (*c*) *Urorosein* is distinct from indigo-red. It is produced from its chromogen by the action of mineral acids. According to Herter, indole acetic acid is its chromogen. It frequently appears when urine is treated with strong hydrochloric acid and allowed to stand, but it appears more readily when an oxidising agent is added as well. It is readily soluble in amylic alcohol, but not in ether. The colour is destroyed by alkalis. It shows an absorption band between the D and E lines (fig. 55, spectrum 8).

6. **Pathological Pigments.**—The most frequently appearing of abnormal pigments are those of blood and bile. The urine may contain accidental pigments due to the use of drugs (rhubarb, senna, logwood, santonin); in carbolic acid poisoning pyrocatechin and hydrochinon are chiefly responsible for the greenish-brown colour of the urine, which increases on exposure to the air. The black or dark-brown pigment called melanin may pass into the urine in cases of melanotic sarcoma. For alcaptonuria, see p. 214.

APPENDIX

HÆMACYTOMETERS

Gowers's Hæmacytometer.—The enumeration of the blood corpuscles is readily effected by the hæmacytometer of Gowers. This instrument consists of a glass slide (fig. 56, C), the centre of which is ruled into $\frac{1}{10}$ millimetre squares and surrounded by a glass rim $\frac{1}{5}$ millimetre thick. It is provided with measuring pipettes (A and B), a vessel (D) for mixing the blood with a saline solution (sulphate of soda of specific gravity 1015), a glass stirrer (E), and a guarded needle (F).

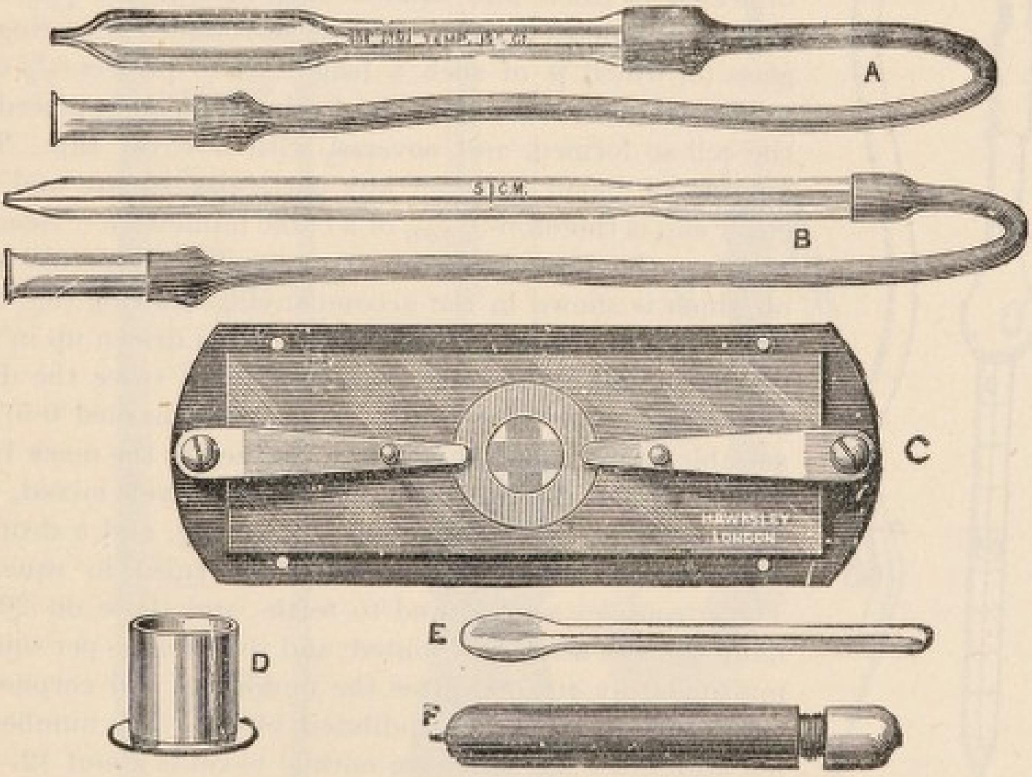


FIG. 56.—Hæmacytometer of Sir W. Gowers.

Nine hundred and ninety-five cubic millimetres of the saline solution are measured out by means of A, and then placed in the mixing jar; 5 cubic millimetres of blood are then drawn from a puncture in the finger by means of the pipette B, and blown into the solution. The two fluids are well mixed by the stirrer, and a small drop of this diluted mixture placed in the centre of the slide C; a cover slip is gently laid on (so as to touch the drop, which thus forms a layer $\frac{1}{5}$ millimetre thick between the slide and cover slip), and pressed down by two brass springs. In a few minutes the corpuscles have sunk to the bottom of the layer of fluid, and rest on the squares. The number on ten squares is then counted, and this multiplied by 10,000 gives the number in a cubic millimetre of blood. The average number of red corpuscles in each square ought therefore in normal human blood to be 45–50.

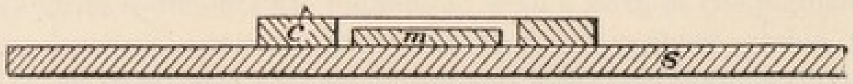


FIG. 57.—Thoma-Zeiss hæmacytometer.

Differential counts to show the relative proportions of the varieties of leucocytes are made in appropriately stained specimens.

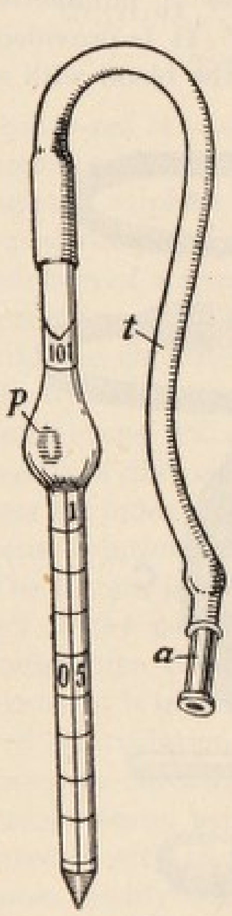


FIG. 58.—Pipette of Thoma-Zeiss hæmacytometer.

Thoma-Zeiss Hæmacytometer.—This instrument consists of a glass slide (*s*) seen in section in fig. 57. In the centre of the slide is a disc of glass *m*, the upper surface of which is ruled into squares each of which is $\frac{1}{400}$ of a square millimetre in area. This is surrounded by a ring of glass (*c*) which is of such a height as to project $\frac{1}{10}$ of a millimetre above *m*. A drop of diluted blood is placed in the cell so formed, and covered with a cover slip. The volume of blood between any particular square and the cover slip is therefore $\frac{1}{4000}$ of a cubic millimetre. Accompanying the instrument are two capillary pipettes, one of which is shown in the accompanying drawing (fig. 58). The finger or ear is pricked, and blood is drawn up in the capillary tube to the line marked 1 (or if twice the dilution is regarded as advisable, to the line marked 0·5); a suitable saline solution is drawn up then to the mark 101; the blood and diluting solution are then well mixed, the glass bead in the bulb aiding the mixing, and a drop of the mixture transferred to the slide ruled in squares. The corpuscles are allowed to settle, and those on 20 or more squares are then counted, and the average per square multiplied by 400,000 gives the number of red corpuscles per cubic millimetre of undiluted blood. The number of red corpuscles per square in normal blood is about 12.

The second pipette provided is like the one just described, but is rather larger, and is similarly employed for counting the leucocytes. In some cases a micrometer slide ruled in larger squares is provided for this purpose. The value of the squares in terms of those provided for the counting of the red corpuscles is known, and so the proportion of coloured and colourless corpuscles can be ascertained. It is, however, preferable to count both kinds of corpuscles in the same specimen, to repeat both counts with another specimen, and take the average result. This can be performed quite readily if the saline solution used for dilution contains a dye. Sherrington recommends the following solution :—

Methylene blue	1·0 gramme
Sodium chloride	1·2 ,,
Potassium oxalate	1·2 ,,
Distilled water	300·0 ,,

For differential counts a mixture of dyes is necessary.

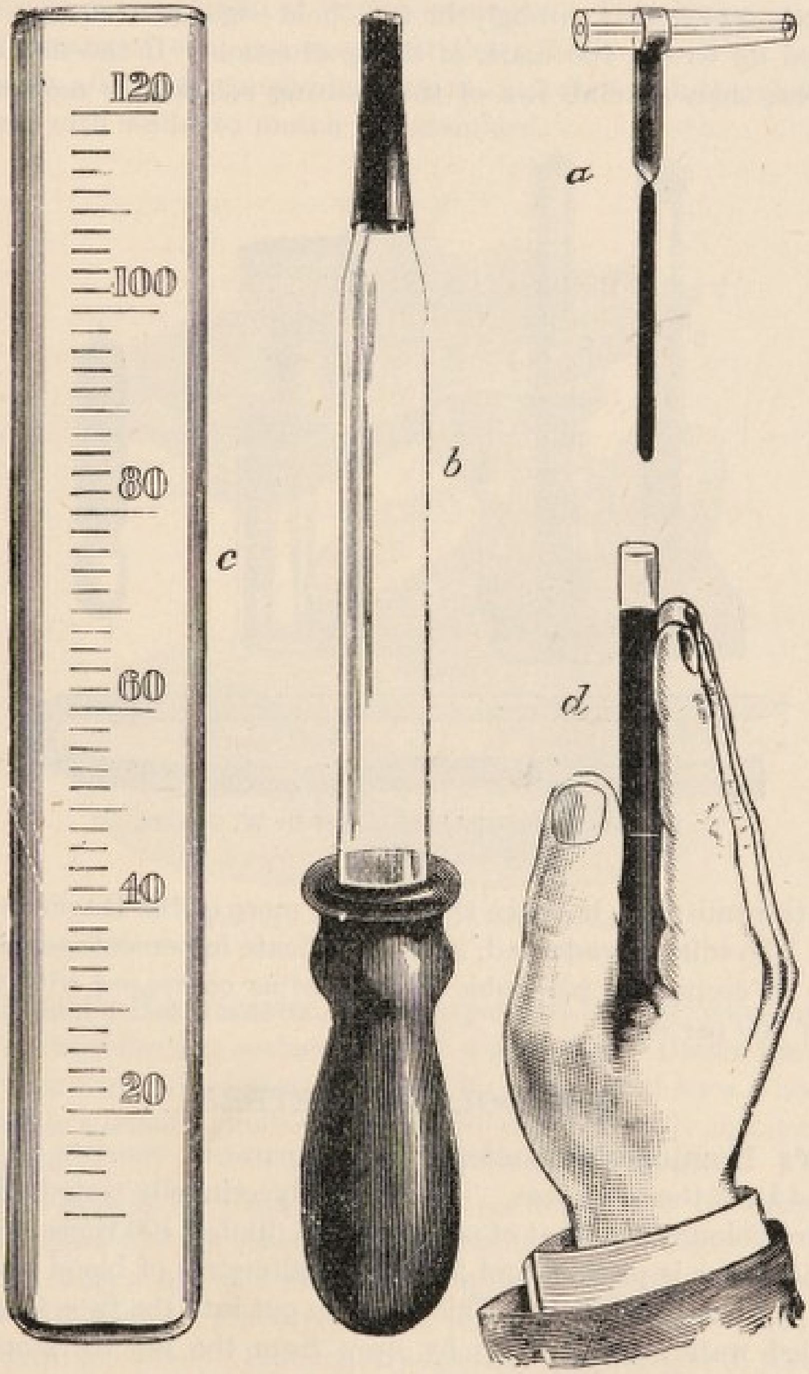


FIG. 59.—Oliver's hæmacytometer.

Oliver's Hæmacytometer.—The following method, devised by Dr George Oliver, is a ready way of determining the total number of corpuscles. It is, however, not possible to determine the relative proportion of red and white corpuscles by this means.

The finger is pricked, and the blood allowed to flow into the small capillary pipette (fig. 59, *a*) until it is full. This is washed out by the dropping tube *b* into a graduated flattened test-tube, *c*, with Hayem's fluid.[1] The graduations of the tube are so adjusted that with normal blood containing 5,000,000 coloured corpuscles per cubic millimetre, the light of a small wax candle placed at a distance of 9 feet from the eye in a dark room is just transmitted as a fine bright line when looked at through the tube held edgeways between the fingers (*d*) and filled up to the 100 mark of the graduation. If the number of corpuscles is less than normal, less of the diluting solution is required for the light to be transmitted ; if above the normal, more of the Hayem's fluid must be added. The tube is graduated, so as to indicate in percentages the decrease or increase of corpuscles per cubic millimetre as compared with the normal standard of 100 per cent.

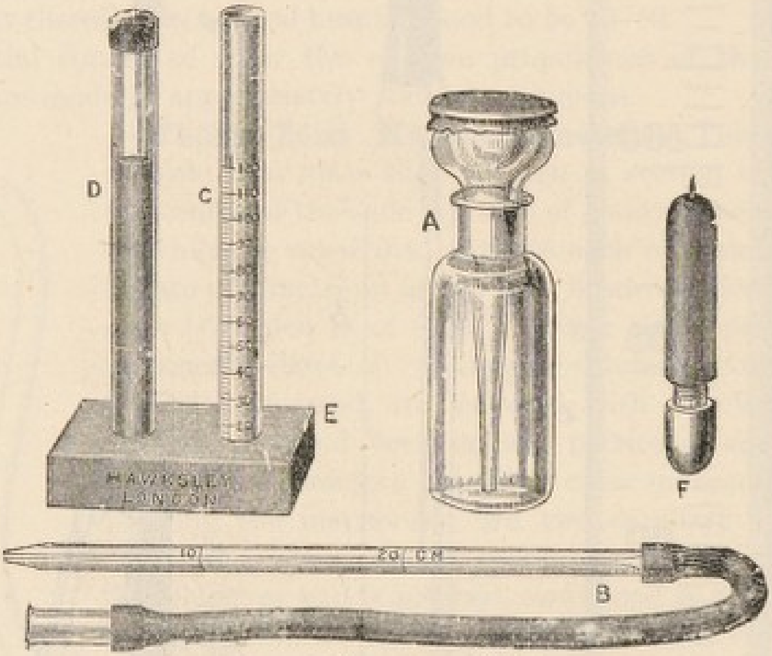


FIG. 60.—Hæmoglobinometer of Sir W. Gowers.

HÆMOGLOBINOMETERS

Gowers's Hæmoglobinometer.—The apparatus consists of two glass tubes, C and D, of the same size. D contains glycerin jelly tinted with carmine to a standard colour—viz. that of normal blood diluted 100 times with distilled water. The finger is pricked and 20 cubic millimetres of blood are measured out by the capillary pipette B. This is blown out into the tube C, and diluted with distilled water, added drop by drop from the pipette stopper of the bottle A, until the tint of the diluted blood reaches the standard colour. The tube C is graduated into 100 parts. If the tint of the diluted blood is the same as the standard when the tube is filled up to the graduation 100, the quantity of oxyhæmoglobin in the blood is normal. If it has to be diluted more largely the oxyhæmoglobin is in excess; if to a smaller extent, it is less than normal. If the blood has, for instance, to be diluted up to the graduation 50, the amount of hæmoglobin is only half what it ought to be—50 per cent. of the normal—and so for other percentages.

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[1] Sodium sulphate 5 grammes, sodium chloride 1 grm., mercuric chloride 0·5 grm., distilled water 200 c.c.

Haldane's Hæmoglobinometer is more frequently used. Instead of tinted gelatin, the standard of comparison is a sealed tube filled with a solution of carbonic oxide hæmoglobin of known strength. This keeps unchanged for years. A stream of coal gas is passed through the blood to be examined. This converts all the hæmoglobin present into carboxyhæmoglobin; this is then diluted with water to match the standard.

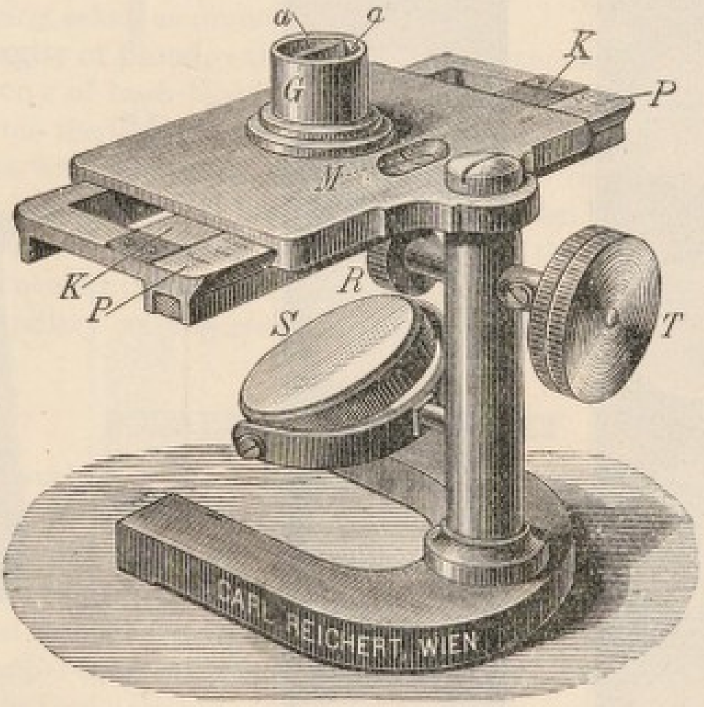

FIG. 61.—Von Fleischl's hæmometer.

Von Fleischl's Hæmometer.—The apparatus (fig. 61) consists of a stand bearing a white reflecting surface (S) and a platform. Under the platform is a slot carrying a glass wedge stained red (K) and moved by a wheel (R). On the platform is a small cylindrical vessel divided vertically into two compartments, *a* and *a′*.

Fill with a pipette the compartment *a′* over the wedge with distilled water. Fill about a quarter of the other compartment (*a*) with water.

Prick the finger and fill the short capillary pipette provided with the instrument with blood. Dissolve this in the water in compartment *a*, and fill it up with distilled water. Having arranged the reflector (S) to throw *artificial* light vertically through both compartments, look down through them, and move the wedge of glass by the milled head (T) until the colour in the two is identical. Read off the scale, which is so constructed as to give the percentage of hæmoglobin.

Oliver's Hæmoglobinometer.—This method consists in comparing a specimen of blood, suitably diluted with water in a shallow white palette, with a

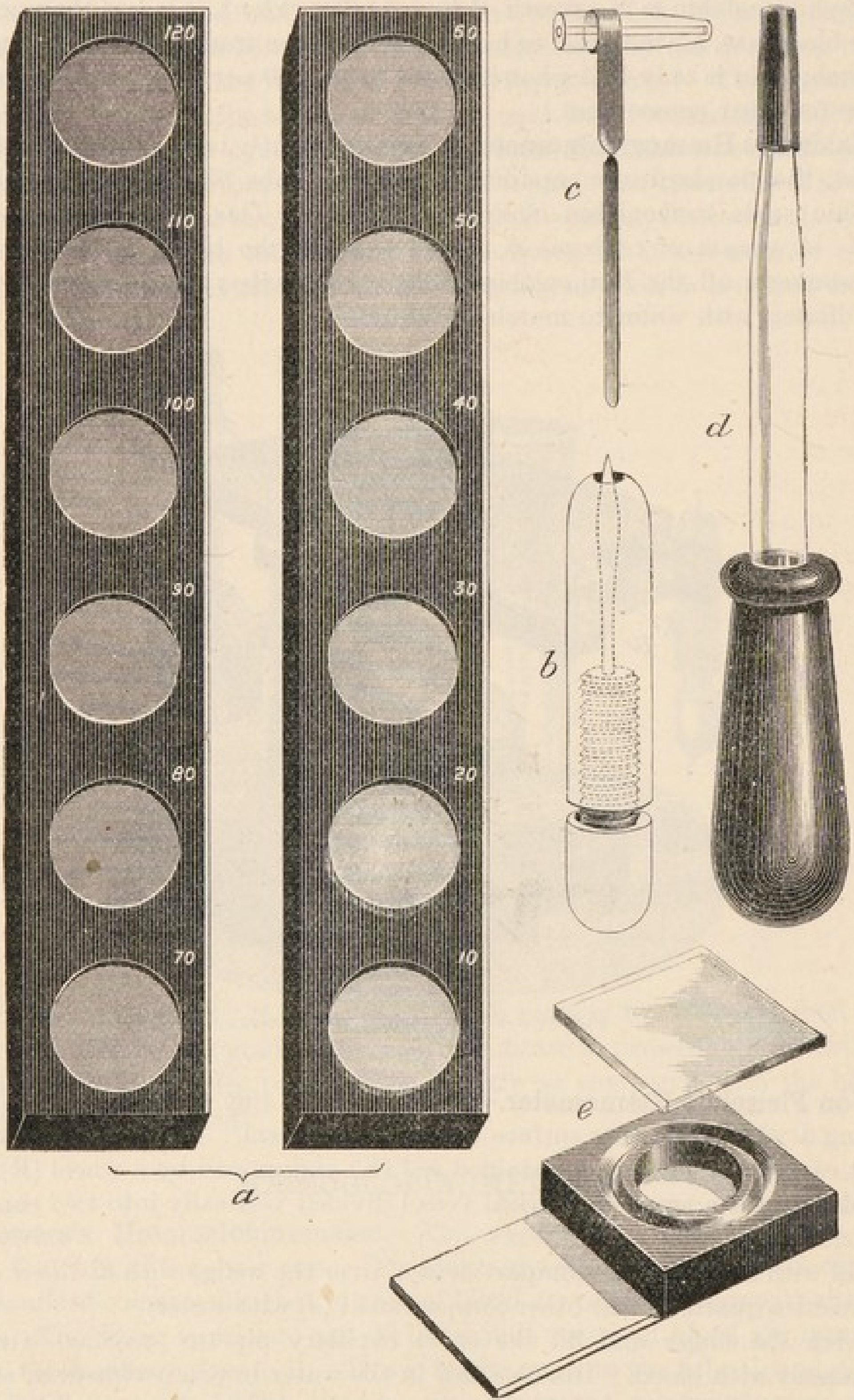

FIG. 62.—Oliver's hæmoglobinometer; *a*, standard gradations; *b*, lancet; *c*, capillary measuring pipette; *d*, mixing pipette; *e*, blood cell and cover glass.

number of standard tests very carefully prepared by the use of Lovibond's coloured glasses. The capillary pipette *c* (fig. 62) is first filled with blood obtained by pricking the finger. This is washed with water by the mixing pipette *d* into the blood cell *e*; the cell is then just filled with water, and the blood and water thoroughly mixed by the handle of *c* being used as a stirrer. The cover glass is then adjusted, when a small bubble should form, a clear sign that the cell has not been overfilled. The cell is then placed by the side of the standard gradations, and the eye quickly recognises its approximate position on the scale. The camera tube provided with the instrument will more accurately define it. Artificial light should be used.

If it is proved that the blood solution is matched in depth of colour by one of the standard grades, the observation is at an end; but if the tint is higher than one grade, but lower than another, the blood cell is placed opposite to the former, and riders (not shown in the illustration) are added to complete the observation. The standard gradations are marked in percentages, 100 per cent. being taken as normal.

Specific Gravity of Blood.—Of the numerous methods introduced for taking the specific gravity of fresh blood, that of Hammerschlag is the simplest. A drop of blood from the finger is placed in a mixture of chloroform and benzene. If the drop falls, add chloroform till it just begins to rise; if the drop rises, add benzene till it just begins to fall. The fluid will then be of the same specific gravity as the blood. Take the specific gravity of the mixture in the usual way with an accurate hydrometer.

Schmalz's capillary pycnometer is more accurate.

POLARISATION OF LIGHT

If an object, such as a black dot on a piece of white paper, is looked at through a crystal of Iceland spar, two black dots will be seen; and if the crystal is rotated, one black dot will move round the other, which remains stationary. That is, each ray of light entering such a crystal is split into two rays, which travel through the crystal with different velocities, and consequently one is more refracted than the other. One ray travels just as it would through glass; this is the *ordinary ray*, the ray which gives the stationary image; the other ray gives the movable image when the crystal is rotated; the ordinary laws of refraction do not apply to it, and it is called the *extraordinary ray*. Both rays are of equal brilliancy. In one direction, however, that of the optic axis of the crystal, a ray of light is transmitted without double refraction.

Ordinary light, according to the wave theory, is due to vibrations occurring in all planes transversely to the direction for the propagation of the wave. Light is said to be plane polarised when the vibrations take place all in one plane. The two rays produced by double refraction are both polarised, one in one plane, the other in a plane at right angles to this one. Doubly refracting bodies are called *anisotropic*; singly refracting bodies, *isotropic*. The effect of polarisation may be very roughly illustrated by a model.

If a string is stretched as in the figure, and then touched with the finger, it can be made to vibrate, and the vibrations will be free to occur from above down, or from side to side, or in any intermediate position. If, however, a disc with a vertical slit be placed on the course of the string, the vibrations will all be obliged to take place in a vertical plane, any side-to-side movement being stopped by the edges of the slit [1] (fig. 63).

Light can be polarised not only by the action of crystals, but by reflection from a surface at an angle which varies for different substances (glass 54° 35′, water 52° 45′, diamond 68°, quartz 57° 32′, etc.). It is also found that certain non-crystalline substances, such as muscle, cilia, etc., are doubly refracting.

Nicol's Prism is the *polariser* usually employed in polariscopes; it consists of a rhombohedron of Iceland spar divided into two by a section through its obtuse angles. The cut surfaces are polished and cemented together in their former position with Canada balsam. By this means the ordinary ray is totally reflected through the Canada balsam; the extraordinary ray passes on and emerges in a direction parallel to the entering ray. In this polarised ray there is nothing to render its peculiar condition visible to the naked eye; but if the eye is aided by a second Nicol's prism, which is called the *analyser*, it is possible to detect the fact that it is polarised.

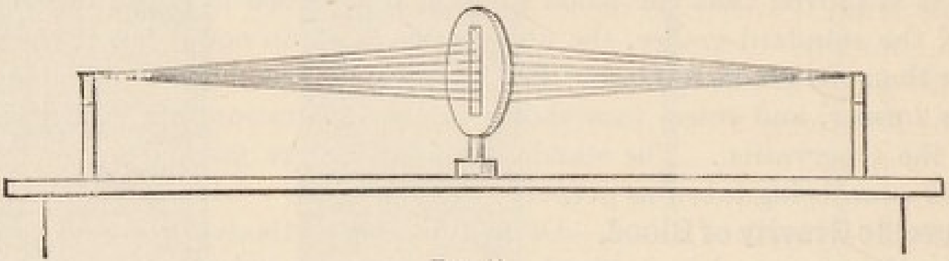

FIG. 63.

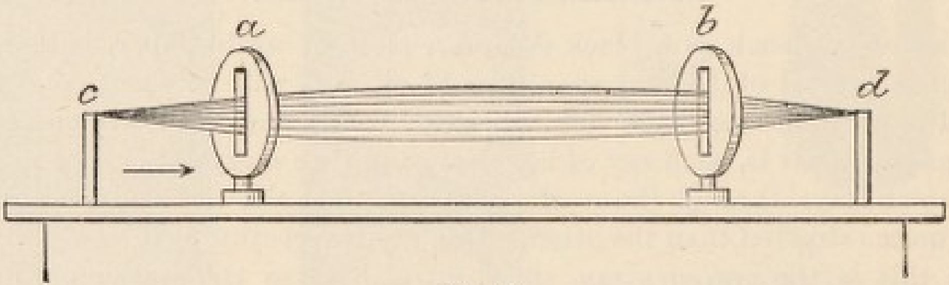

FIG. 64.

This may be again illustrated by reference to our model (fig. 64).

Suppose that the string is made to vibrate, and that the waves travel in the direction of the arrow. From the fixed point *c* to the disc *a*, the string is theoretically free to vibrate in any plane; [2] but after passing through the vertical slit in *a*, the vibrations must all be vertical also; if a second similar disc *b* be placed further on, the vibrations will also pass on freely to the other extremity of the string *d*, if as in the figure (fig. 64) the slit in *b* is also placed vertically. If, however, *b* is so placed that its slit is horizontal (fig. 65) the vibrations will be extinguished on reaching *b*, and the string between *b* and *d* will be motionless.

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[1] Such a model is, of course, imperfect; it does not, for instance, represent the splitting of the ray into two, and moreover the polarisation takes place on each side of the slit; whereas, in regard to light, it is only the rays on one side of a polariser, viz. those which have passed through it, which are polarised.

[2] The imperfection of the model has been explained in the preceding footnote.

c here represents a source of light; the vibrations of the string represent the undulations which by the Nicol's prism *a* are polarised so as to occur in one plane only; if the second Nicol's prism or the analyser *b* is parallel to the first, the vibrations will pass on to the eye, which is represented by *d*; but if the planes of the two nicols are at right angles, the vibrations allowed to pass through the first are extinguished by the second, and so no light reaches the eye. In intermediate positions, *b* will allow only some of the light to pass through it. It must be clearly understood that a Nicol's prism contains no actual slits, but the arrangement of its molecules is such that their action on the particles of ether may be compared to the action of slits in a diaphragm to vibrations of more tangible materials than ether.

The Polarising Microscope consists of an ordinary microscope with certain additions; below the stage is the polarising nicol; in the eye-piece is the analysing nicol; the eye-piece is so arranged that it can be rotated; thus the directions of the two nicols can be made parallel, and then the field is bright; or crossed, and then the field is dark. The stage of the microscope is arranged so that it can also be rotated.

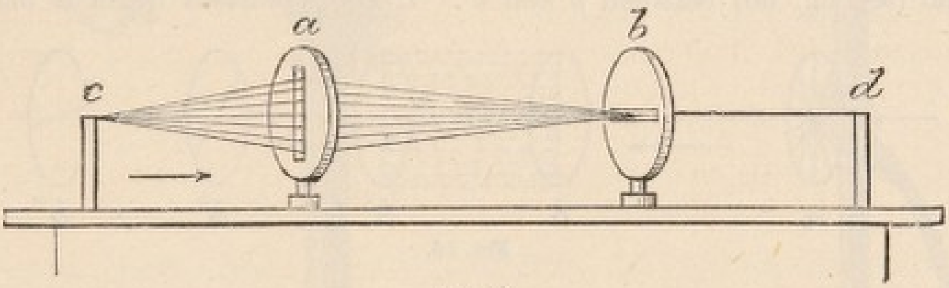


FIG. 65.

The polarising microscope is used to detect doubly refracting substances. Let the two nicols be crossed, so that the field is dark; interpose between the two, that is, place upon the stage of the microscope a doubly refracting plate of which the principal plane is parallel to the first prism or polariser; the ray from the first prism is unaffected by the plate, but will be extinguished by the second; the field therefore still remains dark. If the plate is parallel to the second nicol the field is also dark; but in any intermediate position the light will be transmitted by the second nicol. In other words, if between two crossed nicols, which consequently give a dark field, a substance is interposed which in certain positions causes the darkness to give place to illumination, that substance is doubly refracting or anisotropic.

All crystals except those of the regular system are anisotropic, whilst amorphous substances are isotropic. O. Lehmann has, however, discovered an intermediate stage between anisotropic solid crystals and their isotropic fused condition. In this state certain substances, such as cholesterin compounds, oleates, etc., are anisotropic, but completely fluid. He called this the liquid crystalline state of matter (see also p. 39).

Rotation of the Plane of Polarisation.—Certain crystals such as those of quartz, and certain fluids such as the essence of turpentine, aniseed, etc., and solutions of certain substances such as sugar and albumin, have the power of rotating the plane of polarised light to the right or left. The polarisation of light that is produced by a quartz crystal is different from that produced by a rhombohedron of Iceland spar The light that passes through the latter is plane polarised ; the light that passes through the former (quartz) is circularly polarised, *i.e.* the two sub-rays are made up of vibrations which occur not in a plane, but are curved. The two rays are circularly polarised in opposite directions, one describing circles to the left, the other to the right ; these unite on issuing from the quartz plate : and the net result is a plane polarised ray with the plane rotated to right or left according as the right circularly polarised ray or the left proceeded through the quartz with the greater velocity. There are two kinds of quartz, one which rotates the plane to the right (dextro-rotatory), the other to the left (lævo-rotatory).

Gordon explains this by the following mechanical illustration. Ordinary light may be represented by a wheel travelling in the direction of its axle, and the vibrations composing it are executed along any or all of its spokes (a). If the vibrations all take place in the same direction, *i.e.* along one spoke, and the spoke opposite to it (b), the light is said to be plane polarised. The two spokes as they travel along in the direction of the arrow will trace out a plane (see fig. 66) between b and b'. If this polarised beam is made to travel now through a solution of sugar, the net result is that the plane so traced out is twisted or rotated ; the two spokes, as in bb', do not trace out a plane, but we must consider that they rotate as they travel along, as though guided by a spiral or screw thread cut on the axis, so that after a certain distance the vibrations take place as in b'' ; latter in b''', and so on. This effect on polarised light is due to the molecules in solution, and the amount of rotation will depend on the strength of the solution, and on the length of the column of the solution through which the light passes, or, in the case of a quartz plate, on its thickness.

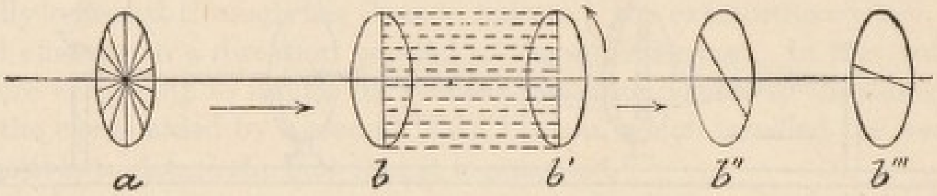


Fig. 66.

If a plate of quartz is interposed between two nicols, the light will not be extinguished in any position of the prisms, but will pass through various colours as rotation is continued. The rotation produced for different kinds of light being different, white light is split into its various constituent colours ; and the angle of rotation that causes each colour to disappear is constant for a given thickness of quartz plate, being least for the red and greatest for the violet. These facts are made use of in the construction of polarising instruments.

POLARIMETERS

Polarimeters are instruments for determining the strength of solutions of sugar, albumin, etc., by the direction and amount of rotation they produce on the plane of polarised light. They are often called saccharimeters, as they are specially useful in the estimation of sugar.

The following is the one most frequently employed at the present time :

Laurent's Polarimeter.—Instead of using daylight, or the light of a lamp, monochromatic light (a sodium flame produced by volatilising common salt in a colourless gas flame) is employed ; the amount of rotation varies for different colours ; and observations are usually recorded as having been taken with light corresponding to the D or sodium line of the spectrum. The essentials of the instrument are a polariser, a tube for the solution, and an analyser. The polarised light before passing into the solution traverses a quartz plate, which, however, covers only half the field, and retards the rays passing through it by half a wave-length. In the 0° position the two halves of the field appear equally illuminated : in any other position, or if rotation has been produced by the solution when the nicols have been set at zero, the two halves appear unequally illuminated. The amount of rotation produced by the solution is ascertained by rotating the analyser until the two halves of the field are once more equally illuminated. This is measured in degrees by a scale and vernier attached to the instrument.

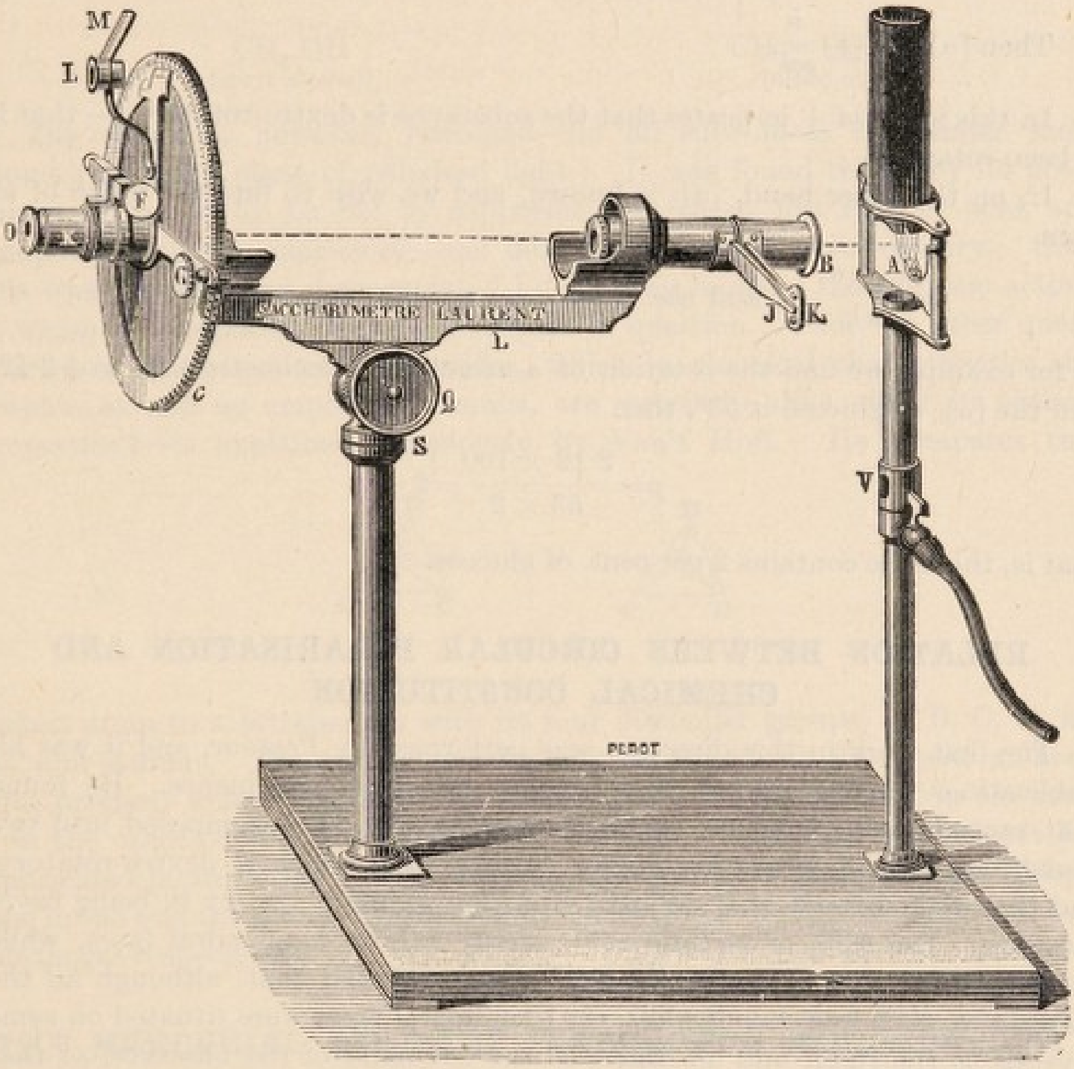


FIG. 67.—Laurent's polarimeter,

The **specific rotatory power** of any substance is the amount of rotation in degrees of a circle of the plane of polarised light produced by 1 gramme of the substance dissolved in 1 c.c. of liquid examined in a column 1 decimetre long.

If a = rotation observed.
w = weight in grammes of the substance per cubic centimetre.
l = length of tube in decimetres.
$[\alpha]_D$ = specific rotation for light with wave-length corresponding to the D line (sodium flame).

Then $[\alpha]_D = \pm \frac{a}{wl}$.

In this formula + indicates that the substance is dextro-rotatory, — that it is lævo-rotatory.

If, on the other hand, $[\alpha]_D$ is known, and we wish to find the value of w, then

$$w = \frac{a}{[\alpha]_D \times l}, \text{ and the percentage amount is } p = \frac{a \times 100}{[\alpha]_D \times l}.$$

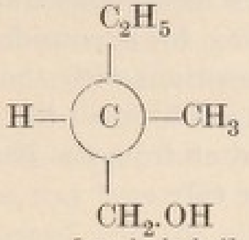
If for example we find the rotation of a urine in a 2-decimetre tube = +2·12° and the $[\alpha]_D$ of glucose is 53°, then

$$p = \frac{2 \cdot 12 \times 100}{53 \times 2} = 2$$

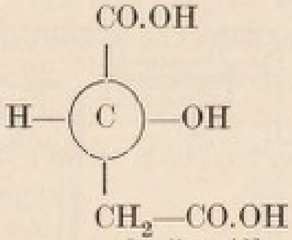
that is, the urine contains 2 per cent. of glucose.

RELATION BETWEEN CIRCULAR POLARISATION AND CHEMICAL CONSTITUTION

The first work in this direction was performed by Pasteur, and it was his publications on this subject that brought him into prominence. He found that racemic acid, which is optically inactive, can be decomposed into two isomerides, one of which is common tartaric acid which is dextro-rotatory, and the other tartaric acid differing from the common variety in being lævo-rotatory. The salts of tartaric acid usually exhibit hemihedral faces, while those of racemic acid are holohedral. Pasteur found that, although all the tartrate crystals were hemihedral, the hemihedral faces were situated on some crystals to the right, and on others to the left hand of the observer, so that one formed, as it were, the reflected image of the other. These crystals were separated, purified by recrystallisation, and those which exhibited dextro-hemihedry possessed dextro-rotatory power, while the others were lævo-rotatory. Pasteur further showed that if the mould *Penicillium glaucum* is grown in a solution of racemic acid, dextro-tartaric acid first disappears, and the lævo-acid alone remains. The subject remained in this condition for many years; it was, however, conjectured that probably there is some molecular condition corresponding to the naked-eye crystalline appearances which produces the opposite optical effects of various substances. What this molecular structure is, was pointed out independently by two observers—Le Bel in Paris, and Van't Hoff in Holland—who published their researches within a few days of each other. They pointed out that all optically active bodies contain one or more asymmetric carbon atoms, *i.e.* one or more atoms of carbon connected with four dissimilar atoms or groups of atoms, as in the following examples :—



[amyl alcohol]



[malic acid]

The question, however, remained—do all substances containing such atoms rotate the plane of polarised light ? It was found that they do not ; this is explained by Le Bel by supposing that these, like racemic acid, are compounds of two molecules—one dextro-, the other lævo-rotatory ; that this was the case was demonstrated by growing moulds, the enzymic action of which is to separate the two molecules in question. Then the other question—how is it that two isomerides, which in chemical characteristics, in graphic as well as empirical formulæ, are precisely alike, differ in optical properties ?—is explained ingeniously by Van't Hoff. He compares the carbon atom to a tetrahedron with its four dissimilar groups, A, B, C, D, at the four corners. The two tetrahedra represented in fig. 68 appear at first sight precisely alike ; but if one be superimposed on the other, C in one and D in the other could never be made to coincide. This difference cannot be represented in any other graphic manner, and probably represents the difference in the way the atoms are grouped in the molecule of right- and left-handed substances respectively.

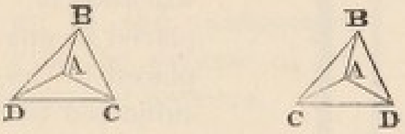


FIG. 68.

THE MERCURIAL AIR-PUMP (BARCROFT'S MODIFICATION)

The instrument consists essentially of the following parts :—1, a small bulb or tube for measuring the amount of blood to be analysed ; 2, a vacuous chamber. When the part of the apparatus from 2 to 4 (fig. 69) is rendered vacuous, the blood is emptied from 1 into 2, and is then kept continuously boiling by means of a water-bath (C) around its lower portion. A condenser (D) packed with ice surrounds the upper part. There is always a stream of aqueous vapour carrying the boiled-out gases to the top of the chamber ; the vapour is condensed and returns to the blood as drops of water, while the gases are free to go into the vacuous pump. In doing so they pass through 3, the drying chamber, which contains sulphuric acid. So much of the gases as has expanded into the pump (4) can then be expelled by lifting the mercury bottle (A), which is connected by strong rubber tubing to the glass tube F. In the figure only the attachments of the rubber tube are shown. The mercury is prevented by a valve (V) from going backwards to the dyring chamber ; it expels the gases down the tube B into a eudiometer tube (E) in which they are collected for measurement and analysis. After boiling for a considerable time, a few exhaustions with the pump are sufficient to deliver all the gases which have boiled off from the blood into the eudiometer tube.

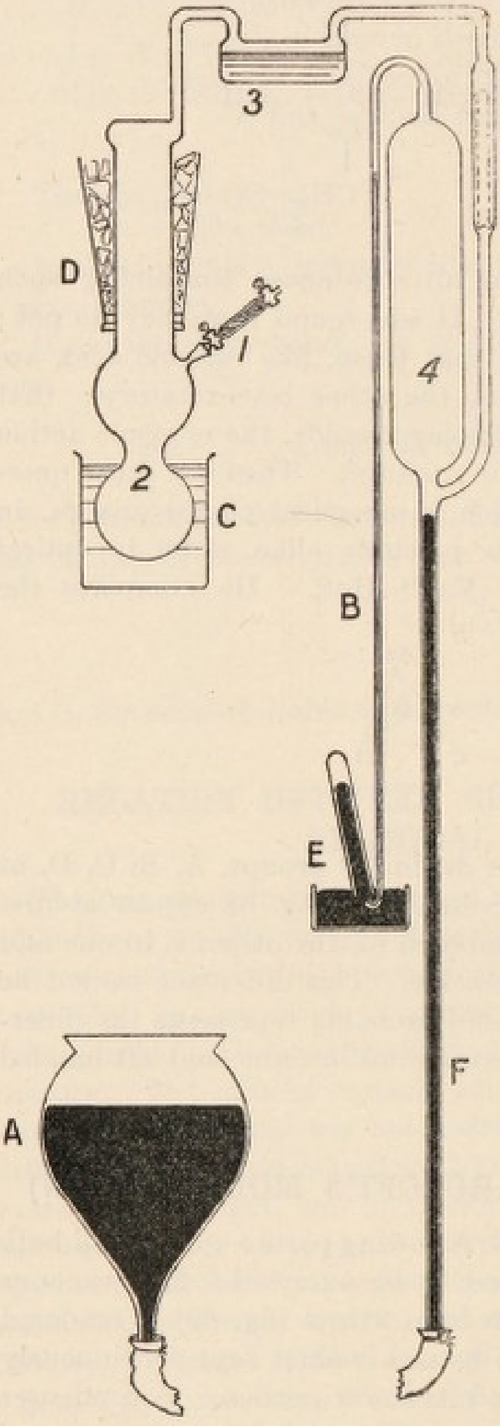


FIG. 69.—Mercurial air-pump for obtaining the gases of the blood (diagrammatic).

BARCROFT'S DIFFERENTIAL APPARATUS FOR ANALYSIS OF BLOOD GASES

The differential method of blood-gas analysis may be used for the measurement either of the quantity of oxygen which the blood gives off when treated with ferricyanide of potassium (see p. 164), or to measure the amount of oxygen which partially reduced blood will absorb. The blood for analysis is placed in one bottle ; a control fluid is placed in the other, and as the two are subjected to the same changes of temperature, vapour pressure, etc., these factors are eliminated.

The apparatus consists of two bottles of equal size connected with one another by means of a manometer filled with clove oil. The bottles should be of about 25 c.c. capacity. The stopper of the bottle is prolonged into a small cup capable of holding potassium ferricyanide. From the stopper a straight tube leads, the bore of which is not so small as to preclude its being cleaned with a pipe cleaner. At the junction of this tube with the manometer is a threeway tap. The student should make himself familiar with the mechanism of the tap ; it contains a **T** boring ; usually there is a spot on the handle of the tap corresponding to the dependent limb of the **T**.

The tap will be spoken of as open when the bottles and the manometer communicate with the external air, and shut when the bottles communicate with the manometer. The whole is mounted on a stand furnished with a clip, by means of which it can hang on the side of a water-bath. The success of the subsequent analyses depends upon the cleanness of the glass of the manometer when the oil is put into it. It should be chemically clean and dry. The utmost care must be taken that water does not get into the manometer.

The oil is best put in with a fine pipette. For this purpose the stopper on the left side is removed; that on the right is turned so as to cut the manometer off from the external air and the air of the bottle. The oil is introduced on the left side. The end of the pipette should penetrate as far as the bend in the tubing at the top of the stand. When as much oil as is judged necessary, or a little more, has been put in, the tap on the right side is opened and the oil allowed to sink into the capillary portion of the manometer till the meniscus reaches the zero on the right side. All superfluous oil should then be removed from the top of the left tube with a little sponge mounted on a wire. The right tap may then be opened again, and the oil allowed to find its own level.

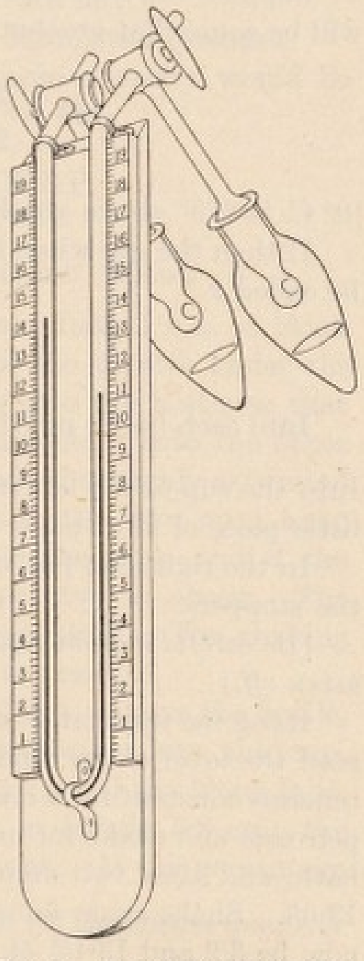


FIG. 70.—Barcroft's differential apparatus.

CALIBRATION OF THE APPARATUS

The volume of gas given off (or absorbed) in the apparatus bears a direct ratio to the difference of pressure set up in the manometer. Supposing, then, that v is the volume of gas given off, and p the difference of pressure.

$$p = K + v.$$

The calibration of the instrument consists in finding the value of the constant K.

This is best done by liberating a known quantity of gas in the apparatus and observing the pressure produced; p and v are then known

$$\text{and } K = \frac{p}{v}$$

The best way of evolving a known quantity of gas in the apparatus is to liberate oxygen from a titrated solution of pure acidulated hydrogen peroxide by mixing potassium permanganate with it.

The solutions necessary are: (1) a decinormal solution of permanganate, (2) some "20-volume" hydrogen peroxide, and (3) sulphuric acid. If the "20-volume" hydrogen peroxide is of full strength, 5 c.c. of it may be made up to 1000 c.c. (Ultimately 1 c.c. of the diluted peroxide should give off about 0·2 c.c. of gas, but it must be borne in mind that only one-half this quantity comes from the peroxide, the other half from the permanganate.)

$$K_2Mn_2O_8 + 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 5H_2O + 5O$$
$$[316\text{ gr.}] \qquad 5O + 5H_2O_2 = 5H_2O + 5O_2\ [111\text{ litres}]$$

or 1 c.c. (0·00316 gr.) permanganate = 1·11 c.c. of oxygen.

Titrate 50 c.c. of the dilute peroxide, mixed with excess of sulphuric acid, with the permanganate. About 8–10 c.c. of the permanganate should be required for the titration if the peroxide is of suitable strength. If it proves to be too weak, as is often the case, a fresh solution must be made up.

Suppose 8·7 c.c. of $K_2Mn_2O_8$ are necessary to reach the end-point (pink colour) in the titration of 50 c.c. of acidulated H_2O_2, then: 1 c.c. of $H_2O_2 = \frac{1{\cdot}11 \times 8{\cdot}7}{50} = 0{\cdot}193$ c.c. of oxygen at normal temperature and pressure (N.T.P.)

Suppose the barometer to be 763 mm., the thermometer to be 15° C., the gas will be somewhat greater in volume than at N.T.P., and will be

$$\cdot 193 \times \frac{288}{273} \times \frac{760}{763} = 0{\cdot}202 \text{ c.c.}$$

[0° C. is 273° above absolute zero, and 15° C. is therefore 288].

If then the gas when liberated from the 1 c.c. of peroxide in the apparatus be called v

$$v = 0{\cdot}202 \text{ c.c.}$$

Into each bottle put 1 c.c. of the peroxide and 2 c.c. of $\frac{N}{100}$ sulphuric acid. Into the cup in the left stopper put 0·3 c.c. of potassium permanganate, and a little piece of filter-paper to cover the orifice.

In the right cup put 0·3 c.c. of water and filter-paper. Put the bottles on the stoppers.

[*Be careful that the taps are always open when the bottles are being put on and taken off.*]

Hang the apparatus on the water-bath for five minutes, then shut the taps; read the level of the fluid on each side (suppose it to be 10·0 and 10·05); if it remains constant for a minute or so, upset the permanganate into the hydrogen peroxide and shake for one minute. At the end replace the apparatus on the bath, and after two minutes more read again. The levels may be 7·0 and 13·05. Shake again for a minute and read two minutes later; the levels may now be 6·9 and 13·15, at which they remain on subsequent shaking.

The side with the permanganate has sunk

from . . . . 10·0 to 6·9 = 3·1 cm.

The other side has risen . . . . . 13·15 to 10·05 = 3·1 cm.

6·2 cm.

$$\text{Then } p = 6{\cdot}2. \qquad v = 0{\cdot}202$$

$$\therefore K = \frac{p}{v} = 0{\cdot}306.$$

The fluid in the bottle should be pink, with excess of permanganate at the end.

Another method of calibration is described by Hoffmann (*Journal of Physiology*, 1913, vol. xlvii. p. 272). The following three exercises illustrate how the instrument may be employed in physiological work.

DETERMINATION OF THE OXYGEN CAPACITY OF BLOOD

Into each bottle put 1 c.c. of blood which has been thoroughly shaken with air (an ordinary 1-c.c. pipette delivers about 0·96 c.c. of blood) and 2 c.c. of ammonia solution (1 c.c. concentrated ammonia made up to 250 c.c. with distilled water). Shake so as to thoroughly lake the blood. Put 0·3 c.c. of ferricyanide into the cup of one stopper, and, if necessary, a slip of filter-paper. Grease the stoppers, and put on the bottles—hang the apparatus on the water-bath and proceed as in the above example. Suppose the final difference of pressure to be 5·8 cm., the amount of gas given off will be :

$$5{\cdot}8 \times 0{\cdot}0306 = 0{\cdot}177 \text{ c.c. at } 15^\circ \text{ C. and } 763 \text{ mm. barometric pressure.}$$

If this were given off by 0·96 c.c. of blood, the oxygen capacity would be obtained by correcting for temperature and pressure :—

$$\frac{0{\cdot}177}{0{\cdot}96} \times \frac{273}{288} \times \frac{763}{760} = 0{\cdot}175 \text{ c.c. at N.T.P.}$$

DETERMINATION OF OXYGEN IN UNSATURATED BLOOD

Place 2 c.c. of the dilute ammonia in each bottle. Take 1 c.c. of blood that is venous in colour[1] in a pipette, put the end of the pipette under the surface of the ammonia, and deliver the blood gently into the bottle so that it lies as a layer underneath the clear solution of ammonia. Into the other bottle similarly deliver 1 c.c. of blood which has been thoroughly saturated with air. Put the bottles on to the stoppers, and with the taps open hang the apparatus on the bath. After five minutes close the taps, and if the zero remains constant, read, and then shake the apparatus as above. The oil will, of course, rise on the side of the unsaturated blood. The shaking should be carried out so as to dirty the cups as little as possible.

Suppose the ultimate difference in level is 2·1 cm. Now, remove the bottle, which contains the fully oxygenated blood, put ferricyanide into the cup, replace the bottle, and determine the total oxygen capacity of the blood in it.

Suppose the final reading with ferricyanide to be as before, 5·8 cm., the student will now have at his disposal two determinations : (1) the percentage saturation, which is $\frac{5{\cdot}8-2{\cdot}1}{5{\cdot}8} \times 100 = 81$ per cent., and (2) the actual quantity of oxygen that was in 1 c.c. of the unsaturated blood, namely, 81 per cent. of the total oxygen capacity, $0{\cdot}189 \times \frac{81}{100} = 0{\cdot}153$ c.c.

STANDARDISATION OF THE GOWERS-HALDANE HÆMOGLOBINOMETER

A Gowers-Haldane hæmoglobinometer should indicate 100 when the oxygen capacity is :

$$1 \text{ c.c. of blood} = 0{\cdot}185 \text{ c.c. oxygen at N.T.P.}$$

To test this it is only necessary to measure the oxygen capacity of the blood with the differential apparatus (it is best to use ox or sheep's blood) and at the same time to determine the oxygen capacity of the same blood with the hæmoglobinometer.

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[1] On no account should stale blood be used.

The cheaper forms of Gowers's hæmoglobinometer when old will be found often to differ very much from the theoretical value: it is therefore desirable that they should be tested.

HALDANE'S APPARATUS FOR GAS ANALYSIS

The apparatus consists of an accurately graduated gas-burette A, provided with a threeway tap at the top. This is surrounded by a water jacket. It is connected by rubber tubing with the levelling tube B, and this and the burette contain mercury. When the levelling tube is raised, A is filled with mercury; when it is lowered the mercury falls in A, and the air to be analysed then is allowed to enter it by one (x) of the connections at the top. The other connections of the tap connect the burette with absorption pipettes, into which the air can be driven by raising B. The absorption pipette E is filled with 20 per-cent. caustic potash, and is connected with a movable reservoir, S, by black rubber tubing. The absorption pipette F is filled with an alkaline solution of pyrogallic acid (10 gr. of pyrogallic acid to the 100 c.c. of saturated caustic potash). This is introduced through the tube K. G and H are partly filled with strong potash solution, which protects the pyrogallate solution from the air. The pressure in the burette is adjusted by using the potash pipette as a pressure gauge and bringing the potash before every reading of the burette to the mark M.

In order to make the readings of the burette independent of changes in temperature, barometric pressure, and percentage of moisture during the analysis, a control tube N stands beside the burette in the water jacket; the threeway tap at P makes it possible to equalise the pressure in N with that of the atmosphere; by means of the T-tube O the potash solution is brought into connection with N, and the potash is adjusted to the mark R by raising or lowering S, P being open to the air. P is then turned, so that the control tube N is connected with the potash tube only, and is not again opened until the analysis is complete. Each time a reading of the burette is made, the potash is brought to the mark R by raising or lowering S. The potash in the absorption pipette is then brought to the mark M by adjusting the levelling tube B. In this way variations of temperature and pressure are compensated for by mechanical means. The lower part of the control tube N is kept full of water; and the gas-burette must have its inner surface wet; the water used for moistening the inner surface of the burette should be slightly acidulated with sulphuric acid. The acidified water is introduced through the free limb of any tap, excess being expelled by raising the levelling tube B. By this means, the air in the burette and in the control tube is always kept saturated with moisture.

In the complete apparatus, a combustion pipette is added also (to estimate carbon monoxide, methane, etc.), but in air analyses, where one has only to deal with oxygen, nitrogen, and carbonic acid, this is not necessary.[1]

In such an analysis the apparatus must first be filled with nitrogen; if the apparatus is used for a succession of analyses, a supply of nitrogen is left at the end of each. If not, the air in the apparatus should be freed from oxygen and carbonic acid by passing it into the pyrogallate and potash pipettes respectively. The next step is to bring the pyrogallate and potash to the marks D, R, and M. The tap closing the control tube N is then closed. The sample of the air to be analysed is then introduced into the burette by lowering the levelling tube; and the tap is turned so as to connect the burette with the potash pipette. The opening of the tap will probably slightly disturb the level M, at which the potash previously stood, and the potash level at R may also shift a little. The potash level is exactly adjusted to R by raising or lowering S; and the exact levelling to the mark M is adjusted by raising or lowering B. The burette is then read to give the total volume of air introduced into the apparatus. The air is then driven into the potash pipette by raising B; it is then brought back again by depressing B, and this is repeated until, on measuring the gas, there is no further diminution in volume; probably half a dozen times will be sufficient; the reading must always be taken when the potash levels are at M and R. The diminution of volume gives the amount of carbonic acid. The burette is then connected with the pyrogallate pipette, and the air driven over into it several times in the same way; some oxygen, however, will still be left in the connection between M and the tap, so this connection is washed out by passing the gas into the potash pipette and back, and then into the pyrogallate pipette twice. Finally, the levels at D, R, and M are adjusted, and the reading indicates the volume of oxygen absorbed.

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[1] For full details see *Methods of Air Analysis*, by J. S. Haldane.

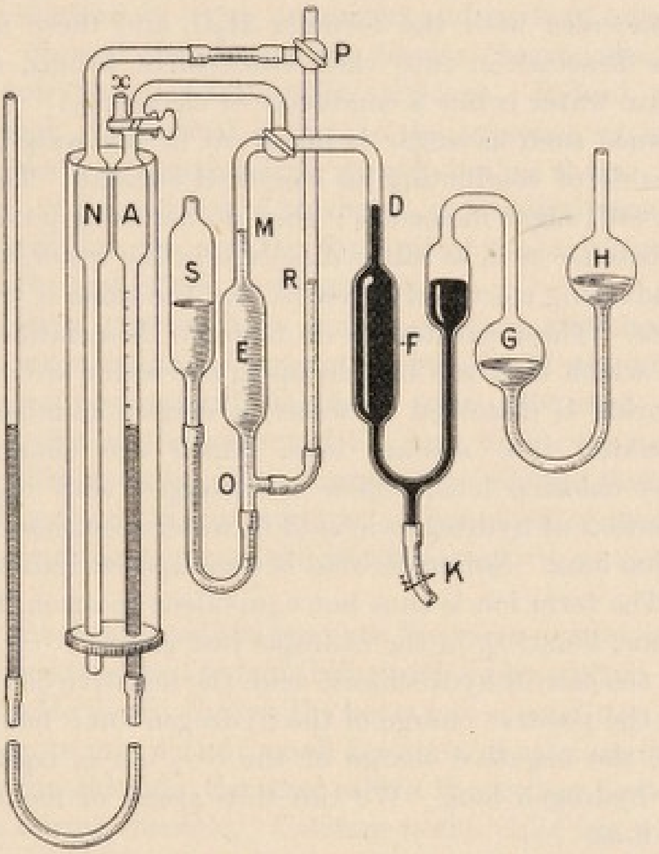

FIG. 71.—Diagram of Haldane's apparatus for air analysis.

SOLUTIONS—DIFFUSION—DIALYSIS—OSMOSIS

The investigations of physical chemists during recent years have given us new conceptions of the meaning of the words that stand at the head of this section. I propose to state what these conceptions are, and briefly to indicate the bearing they have on the elucidation of physiological problems.

Solutions.—Water is the fluid in which soluble materials are usually dissolved, and at ordinary temperatures it is a fluid, the molecules of which are in constant movement ; the hotter the water the more active are the movements of its molecules, until, when at last it is converted into steam, the molecular movements become much more energetic. Perfectly pure water consists of molecules with the formula H_2O, and these molecules undergo practically no dissociation into their constituent atoms, and it is for this reason that pure water is not a conductor of electricity.

If a substance such as sugar is dissolved in the water, the solution still remains incapable of conducting an electrical current. The sugar molecules in solution are still sugar molecules ; they do not undergo dissociation.

But if a substance such as salt is dissolved in the water, the solution is then capable of conducting electrical currents, and the same is true for most acids, bases, and salts. These substances do undergo dissociation, and the simpler materials into which they are broken up in the water are called **ions.** Thus if sodium chloride is dissolved in water, a certain number of its molecules become dissociated into sodium ions, which are charged with positive electricity, and chlorine ions, which are charged with negative electricity. Similarly a solution of hydrochloric acid in water contains free hydrogen ions and free chlorine ions. Sulphuric acid is decomposed into hydrogen ions and ions of SO_4. The term ion is thus not equivalent to atom, for an ion may be a group of atoms, like SO_4, in the example just given.

Further, in the case of hydrochloric acid, the negative charge of the chlorine ion is equal to the positive charge of the hydrogen ion ; but in the case of the sulphuric acid, the negative charge of the SO_4 ion is equal to the positive charge of two hydrogen ions. We can thus speak of monovalent, divalent, trivalent, etc., ions.

Ions charged with positive electricity are called *kat-ions* because they move towards the kathode or negative pole ; those which are charged with negative electricity are called *an-ions* because they move towards the anode or positive pole. The following are some examples of each class :—

Kat-ions. Monovalent : H, Na, K, NH_4, etc.
Divalent : Ca, Ba, Fe (in ferrous salts), etc.
Trivalent : Al, Bi, Sb, Fe (in ferric salts), etc.
An-ions. Monovalent : Cl, Br, I, OH, NO_3, etc.
Divalent : S, Se, SO_4, etc.

Roughly speaking, the greater the dilution the more nearly complete is the dissociation, and in a very dilute solution of such a substance as sodium chloride we may consider that the number of ions is double the number of molecules of the salt present.

The ions liberated by the act of dissociation are, as we have seen, charged with electricity, and when an electrical current is led into such a solution it is conducted through the solution by the movement of the ions. Substances which exhibit the property of dissociation are known as electrolytes.

The conception of electrolytes, which we owe to Arrhenius, is extremely important in view of the question of osmotic pressure which we shall be considering immediately; because the act of dissociation increases the number of particles moving in the solution and so increases the osmotic pressure, for in this relation the ion plays the same part as a molecule.

The liquids of the body contain electrolytes in solution, and it is owing to this fact that they are able to conduct electrical currents.

Another physiological aspect of the subject is seen in a study of the action of mineral salts in solution on living organisms and parts of organisms. Many years ago Ringer showed that contractile tissues (heart, cilia, etc.) continue to manifest their activity in certain saline solutions. Indeed, as Howell puts it, the cause of such rhythmical action is the presence of these inorganic substances in the blood or lymph which usually bathes them. In the case of the heart, the sinus, or venous end of the heart, is peculiarly susceptible to the stimulus of the inorganic salts, and the rhythmical peristaltic waves so started travel thence over the rest of the heart muscle.

Loeb and his fellow workers have confirmed these statements, but interpret them now as ionic action. Contractile tissues will not contract in pure solutions of non-electrolytes (such as sugar, urea, albumin). But different contractile tissues differ in the nature of the ions which are most favourable stimuli. Thus cardiac muscle, cilia, amœboid movement, karyokinesis, cell division are all alike in requiring a proper adjustment of ions in their surroundings if they are to continue to act, but the proportions must be different in individual cases.

In the case of the heart, sodium ions are the most potent in maintaining the osmotic conditions that lead to irritability and contractility; but a solution of pure sodium chloride finally throws the heart into a condition of relaxation: hence it is necessary to mix with it small amounts of calcium ions to restrain this effect; potassium chloride, the third salt in Ringer's or Locke's fluids, also favours relaxation during diastole. Calcium is the chief ion which produces contraction, and by itself produces intense tonic contraction (calcium rigor).

Loeb at one time considered that the process of fertilisation was mainly ionic action, but his later experiments on artificial parthenogenesis have shown that the first change produced by his reagents on the egg-cells of sea urchins and similar animals is the separation of a membrane from their surface; this is caused by fatty acids, saponin and other hæmolytic agents: this superficial *cytolysis* stimulates the egg to commence cleavage, but that process soon ceases; if, however, oxidation is brought about by immersing the egg in hypertonic sea-water for a short time, cleavage continues and well-formed larvæ are produced. The spermatozoon has apparently a similar double action; it produces membrane formation possibly by a fatty acid it carries, and then, having penetrated the membrane, sets up oxidation changes by means of oxidases. In addition to this, certain changes may be produced by other enzymes in the spermatozoon.

Gramme-molecular Solutions.—From the point of view of osmotic pressure a convenient unit is the gramme-molecule. A gramme-molecule of any substance is the quantity in grammes of that substance equal to its molecular weight. A gramme-molecular solution is one which contains a gramme-molecule of the substance per litre. Thus a gramme-molecular solution of sodium chloride is one which contains 58·46 grammes of sodium chloride ($Na = 23{\cdot}000$; $Cl = 35{\cdot}46$) in a litre. A gramme-molecular solution of glucose ($C_6H_{12}O_6$) is one which contains 180 grammes of glucose in a litre. A gramme-molecule of hydrogen (H_2) is 2 grammes by weight of hydrogen, and if this were compressed to the volume of a litre it would be comparable to a gramme-molecular solution. It therefore follows that a litre containing 2 grammes of hydrogen contains the same number of molecules of hydrogen in it as a litre of a solution containing 58·46 grammes of sodium chloride, or one containing 180 grammes of glucose has in it of salt or sugar molecules respectively. To put it another way, the heavier the weight of a molecule of any substance the more of that substance must be dissolved in the litre to obtain its gramme-molecular solution. Or still another way: if solutions of various substances are made all of the same strength per cent., the solutions of the materials of small molecular weight will contain more molecules of those materials than the solutions of the materials which have heavy molecules. We shall see that the calculation of osmotic pressure depends on these facts.

Diffusion, Dialysis, Osmosis.—If two gases are brought together within a closed space, a homogeneous mixture of the two is soon obtained. This is due to the movements of the gaseous molecules within the space, and the process is called *diffusion*. In a similar way diffusion will effect in time a homogeneous mixture of two liquids or solutions. If water is carefully poured on to the surface of a solution of salt, the salt or its ions will soon be equally distributed throughout the whole. If a solution of albumin or any other *colloidal* substance is used instead of salt in the experiment, diffusion will be found to occur much more slowly. If, instead of pouring water on to the surface of a solution of salt or sugar, the two are separated by a membrane made of such a material as parchment paper, a similar diffusion will occur, though more slowly than in cases where the membrane is absent. In time, the water on each side of the membrane will contain the same quantity of sugar or salt. Substances which pass through such membranes are called crystalloids. Substances which have such large molecules (starch, protein, etc.) that they will not pass through such membranes are called *colloids*. Diffusion of substances in solution in which we have to deal with an intervening membrane is usually called **dialysis.** The process of **filtration** (*i.e.* the passage of materials through the pores of a membrane under the influence of mechanical pressure) may be excluded in such experiments by placing the membrane (M) vertically as shown in the diagram (fig. 72), and the two fluids A and B on each side of it. Diffusion through a membrane is not limited to the molecules of water, but it may occur also in the molecules of certain substances dissolved in the water. But very few if any membranes are equally permeable to water and to molecules or ions of the substances dissolved in the water. If in the accompanying diagram the compartment A is filled with pure water, and B with a sodium chloride solution, the liquids in the two compartments will ultimately be found to be equal in bulk as they were at the start, and each will be a solution of salt of half the original strength of that in the compartment B. But at first the volume of the liquid in compartment B increases, because more water molecules pass into it from A than salt molecules pass from B into A. The term **osmosis** is generally limited to the stream of water molecules passing through a membrane, while the term **dialysis** is applied to the passage of the molecules in solution in the water. The osmotic stream of water is especially important, and in connection with this it is necessary to explain the term **osmotic pressure.** At first, then, osmosis (the diffusion of water) is more rapid than the dialysis (the diffusion of the salt molecules or ions). The older explanation of this was that salt attracted the water, but we now express the fact differently by saying that the salt in solution exerts a certain osmotic pressure: the result of the osmotic pressure is that more water flows from the water side to the side of the solution than in the contrary direction. The osmotic pressure varies with the amount of substance in solution, and is also altered by variations of temperature, occurring more rapidly at high than at low temperatures.

If we imagine two masses of water separated by a permeable membrane, as many water molecules will pass through from one side as from the other, and so the volumes of the two masses of water will remain unchanged. If now we imagine the membrane M is not permeable except to water, and the compartment A contains water, and the compartment B contains a solution of salt or sugar; in these circumstances water will pass through into B, and the volume of B will increase in proportion to the osmotic pressure of the sugar or salt in solution in B, but no molecules of sugar or salt can get through into A from B, so the volume of fluid in A will continue to decrease, until at last a limit is reached. The determination of this limit, as measured by the height of a column of fluid or mercury which it will support, will give us a measurement of the osmotic pressure. Membranes of this nature are called **semi-permeable.** One of the best kinds of semi-permeable membrane is ferrocyanide of copper. This may be made by taking a cell of porous earthenware and washing it out first with copper sulphate and then with potassium ferrocyanide. An insoluble precipitate of copper ferrocyanide is thus deposited in the pores of the earthenware. If such a cell is filled with a 1 per-cent. solution of sodium chloride, water diffuses in till the pressure registered by a manometer connected to it registers the enormous height of 5000 millimetres of mercury. Theoretically it is possible to measure osmotic pressure by a manometer in this way, but practically it is seldom done, and some of the indirect methods of measurement described later are used instead. The reason for this is that it has been found difficult to construct a membrane which is absolutely semi-permeable.

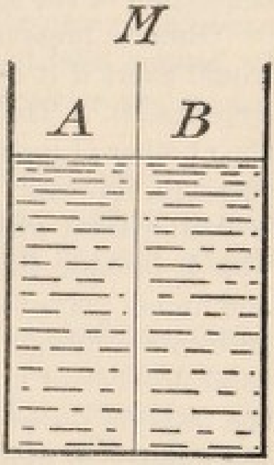

FIG. 72.

Many explanations of the nature of osmotic pressure have been brought forward, but none is perfectly satisfactory. The following simple explanation is perhaps the best, and may be rendered more intelligible by an illustration. Suppose we have a solution of sugar separated by a semi-permeable membrane from water: this is, the membrane is permeable to water molecules, but not to sugar molecules. The streams of water from the two sides will then be unequal; on one side we have water molecules striking against the membrane in what we may call normal numbers, while on the other side both water molecules and sugar molecules are striking against it. On this side, therefore, the sugar molecules take up a certain amount of room, and do not allow the water molecules to get to the membrane; the membrane is, as it were, screened against the water by the sugar, therefore fewer water molecules will get through from the screened to the unscreened side than *vice versa*. This comes to the same thing as saying that the osmotic stream of water is greater from the unscreened water side to the screened sugar side than it is in the reverse direction. The more sugar molecules that are present, the greater will be their screening action, and thus we see that the osmotic pressure is proportional to the number of sugar molecules in the solution: that is, to the concentration of the solution.

Osmotic pressure is, in fact, equal to that which the dissolved substance would exert if it occupied the same space in the form of a gas (Van 't Hoff's hypothesis).[1] The nature of the substance makes no difference; it is only the number of molecules which causes osmotic pressure to vary. The osmotic pressure, however, of substances like sodium chloride, which are electrolytes, is greater than what one would expect from the number of molecules present. This is because the molecules in solution are split into their constituent ions, and an ion plays the same part as a molecule, in questions of osmotic pressure. In dilute solutions of sodium chloride ionisation is more complete, and as the total number of ions is then nearly double the number of original molecules, the osmotic pressure is nearly double what would have been calculated from the number of molecules.

The analogy between osmotic pressure and the partial pressure of gases is complete, as may be seen from the following statements:—

1. At a constant temperature osmotic pressure is proportional to the concentration of the solution (Boyle-Mariotte's law for gases).
2. With constant concentration, the osmotic pressure rises with and is proportional to the temperature (Gay-Lussac's law for gases).
3. The osmotic pressure of a solution of different substances is equal to the sum of the pressures which the individual substances would exert if they were alone in the solution (Dalton-Henry law for partial pressure of gases).
4. The osmotic pressure is independent of the nature of the substance in solution, and depends only on the number of molecules or ions in solution (Avogadro's law for gases).

Calculation of Osmotic Pressure.—We may best illustrate this by an example, and to simplify matters we will take an example in the case of a non-electrolyte such as sugar. We shall then not have to take into account any electrolytic dissociation of the molecules into ions. We will suppose we want to calculate the osmotic pressure of a 1 per-cent. solution of sucrose.

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[1] Moore and Frazer find that the law may be more accurately expressed as follows: The pressure is that which would be exerted if the substance in solution was rendered gaseous at the same temperature and kept to the volume of the pure solvent used (water) instead of the volume of the entire solution.

One gramme of hydrogen at atmospheric pressure and 0° C. occupies a volume of 11·2 litres; two grammes of hydrogen will therefore occupy a volume of 22·4 litres. A gramme-molecule of hydrogen—that is, 2 grammes of hydrogen—when brought to the volume of 1 litre will exert a gas pressure equal to that of 22·4 litres compressed to 1 litre—that is, a pressure of 22·4 atmospheres. A gramme-molecular solution of sucrose, since it contains the same number of molecules in a litre, must therefore exert an osmotic pressure of 22·4 atmospheres also. A gramme-molecular solution of sucrose ($C_{12}H_{22}O_{11}$) contains 342 grammes of sucrose in a litre. A 1 per-cent. solution of sucrose contains only 10 grammes of sucrose in a litre of water; hence the osmotic pressure of a 1 per-cent. solution of sucrose is $\frac{10}{342} \times 22{\cdot}4$ atmospheres, or 0·65 of an atmosphere, which in terms of a column of mercury $= 760 \times 0{\cdot}65 = 494$ mm.

It would not be possible to make such a calculation in the case of an electrolyte, because we should not know how many molecules had been ionised. In the liquids of the body, both electrolytes and non-electrolytes are present, and so a calculation is here also impossible.

We have seen the difficulty of directly measuring osmotic pressure by a manometer; we now see that mere arithmetic often fails us; and so we come to the question to which we have been leading up, viz., how osmotic pressure is actually determined.

Determination of Osmotic Pressure by means of the Freezing-point.—This is the method which is almost universally employed. A very simple apparatus (Beckmann's differential thermometer) is all that is necessary. The principle on which the method depends is the following:—The freezing-point of any substance in solution in water is lower than that of water; the lowering of the freezing-point is proportional to the molecular concentration of the dissolved substance, and that, as we have seen, is proportional to the osmotic pressure.

When a gramme-molecule of any substance is dissolved in a litre of water, the freezing-point is lowered by 1·87° C., and the osmotic pressure is, as we have seen, equal to 22·4 atmospheres: that is, $22{\cdot}4 \times 760 = 17{,}024$ mm. of mercury.

We can therefore calculate the osmotic pressure of any solution if we know the lowering of its freezing-point in degrees Centigrade; the lowering of the freezing-point is usually expressed by the Greek letter Δ.

$$\text{Osmotic pressure} = \frac{\Delta}{1{\cdot}87} \times 17{,}024.$$

For example, a 1 per-cent. solution of sucrose would freeze at $-0{\cdot}052°$ C.; its osmotic pressure is therefore $\frac{{\cdot}052 \times 17{,}024}{1{\cdot}87} = 473$ mm., a number approximately equal to that we obtained by calculation.

Mammalian blood serum gives $\Delta = 0{\cdot}56°$ C. A 0·9 per-cent. solution of sodium chloride has the same Δ; hence serum and a 0·9 per-cent. solution of common salt have the same osmotic pressure, or are *isotonic*. The osmotic pressure of blood serum is $\frac{\cdot 56 \times 17{,}024}{1\cdot 87} = 5000$ mm. of mercury approximately, or a pressure of nearly 7 atmospheres.

The osmotic pressure of solutions may also be compared by observing their effect on red corpuscles, or on vegetable cells such as those in *Tradescantia*. If the solution is *hypertonic*, *i.e.* has a greater osmotic pressure than the cell contents, the protoplasm shrinks and loses water, or, if red corpuscles are used, they become crenated. If the solution is *hypotonic*, *e.g.* has a smaller osmotic pressure than the material within the cell-wall, no shrinking of the protoplasm in the vegetable cell occurs, and if red corpuscles are used they swell and liberate their pigment. *Isotonic* solutions produce neither of these effects, because they have the same molecular concentration and osmotic pressure as the material within the cell-wall.

Physiological Applications.—It will at once be seen how important all these considerations are from the physiological standpoint. In the body we have aqueous solutions of various substances separated from one another by membranes. Thus we have the endothelial walls of the capillaries separating the blood from the lymph ; we have the epithelial walls of the kidney tubules separating the blood and lymph from the urine ; we have similar epithelium in all secreting glands ; and we have the wall of the alimentary canal separating the digested food from the blood-vessels and lacteals. In such important problems, then, as lymph-formation, the formation of urine and other excretions and secretions, and absorption of food, we have to take into account the laws which regulate the movements both of water and of substances which are held in solution by the water. In the body osmosis is not the only force at work, but we have also to consider filtration : that is, the forcible passage of materials through membranes, due to differences of mechanical pressure. Further complicating these two processes we have to take into account another force : namely, the secretory or selective activity of the living cells of which the membranes in question are composed. This is sometimes called by the name *vital action*, which is an unsatisfactory and unscientific expression. The laws which regulate filtration, imbibition, and osmosis are fairly well known and can be experimentally verified. But we have undoubtedly some other force, or some other manifestation of force, in the case of living membranes. It probably is some physical or chemical property of living matter which has not yet been brought into line with the known chemical and physical forces which operate in the inorganic world. We cannot deny its existence, for it sometimes operates so as to neutralise the known forces of osmosis and filtration.

The more one studies the question of lymph-formation, the more convinced one becomes that mere osmosis and filtration will not explain it entirely. The basis of the action is no doubt physical, but the living cells do not behave like the dead membrane of a dialyser ; they have a selective action, picking out some substances and passing them through to the lymph, while they reject others.

The question of gaseous interchanges in the lungs has been another battlefield of a similar kind. Some maintain that all can be explained by the laws of diffusion of gases ; others have asserted that the action is wholly vital ;

but recent research has shown that the main facts are explicable on a physical basis (see p. 171). Take again the case of absorption. The object of digestion is to render the food soluble and diffusible; it can hardly be supposed that this is useless; the readily diffusible substances will pass more easily through into the blood and lymph: but still, as Waymouth Reid has shown, if the living epithelium of the intestine is removed, absorption comes very nearly to a standstill, although from the purely physical standpoint removal of the thick columnar epithelium would increase the facilities for osmosis and filtration.

The osmotic pressure exerted by crystalloids is very considerable, but their ready diffusibility limits their influence on the flow of water in the body. Thus, if a strong solution of salt is injected into the blood, the first effect will be the setting up of an osmotic stream from the tissues to the blood. The salt, however, would soon diffuse out into the tissues, and would now exert osmotic pressure in the opposite direction. Moreover, both effects will be but temporary, because excess of salt is soon got rid of by the excretions.

Osmotic Pressure of Proteins.—It has been generally assumed that proteins, the most abundant and important constituents of the blood, exert little or no osmotic pressure. Starling, however, has claimed that they have a small osmotic pressure; if this is so, it is of importance, for proteins, unlike salt, do not diffuse readily, and their effect therefore remains as an almost permanent factor in the blood. Starling gives the osmotic pressure of the proteins of the blood-plasma as equal to 30 mm. of mercury. By others this is attributed to the inorganic salts with which proteins are always closely associated.[1] Moore, for instance, finds that the purer a protein is, the less is its osmotic pressure; the same is true for other colloidal substances. It really does not matter much, if the osmotic force exists, whether it is due to the protein itself, or to the saline constituents which are almost an integral part of a protein. It is merely interesting from the theoretical point of view. We should from the theoretical standpoint find it difficult to imagine that a pure protein can exert more than a minimal osmotic pressure. It is made up of such huge molecules that, even when the proteins are present to the extent of 7 or 8 per cent., as they are in blood-plasma, there are comparatively few protein molecules in solution, and probably none in true solution. Still, by means of this weak but constant pressure it is possible to explain the fact that an isotonic or even a hypertonic solution of a diffusible crystalloid may be completely absorbed from the peritoneal cavity into the blood.

The functional activity of the tissue elements is accompanied by the breaking down of their constituents into simpler materials. These materials pass into the lymph, and increase its molecular concentration and its osmotic pressure; thus water is attracted (to use the older way of putting it) from the blood to the lymph, and so the volume of the lymph rises and its flow increases. On the other hand, as these substances accumulate in the lymph they will in time attain there a greater concentration than in the blood, and so they will diffuse towards the blood, by which they are carried to the organs of excretion.

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[1] Bayliss has shown that the saline constituents in a protein are not mechanically mixed with it, but are in a state intermediate between mechanical admixture and chemical combination, to which the term *adsorption* is applied. Many dyes used for staining fabrics and histological preparations are also adsorbed.

But, again, we have a difficulty with the proteins ; they are most important for the nutrition of the tissues, but they are practically indiffusible. We must provisionally assume that their presence in the lymph is due to filtration from the blood. The plasma in the capillaries is under a somewhat higher pressure than the lymph in the tissues, and this tends to squeeze the constituents of the blood, including the proteins, through the capillary walls. I have, however, already indicated that the exact mechanism of lymph-formation is one of the many physiological problems which await solution by the physiologists of the future.

COLLOIDS AND COLLOIDAL SOLUTIONS

The proteins, the polysaccharides, and the soaps may be taken as instances of colloids. They do not pass through the membrane of a dialyser, they can be "salted out" of solution, their solutions are opalescent, they crystallise with difficulty if at all, they have a tendency to form jellies, and they exert a very low osmotic pressure.

The study of colloids, a class to which so many important physiological substances belong, is therefore important, and the following paragraphs deal briefly with some of the principal facts now known in relation to this branch of physical chemistry.

A colloid material is spoken of as being capable of assuming two conditions, which are respectively named *sol* and *gel*. If the colloid is fluid, the term sol is used ; if it is solid like a jelly, the word gel is employed. The two conditions are well illustrated in the case of gelatin ; in warm water gelatin is a sol ; when the solution cools we get a gel. In the case of gelatin the condition is easily reversible, but this is not so in relation to all colloids.

If water is the fluid medium employed, the terms "hydrosol" and "hydrogel" are applied ; if alcohol is used, the words "alcoholsol" and "alcoholgel" are employed, and so with other solvents.

Colloid material is often obtainable in another condition still, namely, as a flocculent precipitate. This is seen when proteins are "salted out," or when an albuminous solution is heated beyond its "coagulating point." In some cases an enzyme action will alter the physical and chemical structure of the protein so that it becomes insoluble in the fluid in which it was previously apparently dissolved. This is seen in the familiar examples of the clotting of blood by thrombin, and the curdling of milk by rennin.

There are numerous analogies between these organic colloids and the inorganic colloids. Thus several metals, such as gold, silver, and platinum, are obtainable in colloidal form, and the same is true for certain compounds such as silicic acid. These materials are in an unstable physical condition, passing from the sol to the gel condition under slight provocation. This confers upon them the property of producing what is termed catalysis in chemical substances in contact with them, and the similarities between catalysis and enzyme action are so striking and numerous, that the doctrine that enzyme action is a catalytic one rests on a by no means unstable foundation.

Supposing now the case of a colloid in the condition of a sol, as for instance the proteins are in the plasma or serum of the blood, does that term imply complete solution in the same way as when we use the term in reference to a solution of salt or sugar ? Or have we, on the other hand, rather a condition of suspension, or a kind of attenuated gel ?

The microscopic examination of such fluids, even with the highest powers, reveals no visible particles. The particles which are present if they are not in solution are present either in a smaller or in a more diffuse condition than the particles of an ordinary suspension or emulsion.

An ordinary paper filter has far too large pores to keep back any particles from such fluids. It is necessary to construct a filter of a more efficient character. The kind of filter employed is fashioned on the principle of those used for filtering off small particles such as bacteria from fluids. One of the best is that described by C. J. Martin. The case of the candle of a Pasteur-Chamberland filter is filled with a hot 10 per-cent. solution of gelatin, and this is forced by air pressure through the pores of the porcelain. The hot solution filters through fairly quickly at first, but as the pores get stopped up it runs through more slowly ; when it is nearly cold, the filter case is removed from the compressed-air cylinder, and the filter detached from its case. The gelatin is then washed off from the outside of the filter, and it is ready for use.

Instead of a gelatin filter, one of silicic acid can be made. A stiff solution of sodium silicate is filtered under pressure through the candle ; after a few minutes, when the pores are filled with it, the candle can be detached, filled with 3 per-cent. hydrochloric acid and immersed in a bath of the same acid for a day or two. The acid diffuses into the pores, and decomposes the sodium silicate, depositing a gelatinous precipitate of silicic acid.

If fresh serum or egg-white is placed outside the filter, the filtrate which comes through is clear, colourless, and absolutely free from protein.

Proteoses and crystalloids pass the membrane easily ; meta-proteins slightly; caramel, biliverdin, and dextrins partially. But the following proteins do not pass it at all: egg-albumin, serum-albumin, egg-globulin, serum-globulin, fibrinogen, caseinogen, nucleo-proteins, and hæmoglobin. The colloid carbohydrates starch and glycogen resemble the proteins.

In other words, substances with large molecules which do not pass through membranes by dialysis are also stopped by filtration under pressure through a gelatin or silicic-acid filter, and some are inclined to regard the large size of the molecule as the reason of the non-passage in both cases, and do not agree with Ostwald that the solutions are mechanical mixtures and not true solutions. The small osmotic pressure which such substances as protein exert may be regarded as evidence of true solution. But, as we have already seen, we are by no means certain that absolutely pure proteins do exert osmotic pressure, and further, osmotic pressure appears to be exerted by substances which are admittedly not in true solution. The working hypothesis adopted in this dilemma by the majority of observers is, that in such fluids we are not dealing with true solutions nor with suspensions of fine particles, and the term " colloidal solution " has been invented to express the condition of things, which appears to be something of an intermediate nature.

The similarity between colloidal solution and fine suspension is a marked one. The well-known migration of obvious suspensions (including bacteria) in an electric field is evident also in colloidal solutions; and as Hardy has shown in the case of certain proteins, the sign of the charge in the colloidal state or in suspension may be reversed by very slight alterations in the reaction of the fluid.

Further, both suspensions and colloidal solutions give what is known as the Tyndall phenomenon, scattering light; this test forms the basis of what are termed ultra-microscopic observations.

As compared with ordinary solutions, a very small expenditure of energy is necessary to separate matter in colloidal solution from its "solvent"; and the vapour pressure and freezing-point of the "solvent" are only altered to a negligible degree by the incorporation of the colloid in it. Still, this in itself is not characteristic of colloids, for the same is true for certain pairs of liquids (for instance, dichloracetic acid and isopentane) which form true solutions together.

Precipitation by electrolytes is again a striking feature common to colloidal solutions and suspensions, and the precipitating ions are carried down in amounts which are proportional to the amount of precipitate. The agglutination of bacteria is possibly a phenomenon of the same order. There are, however, differences between the behaviour of inorganic colloids and proteins not only to electrolytes, but also to non-electrolytes, which Waymouth Reid considers still require elucidation, and in the case of electrolytes Pauli points out a certain specificity in the ionic actions, the end result being determined by the algebraic sum of the antagonistic actions of the precipitant and anti-precipitant properties of the kation and anion of a salt.

The view that a colloidal solution approaches near to the state of extremely fine suspension is favoured by the facts that both reduce the surface tension of the fluid containing them, and that both readily form surface films of greater concentration which can be heaped up mechanically and separated by agitation; this, for instance, occurs in emulsification. The case of hæmoglobin shows that this substance, though non-dialysable and capable of being filtered off by an efficient filter, is not on all-fours with other proteins in other particulars, for it is probably dissolved by water (W. Reid). It is possible as research proceeds other exceptions to the general rule may be found, and that the native proteins, although colloids, nevertheless exhibit gradations from those at one extreme which form true solutions, to those at the other which form obvious suspensions only.

SURFACE TENSION

The surface layer of a liquid possesses certain properties which are not shared by the rest of it, for in the interior the arrangement of matter is symmetrical around any point, whereas on the surface the surroundings consist of liquid on one side only, while on the other side is solid or gas, or it may be another liquid. In a gas the molecules are free from one another's attractive influence, and fly about freely with high velocity, producing pressure on the walls of the containing vessel; in a liquid, the mutual attraction of the molecules is great enough to keep the substance together in a definite volume. In order to separate the molecules and convert the liquid into gas, a large amount of energy is required—the so-called latent heat of evaporation. The molecular attractions in a liquid are thus very great, so that any molecule of the surface layer is strongly pulled inwards, and this layer constitutes a stretched elastic skin, and the power thus exerted is termed *surface tension.* The effect of surface tension is most simply seen in a free drop of liquid, such as a raindrop, or a drop of oil immersed in a mixture of alcohol and water of the same density. There is then nothing to prevent the surface layer from contracting as much as possible, and the drop will assume a form in which its volume has the smallest surface, that is, the form of a sphere.

Animal cells are liquid, and when they are at rest, other forces being absent, they also are spherical, and altogether they do not possess as a rule a definite wall of harder material, such as one finds in most vegetable cells ; nevertheless the surface film, exercising the force called surface tension, plays the part of an elastic skin, and is termed the *plasmatic membrane.* This membrane plays an important physiological rôle. In the projection of pseudopodia, for instance, variation in the surface tension occurs in different parts of the circumference of the cell, and at the points where the surface tension is lowered pseudopodia will be thrust out. Protoplasm, however, is not a homogeneous liquid, but contains substances of varying chemical composition ; those substances which have the power of diminishing surface tension always show a tendency to accumulate at the surface. Hence the fats and lipoids, which are powerful depressants of surface tension, are found (probably in a state of an extremely fine emulsion) more abundantly in the plasmatic membrane than in other parts of the cell. The interstitial spaces between the fat globules are filled up with a watery colloidal (protein) solution.

The theory of diffusion of dissolved substances through membranes as applied to cells has been profoundly influenced by the discovery of the composition of the plasmatic membrane. At one time it was believed that diffusion of a colloid material was prevented by the pores of the membrane being too small to allow large molecules to get through them ; it was considered to act as a sort of sieve. But this cannot be the whole explanation, and it is now held that *solution affinities* play the most important part ; that is to say, a membrane is permeable to substances which are soluble in the material of the membrane. Such solubility may imply the formation of actual chemical unions, but more frequently the process is one of adsorption ; this latter process comes specially into play where nutritive materials are assimilated by the cell by means of the protein solution which occupies the interstices between the fat globules. On the other hand, the permeability of the plasmatic membrane by substances such as chloroform and ether is mainly determined by the solubility of these materials in the fatty or fat-like components of the membrane, and this consideration is the foundation of the Meyer-Overton theory of the narcotic effect on cells which these volatile anæsthetics exercise.

THE DETERMINATION OF THE REACTION OF FLUIDS

In any aqueous solution, if the concentration of hydrogen ions (C_H^+) is multiplied by that of hydroxyl ions (C_{OH}^-), the product is constant. In distilled water at 18° C., the two concentrations are equal ($10^{-7.07}$). Therefore

$C_H \times C_{OH} = 10^{-7 \cdot 07} \times 10^{-7 \cdot 07} = 10^{-14 \cdot 14}$. In acid solutions C_H exceeds $10^{-7 \cdot 07}$ and C_{OH} is less. In alkaline solutions the reverse obtains, but in all cases the product is $10^{-14 \cdot 14}$.

The amount of ionisation which acids undergo in solution varies greatly. For instance, in decinormal hydrochloric acid, 91 per cent. is ionised ; therefore C_H is 0·091 times the normal and is equal to $10^{-1 \cdot 04}$. The figure 1·04 (the negative sign is usually omitted) is the logarithm to the base 10 of the concentration of hydrogen ions in grammes per litre, and is conveniently expressed as P_H. On the other hand, decinormal acetic acid is only dissociated into its ions to the extent of 1·3 per cent., and its P_H is 2·89.

In the testing of the reaction of a fluid, various indicators are employed ; these undergo a change of colour on the addition of an acid or alkali, and at a certain point an intermediate tint shows what is termed the neutral point. The fluid, however, is only neutral to the particular indicator employed, and the neutral tint does not imply that the concentrations of hydrogen and hydroxyl ions are equal. Different indicators give the so-called neutral point when the concentrations of ions are widely different. Thus a solution which is neutral to litmus ($P_H = 6 \cdot 5$ approximately) will not be so to methyl orange ($P_H = 4$ approximately). The range of a few indicators is given in the subjoined table :—

	P_H.
Methyl orange	3·1 to 4·4
Töpfer's reagent	2·9 to 4·2
Litmus	5·0 to 8·0
Tropæolin OO	1·4 to 2·6
Phenolphthalein	8·3 to 10·0

The amount of decinormal soda which must be added to an acid fluid to render it neutral is called its "titration acidity" to the indicator employed. Thus normal urine which owes its acidity partly to acid salts, and partly to free acids, has a $P_H = 5$, so that it will be acid to litmus, but alkaline to methyl orange.

The true acidity is ascertained by combining observations with a large number of indicators, or, better still, by an electrometric examination of the fluid. It is only necessary to estimate the concentration of hydrogen ions ; the concentration of hydroxyl ions can always be calculated, because, as already stated, the product of the two is a constant.

The electrometric method we owe to Nernst. It is based on the fact that a metal electrode saturated with hydrogen, immersed in a liquid also saturated with hydrogen, gives rise to a difference of potential between the electrode and the liquid which is dependent on the concentration of hydrogen ions previously present in the liquid. This concentration is calculated from the difference of potential observed. The method presupposes that the saturation with hydrogen referred to produces no change in the hydrogen ion concentration of the original liquid. This assumption is not always correct, especially when one is dealing with biological fluids ; these frequently contain volatile acids or bases which will be partly swept out of solution by the current of hydrogen gas. It will be sufficient to say, without entering into technical details, that there are methods for overcoming this difficulty.

MICROCHEMICAL QUANTITATIVE ANALYSIS

Certain physiological problems have so far baffled experimental investigation, owing to the minute quantity in which many important substances occur in the organs and fluids of the body. This fact makes it in many cases impossible to apply to them the usual quantitative methods of the analytical chemist, or to identify them, when isolated, by the classical methods of elementary analysis. Within recent years, however, the ingenuity of many workers has been applied to the elaboration of microchemical and microphysical methods, which rival in exactness the older methods. A few of these may be mentioned here in outline, as the full description of experimental detail is outside the scope of this book.

1. **Quantitative Micro-elementary Analysis** (**Pregl**).—The principle of the method is the same as that mentioned in Lesson I. 3. Its application to very small quantities of material (5–10 mg.) has been made possible by the construction of sensitive micro-balances (Nernst, Kahlmann), which allow weighings to be made with an accuracy of ± 0·001 mg. Suitable small absorption apparatus for carbon dioxide and water have been constructed by Pregl. The same author has also worked out methods for the estimation of nitrogen in small quantities of organic substances depending on the principle of Dumas's and Kjeldahl's (p. 251) methods. Micro-Dumas, micro-Kjeldahl, micro-sulphur, and micro-halogen estimations have been made possible by the introduction of micro-filtration apparatus made out of capillary tubing or of platinum.

2. **Microchemical Analysis of Blood.**—Bang has described methods for the volumetric estimation of glucose and sodium chloride in 2–3 drops (= 100 mg.) of blood, and has also indicated methods for the estimation of albumin, globulin, hæmoglobin, urea, uric acid, etc., in blood by the use of suitable micro-methods. By Winterstein's method the oxygen can be estimated in 0·05 c.c. of blood. (*See also* Barcroft's method, p. 283.)

3. **Microchemical Analysis of Urine.**—Methods for this purpose have been developed mainly by Folin, by means of which the total nitrogen, ammonia, and urea may be estimated in 1 c.c. of urine. Urea may also be estimated in the same small quantity by Marshall's method, who makes use of the conversion of urea into ammonium carbonate by the enzyme urease contained in soy beans. Further, a quantitative gravimetric method for the estimation of urea in extremely small quantities has been based by Fosse on the insolubility of its xanthydrol compound. Folin's microchemical method for uric acid has already been described (p. 256). The colorimetric method for the estimation of creatinine (p. 257) may also be mentioned in this connection.

4. **Van Slyke's Method** for the estimation of amino-nitrogen (p. 233) has been converted by him into a microchemical method, which requires only 0·5 mg. of amino-nitrogen for analysis.

5. **Spectrometric Methods** for the estimation of cholesterol (and "oxy-cholesterol") and its esters have been worked out by Lifschütz, which appear to be more trustworthy than the older colorimetric methods.

6. **Micro-polarimetric Method of E. Fischer.**—The usual polarimetric method (p. 223) has been converted into a micro-polarimetric method by

reducing the diameter of the tubes used to 1·5 mm., and their contents to 0·1 c.c. The specific gravity of the solutions to be examined is estimated by a micro-pycnometer. Only 5–10 mg. of substance are necessary for an observation.

7. **Microscopic Molecular-Weight Estimations** may be carried out by Barger's method, which depends on the fact that equimolecular solutions of different substances possess the same vapour density. The estimations are carried out in capillary tubes under the microscope, and require only a few milligrammes of substance.

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