CHEMICAL PHYSIOLOGY

THE ESSENTIALS

OF

CHEMICAL PHYSIOLOGY

FOR THE USE OF STUDENTS

BY THE LATE

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PREFACE TO THE THIRTEENTH EDITION

THE Thirteenth Edition of this book is a milestone in its history, for there has occurred since the appearance of the last edition the regrettable death of its originator, Professor Halliburton. Fortunately the writers to whose lot has fallen the duty of preparing the present edition knew him sufficiently long to gain an insight into his ideas on the teaching of Chemical Physiology to medical students, and it will be found that the essential character of the book remains unaltered.

Certain changes have, however, been unavoidable. The method adopted in former editions of dividing the book into elementary and advanced sections has been abandoned, the subject-matter of these parts being fused. This has been accomplished with the elimination of only those exercises which are presently recognised as forming part of an Honours Course of Physiology, and as falling, therefore, outside the scope of this text-book. Moreover, the section on organic chemistry has been deleted. This subject has of late years won for itself a separate place in the medical syllabus, and in any case the amount of space devoted to its treatment in previous editions was too meagre to be of any great value.

By such means has it been found possible to incorporate in the book, without increasing its size, certain new matter. The new exercises have been selected with the aim of enabling the student to test for himself the broad principles of the subject, and also to acquire some practice in those quantitative methods used as aids in clinical diagnosis. It may be added that all the exercises described have actually been carried out by medical students.

Finally, we take the opportunity of thanking Miss A. Shore for her invaluable help and criticism at various stages of the preparation of this edition.

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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	Silicon	Si	28·3
Copper	Cu	63·57	Silver	Ag	107·88
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.

ESSENTIALS OF CHEMICAL PHYSIOLOGY

INTRODUCTION

Chemical Physiology is that branch of physiology which describes in terms of chemistry the phenomena associated with living matter. Its province, therefore, is the study of the chemical composition of the body and of the parts played by the various substances found there in the maintenance of life. It thus differs from **Physiological Chemistry**, that branch of organic chemistry which deals with the chemical composition and the reactions of physiological substances. Since, however, a close understanding of the former must largely depend upon a clear knowledge of the chemical constitution and behaviour of the substances concerned, it will be recognised that the two subjects are closely interwoven. This book really deals with both subjects, although special emphasis is laid on their physiological aspect.

The elements found in the body include carbon, hydrogen, oxygen, nitrogen, sulphur, phosphorus, chlorine, iodine, fluorine, sodium, potassium, calcium, magnesium, iron, lithium, silicon, and traces of manganese, copper, and lead. Of these all but a very few exist in combination with one another. The chief exceptions are oxygen and nitrogen, which occur as such in blood, and hydrogen, a product of bacterial activity in the alimentary canal. The compounds themselves vary considerably in complexity. On the one hand are the comparatively simple inorganic substances, *e.g.*, water, sodium and potassium chlorides, sodium, potassium and calcium phosphates, etc., whilst on the other are the multitudinous organic compounds, of which the chief classes are the carbohydrates, the lipoids, and the proteins.

The unit of structure of the animal, as of the plant, is the cell. The material of which this is constructed is called **Protoplasm**, and it is the ceaseless changes undergone by the latter which are regarded

as the manifestations of life itself. Of the exact nature of protoplasm little is known. Indeed it is only when the cell is killed that its contents can be examined. Such an examination reveals the presence of

1. **Water.**—This may constitute 75 per cent. or more of the total weight.

2. **Proteins.**—These are the most abundant and constant of the solids and contain carbon, hydrogen, oxygen, nitrogen, with sulphur and phosphorus in small but definite amounts. The protein obtained in greatest abundance from cell protoplasm in **nucleo-protein**; that is, a compound of protein with varying amounts of **nuclein.** In this latter (nuclein) phosphorus is present in greater amount than in proteins.

As some index of the chemical complexity of proteins it may be pointed out that, despite a study extending for more than a century, the exact composition of the vast majority of them is not yet definitely known.

White of egg is a familiar example of a protein, and the fact (which is also familiar) that this sets to a solid on heating 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 of other agencies.

3. **Lipoids.**—These are substances which resemble fats in their solubilities and physical appearance. 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 of the terpene series.

4. **Inorganic Salts,** especially phosphates and chlorides of calcium, sodium, and potassium.

5. **Carbohydrates and Fats** are almost invariably present. Since these substances, however, are mainly utilised for the production of heat and of other forms of energy, they are not generally included amongst the components strictly essential for cell life.

The countless changes which take place in the cell-contents while the cell is alive are grouped under the general title of **Metabolism.** Of such changes there are two types. Those leading to the building-up of new material and those whereby material already in existence is broken down. To the former type of cellular activity is given the name **Anabolism,** to the latter, **Katabolism.** During life both these processes are always taking place, anabolism being in the ascendant during the growth period of the animal, but becoming less and less so with its increasing age.

It has been shown above that the protoplasm of the cell contains certain specific inorganic and organic constituents, and it follows that the food of the animal must be such that these constituents can be derived from it. It will be clear, then, that a study of the dietary requirements of the body—in both its qualitative and quantitative aspects—must form an exceedingly important chapter in the subject of chemical physiology.

Another branch of this subject deals with Digestion. With few exceptions the food in the form in which it is swallowed cannot be absorbed by the body. The term Digestion is given to the sum total of the processes by which the food is broken down into substances which are soluble in water and which can be absorbed. Playing very prominent rôles in these processes are substances called Enzymes, organic members of that class of materials termed catalysts. By their intervention digestion takes place much more rapidly than otherwise would be the case. It must not be assumed, however, that the alimentary canal is the only part of the body where enzymes are found. On the contrary, they are prevalent in all parts of the body, and it is, indeed, difficult to conceive of any metabolic activity taking place without the aid of one or more enzymes.

Once the food has been digested the products are absorbed and transported by the blood to the different parts of the body where they are required for certain specific purposes. This is but one of the many functions of the blood. On it, for example, also falls the task of carrying oxygen from the lungs to the tissues; from the tissues it must take the waste (end) products of metabolism to the different organs of excretion, *i.e.*, the lungs, the kidneys, and the skin; it distributes the hormones and internal secretions, substances which influence the co-ordination or which control the metabolic processes of various parts of the body; another of its duties is the protection of the body against the invasion and action of micro-organisms; whilst, in addition, it must possess some mechanism for preventing as far as possible its own escape from the body in case of injury to the blood-vessels themselves. All these functions of the blood have each a chemical basis and must be studied, some in greater degree, others in lesser, by the elementary student.

Having traced the path of the food to its more immediate goal, a study of its ultimate fate naturally follows. Here we come to one of the most difficult sections of our subject. Whether it be utilised for the formation of new tissue, or combusted to yield heat or some other form of energy, the accepted view is that the food absorbed is at some stage incorporated into the protoplasmic mass, a process in which enzymes

again play a significant part. One of the most fruitful methods whereby these various processes of metabolism have been studied in the past has been the examination of the different end-products to which they give rise and which are finally eliminated from the body by its excretory organs. In this way valuable information has been gained regarding the body's metabolic activities under different conditions, as, for example, on diets containing varying amounts of carbohydrate, of fat, and of protein; during the performance of light and heavy work; and especially in those abnormal conditions in which the body is diseased. A thorough study of such end-products, both from a qualitative and a quantitative standpoint, therefore forms an important part of the student's work.

In this very brief outline of the ground covered in the study of chemical physiology there are naturally many gaps. It has been given, however, chiefly for the purpose of emphasising to the student at this early stage the importance of not regarding the subject as one of so many watertight compartments. It must be borne in mind that the functioning of one organ of the body is dependent upon the functioning of other organs. To give but one example. The output of urine by the kidney depends to a large extent on the very variable amount of water lost by the body through the skin.

Even if it were possible to draw up a series of exercises illustrating the many aspects of chemical physiology outlined above, such a scheme would occupy much more time than the average student is able to give to the study of the subject. In this book a number of experiments has been selected to demonstrate certain of its essential features. These are set out at the beginning of each chapter, their relation to that part of the subject under discussion being explained in the theoretical section which follows. One final word. Some of these exercises are quantitative in character. In this connection it should be realised by the student that whilst he cannot hope to become an expert analyst in the short time he can devote to the subject, he should at least endeavour to understand the main principles underlying the various methods described.

CHAPTER I

THE CARBOHYDRATES

Glucose, fructose, lactose, maltose, sucrose, dextrin, starch, and glycogen are provided.

GENERAL TESTS FOR CARBOHYDRATES

I. Heat a small quantity of the carbohydrate on a piece of porcelain. Note the odour of caramel. Continue heating and observe charring. On strong heating the carbon disappears gradually.

II. Dissolve the carbohydrate in a few c.c. of water (by aid of heat if necessary). Cool. On this solution carry out Molisch's reaction thus :—

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

This reaction is given by all carbohydrates and by any substance containing a carbohydrate radical, as, *e.g.*, **some proteins** **(see p. 44)****.**

SPECIFIC TESTS FOR INDIVIDUAL CARBOHYDRATES

Perform the following tests with aqueous solutions of the different carbohydrates mentioned above. Note how each carbohydrate reacts to each test.

1. GLUCOSE.—(a) *Trommer's Test.*—**Run 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 glucose 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). It may be used as a quantitative 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. Uric acid and creatinine also reduce Fehling's solution, and the sodium hydroxide has a destructive action on sugar. These two disadvantages are absent in:—

(c) *Benedict's Test.*—In this test sodium carbonate replaces soda, and sodium citrate is substituted for Rochelle salt. Add a few drops of glucose solution to 5 c.c. of Benedict's (qualitative) reagent, and boil vigorously for a few minutes. The solution becomes filled with a fine precipitate, red, yellow, or green in colour depending on the concentration of the glucose solution.

(d) *Nylander's Test.*—Mix 5 c.c. of glucose solution with 1 c.c. of Nylander's reagent (20 g. of bismuth subnitrate and 50 g. 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. The reaction probably is:—

$$\text{(a) } Bi(OH)_2NO_3 + KOH \longrightarrow Bi(OH)_3 + KNO_3.$$
$$\text{(b) } 2Bi(OH)_3 - 3O \longrightarrow Bi_2 + 3H_2O.$$

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

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

(f) *Fermentation Test.*—Add a fragment of dried yeast to the glucose solution in a test-tube or fermentation tube. If a test-tube be used, fill it with the solution and invert over water or mercury. Maintain at body temperature (circa 37° C.) for twenty-four hours. Alcohol and carbon dioxide are produced from the glucose, the gas collecting at the top of the tube. Their presence may be proved by (a) absorption in soda (CO_2) and by (b) formation of iodoform ($C_2H_5.OH$).

(g) *Picric Acid Test.*—To 5 c.c. of glucose solution add 2 or 3 c.c. of picric acid and make alkaline with 10 per cent. sodium carbonate;

warm. Note the production of the red colour due to the formation of picramic acid, thus :—

$$C_6H_2OH(NO_2)_3 \rightarrow C_6H_2(OH).NH_2(NO_2)_2.$$

This reaction forms the basis of Benedict's colorimetric method for estimating glucose.

(h) *Reaction with Phenyl-hydrazine.*—To half a test-tube (5 c.c.) of glucose solution add 1 dg. of phenyl-hydrazine hydrochloride and twice as much sodium acetate. Effect solution by warming, and place in a water bath at 100° C. for thirty to sixty minutes. On cooling, if not before, a yellow crystalline precipitate is formed which can be filtered off and examined microscopically. For the chemical reactions involved see p. 17.

IMPORTANT.—None of these tests by itself is specific for glucose. *The only specific test for this sugar is the phenyl-hydrazine test in the absence of a positive test for fructose.*

2. **FRUCTOSE.**—Repeat tests (a) to (h) and note that all are positive. Carry out also the following tests :—

(a) *Seliwanoff's Reaction.*—To some fructose solution add one-quarter of its volume of strong hydrochloric acid and a trace of resorcinol, and boil. Observe the highly coloured solution and the formation of a red precipitate which is soluble in alcohol.

(b) *Specific Test for Fructose.*—Add a few drops of dilute hydrochloric acid and a few milligrams of selenium dioxide to a solution of fructose. Boil the mixture and note the rapid precipitation of red selenium.

NOTE.—Glucose, lactose, and maltose do not react in this way. Sucrose reacts only after prolonged boiling causing its hydrolysis to glucose and fructose.

3. **SUCROSE OR CANE SUGAR.**—(a) When mixed with copper sulphate and caustic potash a clear blue solution is produced. On boiling no reduction takes place.

(b) Boil a few c.c. of cane-sugar solution with a drop or two of 25 per cent. sulphuric acid. Cool and *neutralise* with potash or soda. Examine the behaviour of the resulting solution now with Trommer's or Fehling's tests. The copper is reduced owing to the formation of glucose and fructose.

(c) Boil some of the cane-sugar solution with an equal volume of concentrated hydrochloric acid. A red colour is produced by the action of the acid on the fructose formed by hydrolysis. Glucose,

lactose, and maltose do not give this test, though fructose and inulin (which gives fructose on hydrolysis) do.

4. LACTOSE.—Repeat tests 1, (a) to (h). Prepare the phenyl-osazone and compare with that from glucose and fructose.

5. MALTOSE.—Repeat tests 1, (a) to (h). Prepare the phenyl-osazones and compare with those from glucose, fructose and lactose.

6. STARCH.—(a) Note its insolubility in cold water. Mix a little starch with cold water by rubbing it in a mortar to a smooth paste. Pour this paste into boiling water slowly. Keep the solution boiling till an opalescent solution is formed. If too strong it will gelatinise on cooling, and should be diluted with water.

Use this solution for the following tests :—

(b) Treat with Fehling's solution as described above. Probably no reduction will occur. Pure starch is non-reducing.

(c) Add to cold starch solution a drop or two of *very dilute* iodine solution. An intense blue colour is produced, which disappears on heating or on making alkaline, and reappears on cooling or making neutral.

N.B.—Prolonged heating drives off the iodine, and consequently no colour returns on cooling. It may be restored by adding more iodine.

(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.

7. DEXTRIN.—(a) Add iodine solution to a solution of dextrin : a reddish-brown colour is produced. The colour disappears on heating and reappears on cooling. Many commercial dextrins give at first a blue colour which changes through a purple-red to a red-brown on the addition of more iodine.

(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) Commercial dextrin usually gives a slight reduction with Fehling's solution owing to the presence of reducing sugar as an impurity.

8. GLYCOGEN.—(a) A solution of glycogen is opalescent like that of starch (for preparation of glycogen see p. 243).

(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 7 (b), and filter. The glycogen is *completely* precipitated, and the filtrate therefore gives no coloration with iodine. This easily distinguishes it from dextrin.

9. PENTOSES.—The reaction of these may be carried out with gum arabic (which contains arabinose) or with pine-wood shavings (which contain xylose).

Repeat tests 1, (a) to (h) above, and note that all are positive. Prepare the phenyl-osazones as already described for glucose.

The two following tests are characteristic.

(a) *Phloroglucinol Reaction.*—Warm some distilled water with an equal volume of concentrated hydrochloric acid in a test-tube and add phloroglucinol until a little remains undissolved. Add a small quantity of gum arabic, and keep it in the water-bath at 100° C. The solution becomes cherry-red, and a dark coloured precipitate settles out, which gives, when dissolved in amyl alcohol, an absorption band between the D and E lines, *cf.* p. 206. This reaction forms the basis of one method of estimating pentose sugars in which the aqueous sugar is distilled from an acid solution into an acid solution of phloroglucinol. The precipitate is collected and weighed.

(b) *Orcinol Reaction.*—On substituting orcinol for phloroglucinol 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. OXIDATION OF SUGARS BY NITRIC ACID.—Formation of saccharic and mucic acids. Take 1 gram of lactose or galactose and heat it in a porcelain basin with 12 c.c. of nitric acid on a water-bath in the fume chamber 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, glucose, 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. As a confirmatory test for mucic acid, dissolve the precipitate in ammonium hydroxide, filter if necessary, and evaporate to dryness on the water-bath. Dry distillation of the residue yields pyrrol, which may be detected by the red colour produced when a pine shaving (match), moistened with hydrochloric acid, is held at the mouth of the test-tube.

Saccharic acid may be isolated readily as its acid potassium salt. This is relatively insoluble and crystallises without difficulty.

It will be seen that glucose and all sugars containing glucose yield *saccharic acid* on oxidation, whereas those containing galactose yield *mucic acid*. Lactose obviously yields both.

11. ESTIMATION OF CARBOHYDRATES.—A. By reduction of copper salts—Benedict's Method.

BENEDICT'S METHOD.—Benedict's quantitative reagent[1] 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 grams, sodium carbonate (crystalline), 200 grams (or anhydrous sodium carbonate, 75 grams), and potassium thiocyanate, 125 grams, are dissolved in hot water; this, when cool, is made up with distilled water to 800 c.c. and filtered.

18 grams 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 gram of glucose.

Analysis.—3 or 4 grams of anhydrous sodium carbonate are placed in a 300 c.c. 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 reading on the burette is 10 c.c., then this amount of urine contains 0·05 gram of glucose, therefore 100 c.c. contains 0·5 gram. *Should it be found that the first titration gives a result indicating that the percentage is greater than 1 per cent., the urine should be diluted quantitatively so as to bring the concentration between 0·5 and 1·0 per cent.* A further

[1] Benedict's qualitative reagent (p. 6) contains sodium citrate, 175 grams; anhydrous sodium carbonate, 100 grams in about 150 c.c. of water; to this are added 17·3 grams of $CuSO_4$ in 100 c.c. of water, the whole being then made up to 1 litre.

example will make the calculation clear. 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 gram of glucose.
1 c.c. „ „ contains $\frac{0{\cdot}05}{2}$ „
and 100 c.c. „ „ contains $\frac{0{\cdot}05 \times 100}{2}$ „
2·5 grams glucose per cent.

ESTIMATION OF OTHER REDUCING SUGARS IN SOLUTION.—The same method may be used for the estimation of other reducing sugars, the only difference being in the final calculation :—

25 c.c. of Benedict's solution = 0·05 gram of glucose.
= 0·053 „ fructose.
= 0·0676 „ lactose.
= 0·074 „ maltose.

ESTIMATION OF SUCROSE.—Boil 40 c.c. of the sucrose solution with 20 c.c. of half-normal hydrochloric acid for 5 minutes. Cool, neutralise with 20 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 25 c.c. of Benedict's solution = 0·0475 gram sucrose, according to the equation

$$\underset{342}{C_{12}H_{22}O_{11}} + \underset{18}{H_2O} \rightarrow \underbrace{C_6H_{12}O_6 + C_6H_{12}O_6}_{360}$$

that is 0·047 gram sucrose = 0·05 gram glucose.

ESTIMATION OF POLYSACCHARIDES.—All polysaccharides can be estimated by determining the reducing value of known amounts of the polysaccharide after hydrolysis by acid or enzyme, according to the equation

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

Almost always up to 5 per cent. of the glucose formed is lost during the hydrolysis. This should be allowed for and approximate results are given by multiplying the percentage of glucose obtained by the factor 0·927.

B. By use of the polarimeter, **see p. 27.**

As an example of observation of mutarotation and of estimation of a sugar polarimetrically, the following experiment can be carried out.

Prepare a clean, dry polarimeter tube 2 decimetres long, and a dry 50 c.c. graduated flask. Into the latter weigh accurately about 4 to 5 grams of crystalline glucose. Dissolve this as rapidly as possible in water, make up to the mark, and filter into the polarimeter tube. Examine at once with sodium light, and record the observed rotation each half minute. Note how this falls. Remove one glass end-plate and add one drop of strong ammonia, replace cover, invert tube to mix, and examine again. Observe the rotation is now constant. Take six or eight independent readings. Assuming the specific rotation to be $+52{\cdot}5°$, calculate the concentration of the glucose according to the formula

$$[\alpha]_D = \pm \frac{100 \times \alpha}{c \times l}$$

where $[\alpha]_D$ is the specific rotation in sodium light.

α is the observed rotation.

c is the concentration.

l is the length of the tube in decimetres.

Compare the concentration thus found with the amount actually weighed out.

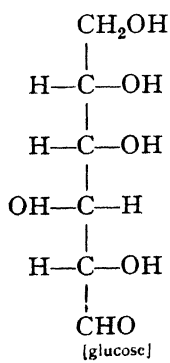
Carbohydrates are found chiefly in vegetable tissues, and many of them form important foods. Some carbohydrates are, however, found in or formed by the animal organism. The most important of these are *glycogen*, or animal starch; *glucose*; and *lactose*, or milk sugar.

The carbohydrates may be conveniently regarded 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 inositol, which are not carbohydrates. Chemically the carbohydrates are primary oxidation products of polyhydric alcohols. 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.

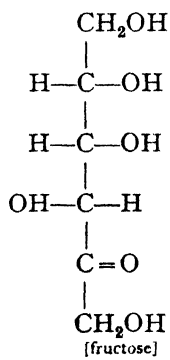
The terms "aldehyde" and "ketone" will be familiar to the student who will remember that the simple aldehydes and ketones are derived from the monohydric alcohols by oxidation.

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 they are reducing agents.

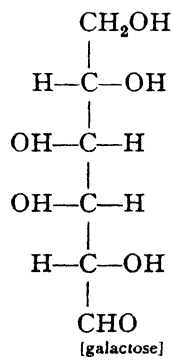
Three hexahydric alcohols, all with the same empirical formula, $C_6H_8(OH)_6$, may be mentioned; they are isomerides, and their names are sorbitol, mannitol, and dulcitol. By careful oxidation the corresponding aldehydes and ketones can be obtained; these are the simple sugars; thus, glucose is the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



[glucose]



[fructose]



[galactose]

They furnish an excellent example of stereochemical isomerism; in this type of isomerism the position of the atoms or groups of atoms in space varies within the molecule. For the present we may regard the constitutional formulæ of three important simple sugars to be as shown on p. 13. 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; subsequently alternative formulæ will be given.

The aldehydic structure of glucose and of galactose is evident, the typical aldehyde group $-C\begin{matrix}O\\H\end{matrix}$ being at the end of the chain. The ketonic structure of fructose is also evident, the typical ketone group $>C=O$ being, of necessity, not terminal.

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 is equal. This number in the case of the sugars already mentioned is six. Hence they are called *hexoses*.

Sugars are also known in which this number is 2, 3, 4, 5, 7, etc., and these are called bioses, trioses, tetroses, pentoses, heptoses, etc. The majority of these are of no physiological interest. It should, however, be mentioned that a pentose has been obtained from certain nucleic acids presently to be described (see p. 72), which are contained in animal organs (pancreas, liver, etc.), and in plants (for instance, prunes, cherries, and 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—the *mono-saccharides*.

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:—

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

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*,

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

The polysaccharides are starch, glycogen, various dextrins, cellu-

lose, 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.	+ Sucrose.	+ Starch.
− Fructose.	+ Lactose.	+ Glycogen.
+ Galactose.	+ Maltose.	+ 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 usually 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 (70-100 mg. per 100 c.c. of blood) and numerous tissues, organs, and fluids of the body, including normal urine. It is the form of sugar found in larger quantities in the blood and urine in the disease known as diabetes mellitus.

Glucose is soluble in hot and cold water and in weak alcohol. It is crystalline, but not so sweet as cane sugar. When heated with alkalis certain complex acids are formed which have a yellow or brown colour. This constitutes *Moore's test* for sugar. In alkaline solutions glucose readily reduces salts of silver, bismuth, mercury, and copper. The reduction of cupric hydrate to cuprous hydrate or oxide constitutes *Trommer's*, *Fehling's*, and *Benedict's tests*, which have been already described. On boiling glucose with an alkaline solution of picric acid a dark red opaque solution is formed due to the reduction of the picric to picramic acid. Another important property of glucose is that under the influence of yeast it is converted into alcohol and carbon dioxide. At the same time many other substances (by-products) are formed, *e.g.*, glycerol. The equation usually given to represent the course of this reaction, viz., $C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2$, is therefore inaccurate in so far as it does not account for all the products of fermentation.

Glucose may be estimated by the quantitative reduction of some alkaline copper solution, usually Benedict's or Fehling's, by the polarimeter, and by measuring the volume of carbon dioxide evolved

[1] For a description of polarised light and polarimeters see p. 25.

after incubation with yeast. Of these three methods the two first are the favourite ones. The specific rotation of glucose ($[\alpha]_D$) is $+52{\cdot}5°$.

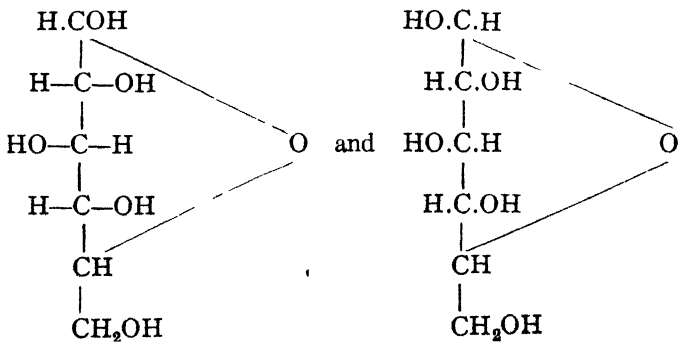
Fructose.—This sugar is also known as lævulose on account of its lævo-rotatory action on polarised light. It is crystallised with greater difficulty than glucose. It gives many of the reactions of glucose. The selenium test mentioned above (p. 7) is, however, specific for fructose, and this, with its optical rotation ($[\alpha]_D = -92°$), serves to distinguish it from all the other reducing sugars liable to be encountered in the body. Chemically fructose behaves as a ketone.

Small quantities have occasionally been reported as occurring in blood, urine, and in muscle.

Galactose is formed by hydrolysis of lactose by acids or by certain specific enzymes whereby one molecule of glucose and one of galactose are produced. It resembles glucose in its general reactions. Its specific rotation ($[\alpha]_D$) is $+80°$.

One distinguishing test is the formation of mucic acid when galactose (or lactose) is oxidised strongly with nitric acid. Mucic acid is a dibasic acid and is sparingly soluble in water. (Glucose on oxidation in a similar manner gives the freely soluble saccharic acid, isomeric with mucic acid.)

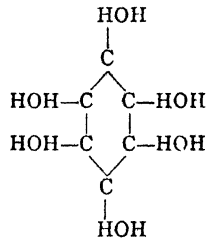
The student should realise that the chemical reactivity of glucose, fructose, and galactose is not as great in neutral solution as the formulæ on p. 13 would suggest. In alkaline or acid solution, however, their activity is greatly increased. This has led to the conception that these monosaccharides exist in aqueous solution in great part as ringed structures, the constitutional formula of glucose being represented by



Further information on this matter is given on p. 23, under glucosides.

Inositol or Inosite was discovered by Scherer in 1850 as a constituent of muscle, and on account of its empirical formula $C_6H_{12}O_6$

was long known as " muscle sugar." It occurs also in other organs of the animal body, and is a fairly constant constituent of roots and leaves of certain plants. In reaction it is neutral, is only faintly sweet, does not reduce Fehling's solution, and gives none of the reactions of the simple sugars. Maquenne ascertained that it has the following formula :—

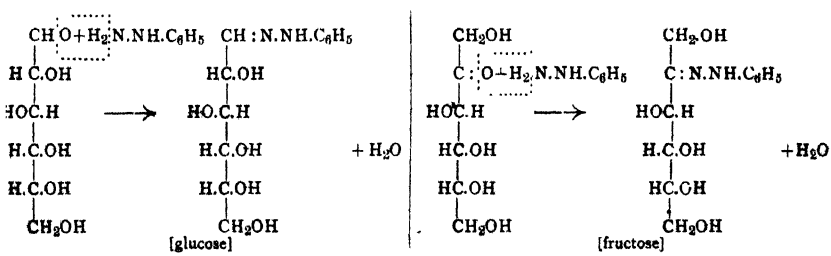


A mere glance at this formula will show that its constitution is very different from those of the sugars given on p. 13. The six carbon atoms, instead of forming an open chain, are linked into a ring, as in the benzene derivatives. It is reduced hexa-hydroxy-hexa-hydro-benzene. It may represent a transition 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 inositol ring would give rise to an open chain, and it has in fact been found that lactic and other aliphatic acids are formed from inositol by the action of certain bacteria. Optically active forms of inositol are known.

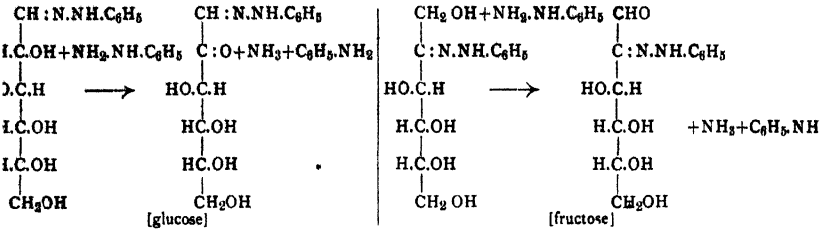
The Use of Phenyl-hydrazine in the Identification of Sugars.— Phenyl-hydrazine and other substituted hydrazines react with *reducing* sugars, both monosaccharides and disaccharides, with the production of typical crystalline coloured substances termed osazones.

The chemical reactions involved are given below for both glucose and fructose. There are three stages in the reaction :—

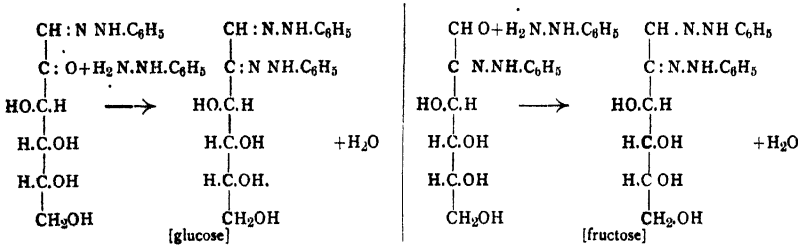
(*a*) *Condensation of the sugar and phenyl-hydrazine.*



(b) Oxidation of the product formed (the hydrazone), with corresponding reduction of the phenyl-hydrazine to aniline and ammonia.



(c) Condensation of the above, with a third molecule of phenyl-hydrazine with the formation of the osazone.



It will be seen that the products in the case of glucose and of fructose are identical; of the other sugars, *e.g.*, maltose, lactose, and galactose, and also of the pentoses, the phenyl-osazones are characteristic. Some of these are given in the coloured plate opposite. **Sucrose does not give an osazone.**

Glucose gives a precipitate of phenyl-glucosazone which crystallises in yellow needles (melting-point 205° C.). (Fig. 1, A.)

Fructose yields an osazone identical with this.

Galactose yields a very similar osazone (phenyl-galactosazone). It differs from phenyl-glucosazone by melting at 190° to 193°, and in being optically inactive when dissolved in glacial acetic acid. A characteristic derivative of galactose is the methyl-phenyl-hydrazone (melting at 180°) which can readily be obtained from the asymmetrical methyl-phenyl-hydrazine. This derivative is usually employed to identify this sugar.

Sucrose, as stated, does not form a compound with phenyl-hydrazine.

Lactose yields phenyl-lactosazone. It crystallises in needles, usually in clusters (melting-point 200° C.). It is soluble in 80 to 90 parts of boiling water. Lactose in urine does not give this test readily. (Fig. 1, C.)

Maltose yields phenyl-maltosazone. It crystallises in much broader

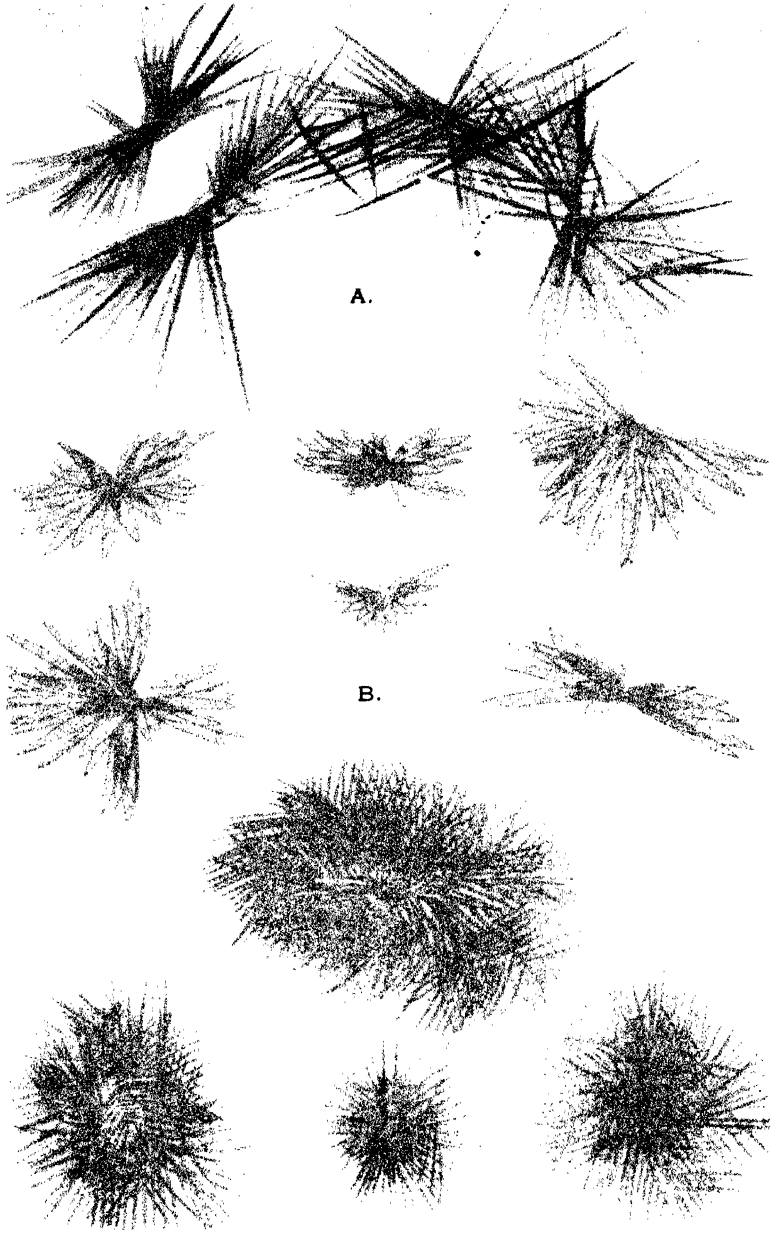


Fig. 1.—Plate of osazone crystals highly magnified.

A, phenyl-glucosazone. *B, phenyl-maltosazone.* *C, phenyl-lactosazone.*

yellow needles 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. (Fig. 1, B.)

To determine the *melting-point* of the osazones (or other organic substances) place a small quantity of the powder in a thin-walled tube attached to a thermometer by capillary attraction. Place this in a bath of glycerol which is gradually heated, and note the temperature at which the crystals melt.

The melting-point is not very reliable in the case of the osazones unless great care is taken in heating.

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 sucrose traces may appear in the urine, but the greater part undergoes hydrolysis or inversion in the alimentary canal.

Pure sucrose is crystalline and dextro-rotatory, ($[\alpha]_D = +67°$). It holds cupric hydrate in solution in an alkaline liquid, giving a blue solution as in Trommer's test. (This is a property of all polyhydroxy compounds, *e.g.*, glycerol.) No reduction occurs on boiling, since in the union of the glucose and fructose molecules the reducing groups of both sugars are rendered inactive. After hydrolysis the solution is strongly reducing.

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

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

With yeast, sucrose is first inverted by means of a special enzyme (*invertase*) produced by the yeast cells, and then follows the 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. 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; its aqueous solution is 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.

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

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

With yeast fermentation takes place only after inversion. This, however, occurs only 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) $$\underset{\text{[lactose]}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow 4\underset{\text{[lactic acid]}}{C_3H_6O_3}$$

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

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 acids 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 the 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 only two-thirds 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 *maltase* of the intestinal juice, it is converted into glucose,

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

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 above (p. 8).

POLYSACCHARIDES

Starch is widely diffused throughout 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 nutritive value in man, but starch is a most important food.

FIG. 2.—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, owing to the cell-walls being ruptured, which if concentrated gelatinises on cooling. This gelatinous mass is called **starch paste.** If very finely ground first, no starch paste is formed owing to the increased solubility of the fine particles of starch. Its most characteristic reaction is the blue colour it gives with iodine in the cold and in neutral or faintly acid solution. On rendering the solution alkaline or on warming, the blue colour disappears to reappear on neutralising the solution or on cooling. As a rule the colour which reappears is not so intense as the original.

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, the complex between the soluble starch and iodine behaving towards heat and alkalinity in the same way as the product from starch.

The molecular weight of starch is unknown with precision. The molecules of the dextrins are smaller. Equations which represent the formation of sugars and dextrins from starch 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.

The dextrins are soluble in water, but insoluble in alcohol and ether. They are amorphous yellowish powders, and are dextrorotatory. On hydrolysis they are converted into glucose.

The action of dextrins on alkaline copper salts is disputed. Possibly the lower dextrins reduce.

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 is glycogen. It is, however, precipitated by basic lead acetate and ammonia. (3) Glycogen is precipitated by 55 per cent. 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. On hydrolysis the products are the same as those given by starch, viz., maltose and glucose.

The isolation and preparation of glycogen from and the estimation of glycogen in tissues are described on p. 242.

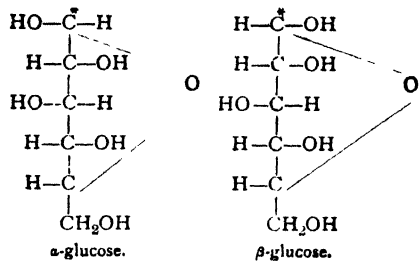
Cellulose.—This is the colourless material of which the cell-walls and woody fibres of plants are composed. By treatment with strong mineral acids, it is, like starch, but with much greater difficulty, converted into glucose. The various enzymes of the intestine have little or no action on cellulose. Boiling ruptures the cellulose envelopes of the starch grains, and so allows the digestive juices to reach the starch proper; hence the necessity of boiling starch before it is taken as food. Certain bacteria of the intestinal flora are capable of effecting small amounts of hydrolysis. 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 our study of the proteins that this method, known as "salting out," is one largely employed there for precipitating and distinguishing different classes of proteins. THE STUDENT IS THEREFORE WARNED

THAT A PRECIPITATE OBTAINED UNDER SUCH CONDITIONS DOES NOT NECESSARILY INDICATE THE PRESENCE OF PROTEIN.

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.

It is difficult to account for many of the properties of the hexoses and for this *mutarotation* if the formulæ of glucose, fructose, and galactose given on p. 13 are accepted. An explanation is more readily obtained if one assumes that two isomeric forms of each of the hexoses exist in aqueous solution; these two modifications are termed respectively α and β. It will be seen that the aldehyde group is potentially functional. This group is marked * in the two glucoses formed below.



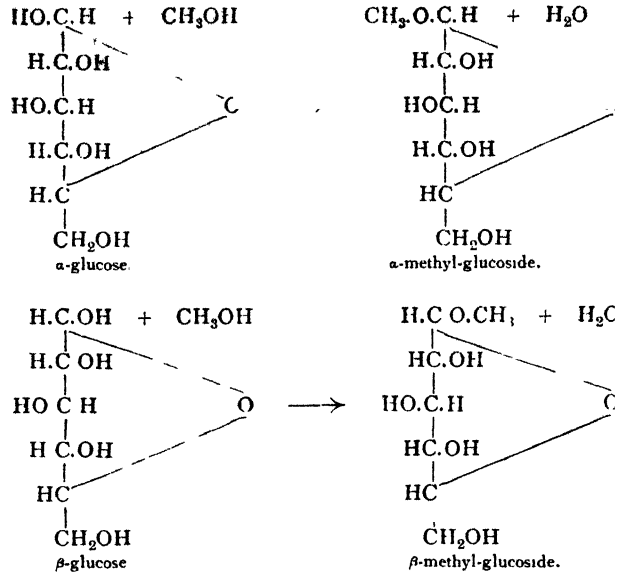
α-glucose. β-glucose.

The α form of glucose has a specific rotation $+110°$, the β form $+19°$. Each may exist separately as crystalline modifications, but on solution in water a tautomeric change occurs with the partial conversion of one into the other until a mixture is obtained whose permanent $[\alpha]_D$ is $+52{\cdot}5°$. Tautomerism of this nature is by no means uncommon in organic compounds.

Glucosides.—If an aqueous solution of glucose be treated with methyl sulphate in the presence of caustic soda, or if glucose be treated with methyl alcohol in the presence of anhydrous hydrogen chloride, there is formed a crystalline product which can be separated into two distinct compounds each of which is the condensation product of one molecular proportion of glucose and one of methyl alcohol.

In one case one molecule of α-glucose, in the other one molecule of β-glucose, has condensed with the alcohol. These two products are examples of glucosides and are the two methyl glucosides, viz.,

α-methyl glucoside and β-methyl glucoside. The condensations may be represented thus :—



It will be seen that α-glucose gives rise to an α-glucoside and β-glucose to a β-glucoside. Also it will be realised that **hydrolysis** of an α-glucoside will form α-glucose and **hydrolysis** of a β-glucoside β-glucose. As condensation is effected at the potential reducing group glucosides will be NON-REDUCING unless the molecule other than the glucose fraction contains a free reducing group.

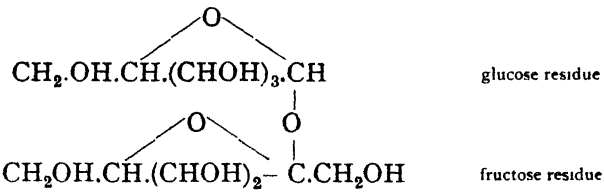
These two glucosides are the simplest known and are cited as types of numerous naturally occurring compounds in which alcohols, phenols, acids, and even other sugars are condensed not only with glucose to give glucosides but with fructose to give fructosides, with galactose to give galactosides and with maltose and lactose to give maltosides and lactosides respectively. Thus amygdalin in bitter almonds is a compound of glucose with mandelic nitrile (benzaldehyde cyanhydrin); 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.

As examples of glucoside formation between two molecules of sugars we may recall that maltose can be hydrolysed by acid or by an enzyme (maltase) into two molecules of glucose. Maltose is therefore the condensation product of two molecules of glucose and is a glucoside.

It is, in fact, **glucose α-glucoside.** As condensation between the two hexoses takes place at the reducing group (terminal carbon atom) of the one glucose molecule but not at the reducing group of the other, maltose still possesses one reducing group and is a REDUCING sugar.

Lactose on hydrolysis yields glucose and galactose, and is probably formed from these in the mammary gland, to be secreted in the milk. Lactose is **glucose β-galactoside** and, like maltose, has one aldehyde group free and is a reducing sugar.

Sucrose, on the other hand, while yielding both glucose and fructose on hydrolysis, is NON-REDUCING. Condensation must therefore have taken place in such a way that neither the aldehyde group in the glucose part nor the ketone group in the fructose moiety is free and functional in the disaccharide. The following possibly represents the structure of sucrose :—



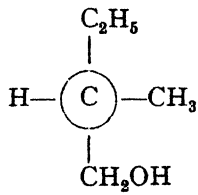
It will be seen that the fructose residue of the sucrose molecule is represented as having a five-membered oxygen-containing ring. Derivatives of these modifications are known in the case of the three hexoses mentioned and are characterised by their extraordinary chemical reactivity. They may play a part in metabolism which is far from unimportant, though any such suggestion is little more than speculation.

As all the naturally occurring carbohydrates, the majority of proteins and amino acids and many other substances found in or formed by the body are optically active, and as advantage is taken of this to detect and estimate these substances it is essential to outline the

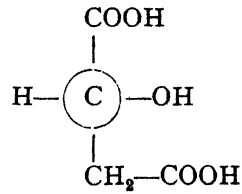
RELATION BETWEEN CIRCULAR POLARISATION AND CHEMICAL CONSTITUTION

The first work in this direction was performed by Pasteur, and it was his publications on this subject that first brought him into prominence. He found that racemic acid, which is optically inactive, can be separated into two isomerides, one of which is tartaric acid and is dextro-rotatory, while the other is also tartaric acid but which differs from the first variety in being lævo-rotatory.

Pasteur further showed that if the mould *Penicillium glaucum* is grown in a solution of racemic acid, dextro-tartaric acid 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 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 carbon atoms rotate the plane of polarised light? It was found that they do not. This was explained by Le Bel by supposing that these, like racemic acid, are compounds of two molecules—one dextro the other lævorotatory; that this was the case was demonstrated by growing moulds, the action of whose enzymes is to separate the two molecules in question. Then the other question—how it is 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. 3 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 spatially in the molecule of a stereoisomeric substance.

FIG. 3.

To detect and measure any rotation of polarised light by a substance, use is made of a polarimeter.

The following is a brief description of the polarimeter 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. As the amount of rotation varies for different colours, the 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 containing the solution, and an analyser. The polarised light before passing into the solution traverses a quartz plate,

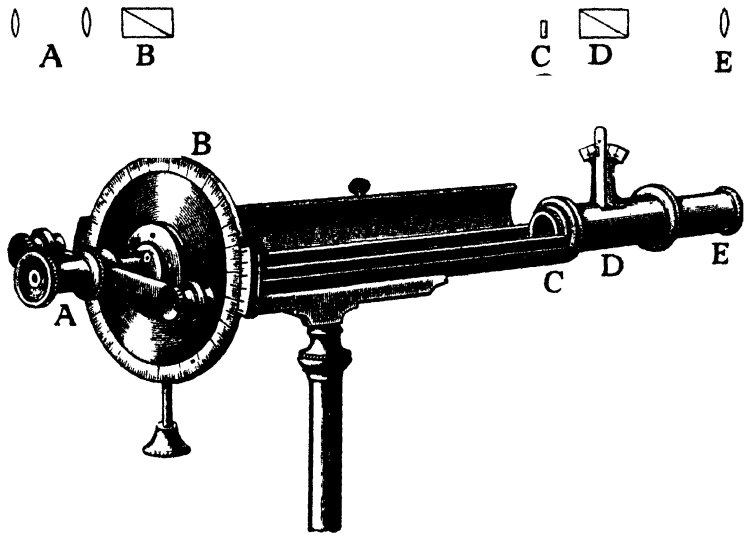


FIG. 4.

which, however, covers only half the field, and retards the rays passing through it by half a wave-length. In the zero position the two halves of the field appear equally illuminated [1]: 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.

The **specific rotatory power** of any substance is the amount of

[1] In the most delicate instruments the field is divided into three parts or into two concentric discs.

rotation in degrees of a circle of the plane of polarised light produced by 1 gram of the substance dissolved in 1 c.c. of liquid examined in a column 1 decimetre long.

If α = rotation observed,
w = weight in grams of the substance per cubic centimetre,
l = length of tube in decimetres,
$[\alpha]_D^t$ = specific rotation for light with wave-length corresponding to the D line (sodium flame) at temperature t.

Then $[\alpha]_D^t = \pm \frac{\alpha}{wl}$.

In this formula + indicates that the substance is dextro-rotatory, − that it is lævo-rotatory.

If, on the other hand, $[\alpha]_D^t$ is known, and we wish to find the value of w, then

$$w = \frac{\alpha}{[\alpha]_D^t \times l}, \text{ and the percentage amount is } p = \frac{\alpha \times 100}{[\alpha]_D^t \times l}.$$

In the practical application of this method there are one or two points to be noted. One of these is the phenomenon of mutarotation, the other is the temperature at which the observation is made, as this affects in some cases, *e.g.*, fructose, the amount of rotation to a very great extent. With glucose, however, this is not a factor of very great moment, provided it be not far removed from room temperature.

Regarding mutarotation, it may be taken that all the common sugars, except sucrose, exhibit this phenomenon, as in solution such sugars exist as an equilibrated mixture of the two stereoisomers, the α and β modifications (*cf.* p. 23).

The speed of mutarotation varies with the purity of the reagents, and is instantaneously completed in the presence of a trace of alkali.

The application of polarimetry to the estimation of reducing sugars in urine is given later (p. 279).

CHAPTER II

THE FATS AND LIPOIDS

Suet, lard, olive oil, glycerol, and cholesterol are provided.

1. Heat a small portion of suet or lard on a piece of porcelain. Note that it melts and that on stronger heating it burns, chars, and evolves acrid fumes.

2. Test the solubility of each in water and in organic solvents such as ether and alcohol. Drop some of this solution on to paper and observe the greasy stain left.

3. Test the reaction to litmus of alcoholic solutions of fats (a) when fresh, (b) when rancid.

4. SAPONIFICATION BY ALKALI.—By boiling with alkali, fat yields a solution of soap. On adding some sulphuric acid to this the fatty acid separates 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 or soda in alcohol [1] contained in a small flask and heated carefully *on a water bath* nearly to boiling-point. Continue to boil until saponification is completed as seen by dropping some of the solution into a test-tube containing a few c.c. of water ; the solution of soap will be clear ; no oil globules should separate out. If there is any separation of oil globules, continue boiling.

Drop the cooled soap solution into some cold 25 per cent. sulphuric acid contained in a small beaker ; the fatty acids separate out and float on the surface as a cake.

5. REACTION OF FATTY ACIDS.—Wash the fatty acid obtained in experiment 4 repeatedly with water, until the wash water no longer gives the reaction for sulphates, 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

[1] 30 grams of potash or soda are dissolved in 20 c.c. of water, and 200 c.c. of 90 per cent. alcohol are added.

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 is evolved. Note with the third portion that it produces a greasy stain on paper.

6. REACTIONS OF SOAP.—A solution of soap may be prepared by heating a small amount of stearic acid with a few c.c. of water and adding very dilute 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 solid sodium chloride and shake ; the soap is salted out as it is insoluble in brine.

(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. Treat a chloroform solution of an unsaturated fat (olein) with a few (2 or 3) drops of iodine solution. Shake and note the disappearance of the colour owing to addition of the halogen at the double bond of the oleic acid radical of the fat.

9. 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 a drop or two of glycerol in a dry test-tube, add some 2 to 3 grams of solid acid potassium sulphate and heat gently. Acrolein is given off and is recognised by its characteristic unpleasant odour, and by the fact that, if in large amount, it blackens a piece of filter paper previously moistened with ammoniacal silver nitrate solution. Repeat the test on some lard.

10. ACTION ON COPPER HYDROXIDE.—Prepare some cupric hydroxide and add to it in aqueous suspension a little glycerol. Note the solution of the hydroxide in the cold, and no reduction on boiling *cf.* p. 19.

11. OXIDATION OF GLYCEROL.—Boil 2 to 3 c.c. of a 1 per cent. solution of glycerol in water with 3 drops of normal sodium hypochlorite or hypobromite for a minute. Cool, and add 1 c.c. of strong hydrochloric acid, and boil until the free chlorine is evolved. To the solution now add an equal volume of fuming hydrochloric acid and a trace of orcinol. On boiling once more the solution becomes green-blue

or violet in colour. The colour can be extracted with amyl alcohol. What products are formed from the glycerol?

12. **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.

13. Some properties and reactions of **CHOLESTEROL.**—(a) Dissolve a few crystals of cholesterol in boiling alcohol. Thoroughly cool the solution, when the cholesterol should commence to crystallise. Transfer the crystals to a microscope slide and examine under the microscope. Note the characteristic four-sided plates with the notched angle.

When the alcohol on the slide has evaporated, run on the crystals a drop of concentrated sulphuric acid and notice they turn red at the edges. Now add a drop of iodine solution. The crystals take on a violet colour which gives place to green, blue, and finally black.

(b) *Salkowski's Reaction.*—Dissolve a little cholesterol in a few c.c. of chloroform. To the solution add an equal quantity of strong sulphuric acid, shake, and then allow to stand. The upper layer (chloroform) becomes red, the lower (sulphuric acid) yellow with a green fluorescence.

(c) *Liebermann's Reaction.*—Dissolve a crystal of cholesterol in 2 c.c. of chloroform in a dry test-tube, add 2 to 3 drops of acetic anhydride and then concentrated sulphuric acid drop by drop. A red colour which becomes blue and finally bluish-green is obtained.

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 Chapter IV. The fat of adipose tissues certainly comes from ingested fat, but, as proved by the classic work of Lawes and Gilbert, it may also be synthesised within the organism of the animal from carbohydrate and protein. It should be noted that fat forms the best means available to the organism for storing energy.

The contents of the fat cells of adipose tissue are fluid during life, the normal temperature of the body (37° C., or 98·4° F.) being considerably above the melting-point (25° C.) of the mixture of the fats found there. These fats are chiefly three in number, and are called palmitin, stearin, and olein. They differ from one another in chemical composition and in certain physical properties, such as melting-point and solubilities. Olein solidifies at −5° C., palmitin at 45° C., and stearin at 53° to 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 choloroform, but insoluble in water.

Chemical Constitution of the Fats.—Fats are typical esters and as such are compounds formed by the condensation of a fatty acid and an alcohol with the elimination of a molecule of water. The fatty acids of the three fats above mentioned are respectively palmitic, stearic, and oleic acid, whilst the component alcohol is in all cases glycerol (glycerin). Of the acids mentioned, palmitic and stearic belong to the series of saturated fatty acids of which the first members are formic and acetic acids. Palmitic is the sixteenth member of the series, having the formula $C_{15}H_{31}COOH$ or $CH_3(CH_2)_{14}COOH$, whilst stearic is the eighteenth, its formula being $C_{17}H_{35}COOH$ or $CH_3(CH_2)_{16}COOH$.

Oleic acid, on the other hand, is a member not of the series of saturated fatty acids but of the unsaturated series, of which acrylic acid $CH_2=CHCOOH$ is the first. Oleic acid is the eighteenth member of the group having the formula $C_{17}H_{33}COOH$ or $CH_3(CH_2)_7CH:CH(CH_2)_7COOH$. It will be noticed that two of the carbon atoms in the chain are united by a double bond, 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 their colour reactions with osmic acid (p. 30) and Sudan III. (red coloration) and the absorption of iodine 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. Similarly the ethylene linkage and more highly unsaturated groupings can be saturated by the addition of hydrogen. This process is used commercially and is known as the " hardening " of fats. The fats palmitin and stearin containing no unsaturated fatty acids do not give, therefore, the osmic acid and Sudan III. reactions, nor do they take up iodine.

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

Acetic acid .	$CH_3CO\,OH$	or $CH_3CO.OH$
Palmitic acid .	$CH_3(CH_2)_{14}CO.OH$	or $C_{15}H_{31}CO.OH$
Stearic acid .	$CH_3(CH_2)_{16}CO.OH$	or $C_{17}H_{35}CO.OH$
Oleic acid .	$CH_3(CH_2)_7CH : CH(CH_2)_7CO\,OH$	or $C_{17}H_{33}CO.OH$

Each consists of a complex group united to the hydroxyl group. This 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

It will be noted that the naturally occurring fatty acids contain an *even* number of carbon atoms, a matter probably of much importance in the digestion and oxidation of these foods by the animal.

Glycerol (popularly known as glycerin) is a trihydric alcohol, $C_3H_5(OH)_3$, namely, trihydroxy propane. The hydrogen in the hydroxyl atoms is replaceable by other organic radicals. As an example take the radical of acetic acid called the acetyl group (CH_3CO-). The following formulæ represent the derivatives that can be obtained by replacing one, two, or all three hydroxyl hydrogen atoms in this way :—

$CH_2.OH$	$CH_2.OH$	$CH_2.OH$	$CH_2.O.OC.CH_3$
$\vert$	$\vert$	$\vert$	$\vert$
$CH.OH$	$CH.OH$	$CH.O.OC.CH_3$	$CH.O.OC.CH_3$
$\vert$	$\vert$	$\vert$	$\vert$
$CH_2.OH$	$CH_2.O.OC.CH_3$	$CH_2\,O.OC.CH_3$	$CH_2.O.OC.CH_3$
[glycerol]	[monacetin]	[diacetin]	[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 three hydroxyls are replaced by radicals of the fatty acid. This is shown in the following formulæ :—

Palmitin	$C_3H_5O_3(C_{15}H_{31}CO)_3$
Stearin	$C_3H_5O_3(C_{17}H_{35}CO)_3$
Olein	$C_3H_5O_3(C_{17}H_{33}CO)_3$

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

$$\begin{array}{l} CH_2O.R_{,} \\ | \\ CHO.R_{,,} \\ | \\ CH_2O.R_{,,,} \end{array}$$

where $R_{,}$, $R_{,,}$, and $R_{,,,}$ may be the same or different acid radicals; that is, a neutral fat may be composed of three different acids condensed with one molecule of glycerol.

It must not be assumed that palmitic, stearic, and oleic acids are the only acids which form naturally occurring glycerides and which are therefore the only ones of importance to the animal because of the reasons given above. For example, butyric acid is present as a glyceride in butter to the extent of 6 per cent.; *n*-caproic $CH_3(CH_2)_4COOH$ and *n*-caprylic acid $CH_3(CH_2)_6COOH$ as glycerides in butter, coco-nut oil and palm-nut oil; capric acid $CH_3(CH_2)_8COOH$ as a glyceride in the milk of cows and goats. Instances of other fatty acids will be mentioned later when we deal with the chemistry of lecithin and kephalin.

Decomposition Products of the Fats.—The fats may be decomposed into the substances out of which they are built.

Under the influence of superheated steam, boiling mineral acids and alkalis and other catalysts employed in commercial processes, a fat combines with water and splits into glycerol and fatty acid. In the body fat-splitting is accomplished by an organic catalyst or enzyme known as lipase. The following equation represents what occurs in a fat, taking tripalmitin as an example :—

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

When an alkali is used as the hydrolytic agent, the fatty acid liberated combines with the alkali to form a soap. This form of hydrolysis has therefore been called **saponification.** Suppose, for instance, that potassium hydroxide is used, we get—

$$\underset{\text{[palmitin—a fat]}}{C_3H_5(OOCC_{15}H_{31})_3} + 3KOH \rightarrow \underset{\text{[glycerol]}}{C_3H_5(OH)_3} + \underset{\text{[potassium palmitate—a soap]}}{3C_{15}H_{31}COOK}$$

It used to be considered that non-hydrolysed fats were not absorbed from the gut, and this idea formed the basis of the use of liquid paraffin therapeutically to render the fæces less hard. More recently it has

shown, however, that part of the liquid paraffin so administered to an animal can be recovered from its liver, showing that some has been absorbed from the intestine. In all probability a certain amount of non-hydrolysed fat is also absorbed directly into the blood.

Emulsification. — Another change that fats undergo is very different from that of saponification. It is a physical rather than 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 chapter.

Estimation of Fat.—This may be carried out by extracting the fat with some solvent such as ether, distilling off the ether and weighing the residue. In many cases, however, it is preferable to estimate the fatty acid constituents of the fat. For this purpose the following figures are obtained:—(*a*) The acid value, *i.e.*, the number of milligrams of potassium hydroxide required to neutralise the *free* acid in 1 gram of the fat; (*b*) the saponification value—the number of milligrams of potassium hydroxide required to saponify completely 1 gram of the fat; (*c*) the iodine value—the amount of iodine required to saturate the acids in 100 grams of the fat; (*d*) the amount of potassium hydroxide required to neutralise the volatile fatty acid in 5 grams of fat. Several other methods are in use, and the majority of the analyses have to be carried out under special and constant conditions.

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 which 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 (Chapter IX.).

They may be classified in the following way:—

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

(2) Those which are free from phosphorus but contain nitrogen. These when hydrolysed yield the reducing sugar called galactose, 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 is found in egg-yolk, but " cuorin " separated from heart-muscle has been shown to be a mixture and does not belong to this group.

(*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.

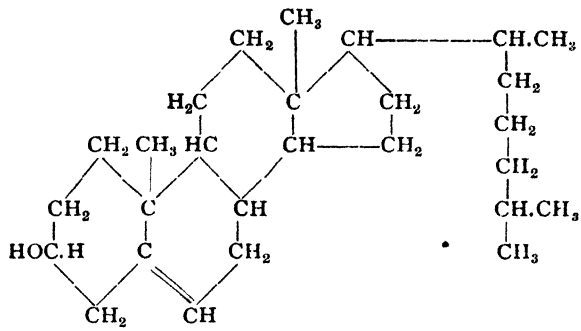
This classification is obviously capable of extension as new phosphatides are discovered.

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

Cholesterol is the best-known member of that increasingly important group of chemical substances called the sterols, of which other members are coprosterol, sitosterol, stigmasterol, and ergosterol. It is found in small quantities in all forms of protoplasm. It was first isolated from gall-stones by Conradi in 1775 and derives its name from the fact that it is a constituent of bile. It is present there, normally, in small quantities, but it may occur in that fluid in excess when it is liable to form the concretions known as gall-stones. In the tissues and in the blood it exists both free and combined with fatty acids as esters. It is abundant in brain, from which tissue it can readily be extracted by the use of cold acetone (*cf.* p. 243). In lanolin, the fat of sheep's wool, it is present as the oleate, palmitate, and stearate.

It is a monohydric unsaturated alcohol, and has the empirical formula $C_{27}H_{45}OH$. Recent research has shown it to belong to the

terpene series, and whilst its chemical structure is not yet definitely established, Windaus is of the opinion that it is



It was once thought to be merely a waste product of metabolism, but it is increasingly evident that such is not the case. It is, for instance, recognised that the four-ringed nucleus of cholesterol is also present in the structure of such substances as the bile acids, the male and female sex hormones, and that it forms an integral part of those compounds so far found to possess the highest carcinogenic activity.

It is believed that cholesterol exerts 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, *e.g.*, membranous bags containing hæmaglobin, 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.

Cholesterol is optically active, it is soluble in fat solvents, fatty acids, bile acids and bile, but not in water. It crystallises from alcohol as colourless, glistening, rhombic plates with a notched corner. Owing to the presence of an unsaturated double bond it can combine with iodine.

Coprosterol $C_{27}H_{47}OH$, a constituent of fæces, is formed by the reducing action of bacteria in the lower intestine.

Widely distributed in the fats of both plants and animals and

usually in association with cholesterol is another sterol, *ergosterol*, $C_{27}H_{41}OH$, first discovered by Tanret in ergot. This compound has recently become of greater importance since, on irradiation, it is transformed successively into a number of isomers, *e.g.*, lumisterol, tachysterol, vitamin D, the last of which compounds has been shown to be capable of curing or preventing rickets.

Closely resembling cholesterol are the **phytosterols** of plants. Of these *sitosterol*, $C_{29}H_{49}OH$, is the best-known example.

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 with hot acetone 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:—

(1) A reducing sugar, galactose.

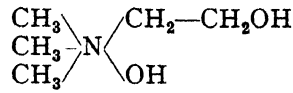
(2) A base termed sphingosine ($C_{17}H_{35}NO_2$), which is an unsaturated mono-amino-dihydroxy alcohol.

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

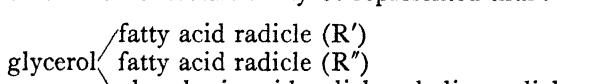
Kerasin also yields galactose and sphingosine, but its fatty acid is different; it is lignoceric acid, $C_{24}H_{48}O_2$.

The Phosphatides.—The best known of these is *lecithin*. This is a very labile substance, being easily oxidised on exposure to the

air and turning brown in the process. 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 the ammonium-like base, 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



Choline is thus hydroxyethyl-trimethyl-ammonium hydroxide. And the constitution of lecithin may be represented thus:—



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 (hydroxylethylamine). 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 sphærocrystals, which in suspension rotate the plane of polarised light to the left. Choline, sphingosine, and fatty acids have been found among its cleavage products. It, however, differs from lecithin by containing no glycerol.

The Luteins or Lipochromes.—The yellow or orange-red pigments which usually accompany fats in the animal organism, were called *luteins* by Thudichum, who was the first to recognise, by spectrum analysis, their identity with the yellow-flower pigments which are now called carotinoids. The two best-known representatives are *carotin* ($C_{40}H_{56}$), which is an unsaturated hydrocarbon, and *xanthophyll* ($C_{40}H_{56}O_2$), an oxide of carotin. Their isolation from animal tissues presents great difficulties, owing to the small amounts present (only 0·45 g. carotin was obtained from 10,000 cows' ovaries) and owing to their solubility in the usual solvents for fat, from which they cannot

be easily separated. They are, however, in distinction from fats, not affected by saponification. In carbon disulphide solution they possess typical absorption bands in the blue end of the spectrum. The lutein of the ovaries, of milk and butter, consists of carotin. The lutein of egg-yolk is isomeric with xanthophyll. Serum-lutein (cow) consists mainly of carotin, which seems to be present in a water-soluble combination with serum-albumin. These pigments cannot apparently be synthesised by the animal, but are taken up from the food, the colour of the egg, milk, or butter, for example, depending greatly upon the amount of the pigments available in the vegetable food given.

CHAPTER III

THE PROTEINS

Preliminary Tests (to be carried out on the albumin, caseinogen, gelatin, keratin provided).

1. (a) Heat a small portion of any one of the proteins provided on a piece of procelain. Note that it chars and gives off an odour characteristic of burning flesh or feathers.

(b) To show that protein contains NITROGEN :—

(i) 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 (i) by its odour ; (ii) by turning moistened red litmus paper blue ; (iii) by yielding white fumes when a glass rod dipped in hydrochloric acid is held over the mouth of the tube.

(ii) 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 this is, in a dry test-tube, first gently heated and finally heated more strongly for about half a minute to red heat. Dip the tube whilst still glowing into a mortar containing about 10 c.c. of distilled water ; the tube will break and its contents mix with the water. Grind up the mass with a pestle. Filter and label the filtrate A ; divide it into two parts and reserve one for the test for sulphur below. To the other 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. Should no precipitate be apparent, although the colour change

occurs, filter through a white filter paper when the precipitate will be seen as highly coloured particles on the paper. This test is due to the fact that some of the nitrogen is fixed as sodium cyanide, and this forms Prussian blue ($Fe_4(FeC_6N_6)_3$) with the reagents added.

(c) To show that protein contains **SULPHUR** and that this element may be loosely combined or firmly combined :—

(i) To the reserved portion of filtrate A (*cf.* 1 (b) above) add a freshly prepared solution of sodium nitroprusside ($Na_2Fe(CN)_5NO$). The formation of a reddish-violet colour indicates the presence of sulphur.

(ii) To prove the presence of loosely combined sulphur proceed thus :—Add 2 drops of a neutral lead acetate solution to a few c.c. of caustic soda. The precipitate of lead hydroxide which first forms soon dissolves. Heat a small portion of the albumin with this alkaline solution. The mixture turns black due to the formation of lead sulphide, part of the sulphur present in albumin being split off from it by the caustic soda as sodium sulphide (*cf.* p. 53).

2. Tests showing that other elements and groups may be present in certain protein molecules :—

(a) **PHOSPHORUS.**—To a little caseinogen in a test-tube add a mixture of sulphuric and nitric acids. Heat the mixture gently to destroy the organic matter (Neumann's method). When the solution is clear add half its volume of concentrated nitric acid and then ammonium molybdate in excess. Boil the mixture when a yellow crystalline precipitate of ammonium phospho-molybdate ($(NH_4)_3PO_4 + 10MoO_3$) is produced.

(b) **CARBOHYDRATE.** (See Colour Reactions, p. 5.)

General Tests.—The following tests are to be carried out on a mixture of one part of white of egg to ten of water. (The more important proteins present in egg-white are albumin, globulin, and ovomucoid.)

3. Reactions involving Precipitation of the Protein.—(a) *Heat Coagulation.*—Test the given solution of protein with litmus paper and note the reaction. Faintly acidify by adding a few drops of 2 per cent. acetic acid and boil. The protein is thus rendered insoluble (coagulated protein).

(b) *Precipitation by Alcohol.*—**Faintly acidify the original solution (*vide* (a)) and add excess of ethyl alcohol. The first precipitate which forms re-dissolves on shaking. After prolonged contact with alcohol the precipitated protein will no longer re-dissolve.**

(c) *Precipitation by Neutral Salts, e.g.,* **ammonium or sodium sulphate, sodium chloride, etc. (see § 5).**

(d) *Precipitation by Salts of Heavy Metals.*—**Into each of five test-tubes run some 2 c.c. of the original solution. To the first add a solution of mercuric chloride drop by drop. Note the formation of a heavy white precipitate of the mercury compound. Repeat the experiment with solutions of copper sulphate, ferric chloride, neutral and basic lead acetate.**

(e) *Precipitation by Mineral Acids.*—**Carefully run a few c.c. of the protein solution down the side of a test-tube containing 5 c.c. of concentrated nitric acid to form a layer on the acid. A white precipitate will form at the junction of the two liquids.**

(f) *Precipitation by "Alkaloidal" Reagents in Acid Solution—*

(i) *Hydroferrocyanic Acid.*—**Acidify the original solution with a few drops of glacial acetic acid, and then drop by drop add a solution of potassium ferrocyanide. A voluminous precipitate forms.**

(ii) *Picric Acid.*—**Addition of an aqueous solution of picric acid forms a yellowish precipitate.**

(iii) *Tungstic Acid.*—**Acidify the original solution with a few drops of dilute hydrochloric or sulphuric acid and then add a 10 per cent. solution of sodium tungstate. A bulky white precipitate is formed.**

(iv) *Trichloracetic Acid.*—**In this case use an equal volume of 10 per cent. aqueous trichloracetic acid.**

(v) *Sulphosalicylic Acid.*—**Repeat experiment (iv), using a 20 per cent. aqueous solution of sulphosalicylic acid.**

(vi) *Tannic Acid.*—**The addition of a few drops of a dilute alcoholic solution of tannic acid to the original solution of protein slightly acidified gives usually a brownish precipitate.**

4. Colour Reactions. **(See p. 64 for an explanation of these tests.)**

(a) *Biuret (Rose's or Piotrowski's) Test.*—**Add** *one drop* **of a 1 per cent. solution of cupric sulphate to the original solution and then**

excess of caustic alkali. A violet solution is obtained. The colour obtained should always be compared with that produced by the addition of excess of caustic alkali to one drop of 1 per cent. copper sulphate.

Repeat experiment (a) with a solution of commercial peptone, and note that a rose-red solution is obtained.

(b) *Xanthoproteic Reaction.*—On boiling the white precipitate produced by the addition of nitric acid to the solution of protein (see 3 (e)), it turns yellow; now *cool* and add concentrated ammonium hydroxide to form a layer on the acid. The yellow turns to orange.

(c) *Millon's Test.*—The addition of a few drops of Millon's reagent (an aqueous solution of the two nitrates of mercury containing excess of nitric acid) to the protein solution gives a white precipitate, which on boiling turns brick-red.

(d) *Rosenheim's Formaldehyde Reaction.*—To the solution of commercial peptone add 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.

(e) *Adamkiewicz Reaction.*—Here glyoxylic acid is substituted for the formaldehyde of the previous test. *Note.*—Some specimens of glacial acetic acid contain glyoxylic acid and may therefore be used for this test.

(f) *Sakaguchi Reaction.*—To 3 c.c. of the solution add 1 c.c. of 5 per cent. sodium hydroxide and 2 drops of a 1 per cent. solution of α-naphthol in alcohol. Add one drop of 10 per cent. sodium hypochlorite and shake. A bright red colour soon develops.

(g) *Molisch's Reaction.* (See Tests for Carbohydrates, p. 5.)

5. **ACTION OF NEUTRAL SALTS.**—(a) Saturate the solution of egg-white with magnesium sulphate by adding crystals of the salt and grinding thoroughly in a mortar. A white precipitate of egg-globulin is produced. Filter. The filtrate contains egg-albumin. The precipitate of *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*. Filter. Completely saturate the filtrate with ammonium sulphate (add solid ammonium sulphate and shake) when a precipitate consisting of *albumin* will be thrown down.

(c) **Completely saturate a solution of commercial peptone with ammonium sulphate. A precipitate is produced of the proteoses present in it. Filter. The filtrate contains the true peptone and gives the biuret reaction (see above), although 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.*

6. 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 water-bath at about the temperature of the body (36° to 40° C.).

After five minutes remove test-tube A, and boil. The protein is no longer coagulated by heat, having been converted into *alkali-metaprotein.* **After cooling, colour with litmus solution and neutralise with 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, an indicator such as litmus, and neutralise as before. Note that the alkali-metaprotein now requires more acid for its precipitation 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 precipate is formed, soluble in excess of acid or alkali. Acid-metaprotein is formed more slowly than alkali-metaprotein.**

Other acids, such as acetic or oxalic, may be employed instead of sulphuric acid for making acid-metaprotein, thus :—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° to 50° C. for a few minutes. Then gradually heat the solution to boiling-point; no coagulum results.

7. 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 on it carry out all the protein tests described on pp. 43, 44 and the sulphur tests (1 (c)). Saturate a few c.c. of the dilute gelatin solution with ammonium sulphate. Note the precipitation which occurs. Carefully note your results and determine which amino-acids are either absent or present, in small amount only, in gelatin.

8. KERATIN.—Suspend some horn shavings or hair in water, and try all the colour tests for protein with this. Also repeat the sulphur tests with this protein.

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

10. 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 humor or the Whartonian jelly of the umbilical cord is much richer in interstitial substance than tendon, and, if treated in the same way, a much larger yield of mucoid is obtained.

The **Proteins** (derived from the Greek *protos*, meaning first) are the most important substances which occur in the animal or vegetable organism. They form, with water, the material out of which is built the protoplasm of the cell, and the most characteristic sign that the cell is living is the actual metabolism of the protein of which it is composed. They are highly complex compounds of carbon, hydrogen, oxygen, nitrogen, and sulphur; they occur in a viscous condition, or in colloidal solution, in nearly all parts of the body. Whilst the different members of this group of substances have many characteristics in common, *e.g.*, the large size of their molecules and certain colour tests with specific chemical reagents, yet, on the other hand, individually they show considerable differences.

The proteins in the food form the source of the proteins of the body tissues, but the latter are usually different in composition from the former. The food proteins in digestion are broken up into simpler substances, usually called *cleavage products*, and it is these which, as will be seen later, pass into the blood-stream to be anabolised by the body cells to form proteins peculiar to these cells. As a result of katabolic processes in the body the proteins are once more broken down, urea, carbon dioxide, water, sulphuric acid (combined as sulphates), and creatinine being the principal final products which are discharged from the body by way of the urine and other excretions. The intermediate substances between the proteins and such final katabolites as urea will be discussed later (Chapter X.).

The following figures from Hoppe-Seyler will show how different the proteins are even in elementary composition:—

	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

Recent research has since shown that the variations are even greater than those given.

Differences between proteins are also seen when the cleavage products are separated and estimated. These products differ both in kind and in amount, but nearly all of them are substances which are termed *amino-acids*. We know now that the proteins are composed of a greater or lesser number of these amino-acids linked together, and there is hope that in the future this knowledge will lead to an accurate knowledge of the constitution of the protein molecule, from which will follow its actual synthesis.

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 one of *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 shorter linkages of amino-acids which are called *polypeptides*, and finally the individual amino-acids are obtained separated from each other.

AMINO-ACIDS

(i) **Mono-amino-acids.**—An amino-acid is essentially a derivative (1) of a fatty acid and (2) of ammonia. If we take the formula of acetic acid, CH_3COOH, and replace one of the three hydrogen atoms of the CH_3 group by an NH_2, we get the formula

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

The organic radical NH_2 is the amino group, and the correct chemical name for the compound represented by the new formula is amino-acetic acid. More commonly it is known as glycine or glycocoll and is the simplest amino-acid.

Examining the formula for glycine again it can be seen that besides regarding it as a derivative of acetic acid, we can look upon it as being obtainable from ammonia, one of the hydrogen atoms of that compound being replaced by the group $-CH_2COOH$. From this it will be understood that glycine, besides possessing acidic properties also possesses basic properties. Glycine is but one of a whole series of compounds, some twenty-five or twenty-six in number, all of which have this characteristic in common of possessing both acidic and basic properties, and which therefore can form salts both with bases and with acids. Such compounds are said to be **amphoteric** or **ampholytic.** All amino-acids are ampholytes, and it will be seen later that the proteins and their cleavage products in virtue of their being compounds containing linked amino-acids are also ampholytes.

The next simplest member of the series is alanine, derived from propionic acid. The formula for alanine is

$$\begin{array}{l} \overset{\beta}{CH_3}\ \overset{\alpha}{CH}.COOH \\ \quad\quad\quad\ | \\ \quad\quad\quad NH_2 \end{array}$$

It should be noted that the amino group is attached to the α-carbon atom and its correct name is α-amino-propionic acid. Sometimes

the amino-acid molecule contains more than one amino group, but invariably one of these is attached to the α-carbon atom.

If instead of propionic acid we take β-hydroxy-propionic acid we obtain the corresponding amino-derivative (α-amino-β-hydroxy-propionic acid) termed **Serine.**

A fourth amino-acid is similarly obtained by the introduction of the NH_2 group into valeric acid, $CH_3CH_2CH_2CH_2COOH$. α-Amino-valeric acid, $CH_3CH_2CH_2CHCOOH$, is called **Valine.**

NH_2

Passing to the next fatty acid in the series, caproic acid, $C_5H_{11}.COOH$, we may obtain from it in an exactly similar way $C_5H_{10}(NH_2)COOH$, which is α-amino-caproic acid or **leucine.** Impure leucine crystallises in spheroidal clumps of crystals, as shown in Fig. 5. With pure leucine, needle-like crystals are only obtained. Since the parent substance, caproic acid, itself can exist in isomeric modifications, its amino-derivatives must show a similar phenomenon. Many of these have been prepared synthetically, and it has been shown that the amino-caproic acid called leucine, formed by hydrolysis from most proteins, is the lævo-rotatory variety, and should be more accurately named α-amino-isobutyl-acetic acid. Normal α-amino-caproic acid is, however, stated to be obtained from the proteins of brain, and was called, on account of its sweet taste, *glycoleucine* by its discoverer (Thudichum). Abderhalden calls it *norleucine*. The graphic formulæ of the two substances are:—

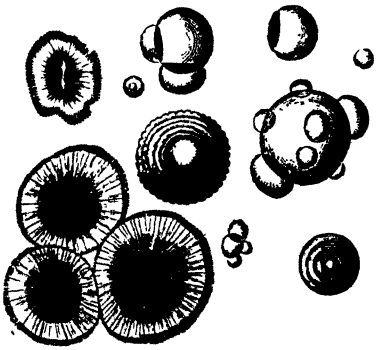
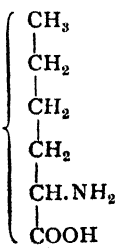


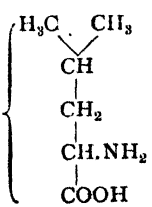
FIG. 5.—Leucine crystals.

Normal α-amino-caproic acid



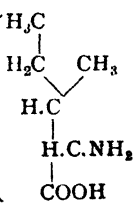
[glycoleucine]

Iso-butyl α-amino-acetic acid



[leucine]

β-methyl β-ethyl α-amino-propionic acid



[isoleucine]

All the five amino-acids mentioned (glycine, alanine, serine, valine, and leucine) are found among the final cleavage products of most proteins (see table, p. 55).

(ii) **Dicarboxylic Amino-acids.**—A second group of amino-acids is obtained from fatty acids, which contain two carboxyl 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 formula of glutamic acid is

$$HOOC.CH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}H.COOH$$

(iii) **Aromatic Amino-acids.** — This is a very important group, comprising amino-acids with an aromatic nucleus, and of these we will mention three, namely, phenyl-alanine, tyrosine, and tryptophan.

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

Propionic acid has the formula $CH_3.CH_2.COOH$.

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

FIG. 6.—Tyrosine crystals.

Phenyl-alanine is β-phenyl-α-amino-propionic acid

$$C_6H_5.CH_2.CH.(NH_2)COOH.$$

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

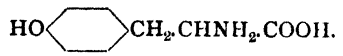
If one H in benzene is replaced by the side chain $CH_2CH(NH_2)COOH$, we obtain phenylalanine :—


$CH_2CH(NH_2)COOH,$

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

Tyrosine is a simple deriva-

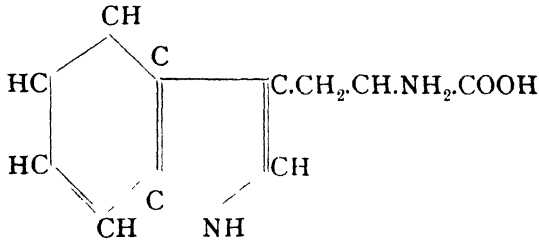
tive of phenyl-alanine; it is *p*-oxy-phenyl-alanine; that is, instead of the phenyl group (C_6H_5) in the formula of phenyl-alanine, we have now the oxy-phenyl group; the formula for tyrosine is thus



Tyrosine crystallises in collections of very fine needles (see Fig. 6).

It is characterised by its insolubility in water (1 part of tyrosine is soluble in 2490 parts of water at 17°) and is accordingly one of the amino-acids most easily isolated from a protein hydrolysate.

Tryptophan is more complex; it is α-amino-β-indolyl-propionic acid. It is the particular amino-acid of the protein molecule which is the parent substance of the well-known products of bacterial metabolism, indole and scatole (or methyl indole). The formula for tryptophan is:—



Tryptophan is the radical in the protein molecule which is responsible for the colour produced in the Adamkiewicz reaction.

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

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

These amino-acids, together with cystine, are sharply differentiated from all other amino-acids of the protein molecule by being precipitated by phosphotungstic acid (*cf.* p. 57).

Lysine is α-ε-diamino-caproic acid. Caproic acid is

$$CH_3CH_2CH_2CH_2CH_2COOH.$$

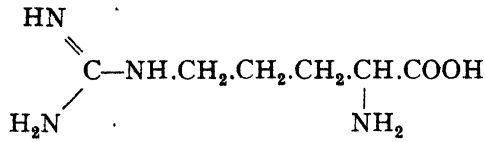
Mono-amino-caproic acid or leucine, we have already seen, is $CH_3CH_2CH_2CH_2CH(NH_2)COOH$. Lysine, accordingly, is $(NH_2)CH_2.CH_2CH_2CH_2CH(NH_2)COOH$.

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

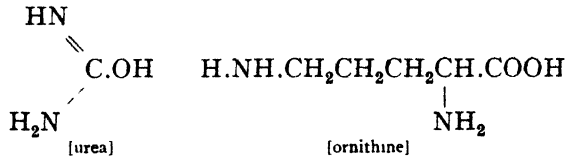
$CH_3CH_2CH_2CH_2COOH$ is valeric acid.

$(NH_2)CH_2CH_2CH_2CH(NH_2)COOH$ is $\alpha\delta$-diamino-valeric acid or ornithine.

Arginine is somewhat more complex and contains the ornithine radical. Its formula is

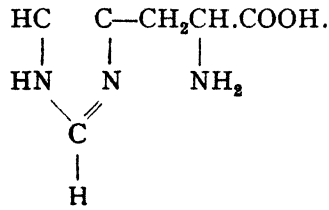


On boiling arginine with baryta water, it undergoes cleavage (at the linkage shown by the dotted line) into urea and ornithine. Such a reaction involves the addition of a molecule of water.



In the animal body arginine may suffer a similar change, **arginase,** an enzyme present in the liver, being responsible in this case for the cleavage taking place.

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



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 :—

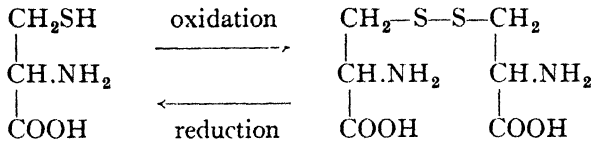
Lysine	$C_6H_{14}N_2O_2$
Arginine	$C_6H_{14}N_4O_2$
Histidine	$C_6H_9N_3O_2$

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

(v) **Amino-acids containing Sulphur.**—On turning back to p. 47 and re-examining the figures which Hoppe-Seyler gave for the percentage composition of the protein molecule, it will be found that sulphur forms a small but definite constituent of protein. On complete hydrolysis of the protein by any of the usual methods, this sulphur appears as a constituent of either or both of two amino-acids, **cystine** and **methionine**.

Cystine itself is an oxidation product of another sulphur-containing amino-acid **Cysteine**. This latter amino-acid is closely related to **serine**, shown above to be α-amino-β-hydroxy-propionic acid.

By replacing the β-hydroxyl group of serine by a sulphydryl (–SH) group we obtain cysteine. Cysteine is then α-amino-β-thiol-propionic acid. Cysteine is stable only in the form of its hydrochloride or in an acid solution. If an acid solution of cysteine be made alkaline and air is passed through it, the cysteine is rapidly oxidised to cystine, di-(β-thio-α-amino-propionic acid).



The reverse process, *i.e.*, the reduction of the cystine to cysteine, can be accomplished by reducing the cystine with tin and hydrochloric acid and then removing the tin with hydrogen sulphide. Cystine is the amino-acid responsible for the "loosely bound sulphur" test, described in the practical section (p. 42), and the fact that proteins of the epidermal tissues, *e.g.*, keratin, respond strongly to this reaction is indicative of their high content of cystine (see table, p. 55). Cysteine is of interest, since it is an integral part of *glutathione*, the compound responsible for the vivid purple coloration obtained when tissues or tissue extracts, made slightly alkaline with ammonia, are treated with a dilute solution of sodium nitroprusside.

Glutathione is a tripeptide containing the three amino-acids, cysteine, glutamic acid, and glycine. It can, therefore, like cysteine, be readily oxidised to give a product which, under certain conditions, can again be reduced to the original form. These reactions may be represented thus :—

$$\underset{\substack{\text{glutathione}\\(\text{reduced form})}}{2\text{G.SH}} \rightleftarrows \underset{\substack{\text{glutathione}\\(\text{oxidised form})}}{\text{G—S—S—G}} + \text{H}_2$$

The ease with which these oxidation and reduction reactions can take place makes it appear highly probable that the compound is closely bound up with the oxidative processes of the cell.

The second amino-acid in which sulphur occurs is **methionine.** It is one of the most recently discovered amino-acids, only being isolated by Mueller in 1922. It is rather curious that this should have been so, for when leucine, discovered in 1820, separates from a neutralised protein hydrolysate, it invariably carries down methionine with it. Methionine is α-amino-γ-methylthiol-butyric acid

$$\begin{array}{l}\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH.COOH}\\ \qquad\qquad\quad |\\ \qquad\qquad\quad \text{NH}_2\end{array}$$

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

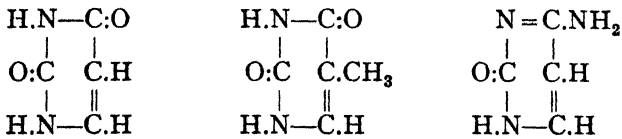
$$\begin{array}{l}\text{H}_2\text{C—CH}_2\\ \quad |\quad\ |\\ \text{H}_2\text{C}\quad \text{CH.COOH}\\ \quad \diagdown\ \diagup\\ \qquad \text{N}\\ \qquad \text{H}\end{array}$$

In addition to the various types of amino-acids already discussed, there are invariably present certain other nitrogenous compounds associated with the protein molecule, which, though they are not strictly speaking amino-acids, are of such importance that brief reference must be made to them here. They are the pyrimidine and the purine bases.

Pyrimidine Bases.—These are derivatives of pyrimidine, which is 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]

Purine Bases.—These, like the preceding, are obtained from the nucleic acid complex of nucleo-proteins, and will be described on p. 72.

We may summarise up to this point by enumerating the principal members of these various groups of nitrogenous compounds :—

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 *aromatic* group : phenyl-alanine, tyrosine, and tryptophan.
2. **The diamino-acids :** lysine, ornithine, arginine, and cystine.
3. **Pyrrolidine derivatives :** proline, oxyproline.
4. **Pyrimidine bases :** cytosine, thymine, uracil.
5. **Purine bases :** guanine, adenine.

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 from some of the proteins. The numbers given are percentages :—

	Lact-Albumin.	Egg-Albumin.	Caseinogen (Cow's Milk).	Gelatin.	Keratin (Horse Hair).	Salmine (Rhine Salmon).	Edestin, a Globulin (Cotton Seed).	Zein (Maize).	Gliadin (Wheat).
Glycine . .	0	0	0·45	25·5	4·7	...	3·8	0	0
Leucine . .	19·4	6·1	9·7	7·1	7·1	+	20·9	19·6	6·6
Glutamic acid	12·9	8·0	21·77	5·8	3·7	..	6·3	26·2	43·7
Tyrosine .	1·9	1·1	4·5	...	3·2	..	2·1	3·6	1·2
Arginine .	3·2	2·14	3·81	8·2	4·5	87·4	11·7	1·6	3·2
Tryptophan .	2·4	+	1·5	...	...	...	+	0	1·0
Cystine . .	0·4	0·2	...	...	8·0	...	0·3	...	0·5

Such numbers, of course, are not to be committed to memory, being cited to give the reader some idea of the differences which exist between the various proteins. There are several blanks left, 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, tryptophan, and cystine from gelatin.
4. The high percentage of the sulphur-containing substance (cystine) in keratin.
5. The high percentage of glutamic acid in vegetable proteins.

Methods of Analysing Proteins.—The aim of the chemist is 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 ideal has not yet been attained on account of the secondary reactions taking place during hydrolysis, such as the formation of brown and black pigments, the splitting off of carbon dioxide, etc. Even with the best methods at his disposal, Fischer and his colleagues succeeded in separating at the utmost only 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. The new method recently introduced by Dakin, involving the extraction of the products of hydrolysis by butyl alcohol, has given better results. As a result of the use of this method, practically the entire amino-acid content of gelatin is known.

In 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 used by Osborne and others.

The main principles of *Hausmann's method* are 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. This is the nitrogen contained 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 of results of analyses by 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 proteins, *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 in the diamino-N were revealed. The method has also proved useful for the differentiation of proteoses, while interesting deductions as to the food value of various proteins have been drawn from its results.

Van Slyke's Method.—A further differentiation of the units in the protein molecule has been made possible by a method elaborated by Van Slyke. It is an application of the well-known reaction of nitrous acid on substances containing an amino group :—

$$R.NH_2 + HONO \longrightarrow R.OH + N_2 + H_2O.$$

Details of the actual method are given on p. 151.

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 as in proline, oxyproline, tryptophan, 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, the whole operation being carried out with 2 or 3 grams 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 mono-amino-acids contained in the phosphotungstic acid 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 (tryptophan).

During the hydrolysis as described above, 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:—

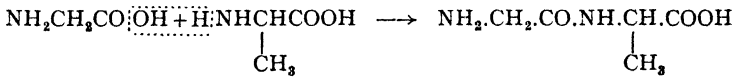
	Amide-N.	Humin-N.	Cystine-N.	Arginine-N.	Histidine-N.	Lysine-N.	Mono-amino-N.	Non-amino-N.	Total N.
Wheat gliadin .	25·52	0·86	1·25	5·71	5·20	0·75	51·98	8·50	99·77
Edestin . .	9·99	1·98	1·49	27·05	5·75	3·86	47·55	1·70	99·37
Keratin (from hair) . .	10·05	7·42	6·60	15·33	3·48	5·37	47·50	3·10	98·85
Gelatin . .	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 practically 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 this protein contains larger amounts of proline and oxyproline than the older analyses had revealed. The large amount of lysine in hæmoglobin is also unexpected.

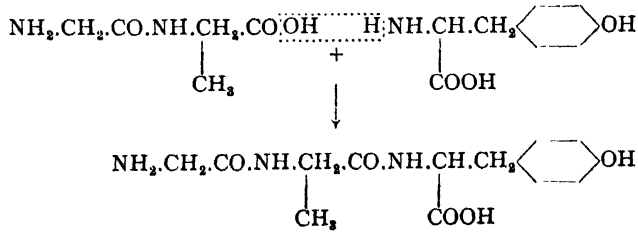
THE STRUCTURE AND THE MOLECULAR WEIGHT OF THE PROTEIN MOLECULE

We have seen how, by a variety of agents, the protein molecule can be broken up ultimately into a number of units consisting of amino-acids of various types. These amino-acids, of which there are some twenty-five or twenty-six already known, may be regarded as the building-stones from which the proteins arise in nature. The question we must now ask ourselves is how are these units linked together.

One of the first investigators to attempt an answer to this problem was Emil Fischer. He showed that one amino-acid may be combined with itself or with other amino-acids in the following way :—

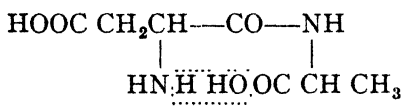


the union being effected through the carboxyl group of the one acid and the amino group of the other, a molecule of water being eliminated in the process. The compound so formed from glycine and alanine belongs to the group of substances called **dipeptides,** and is named glycyl-alanine. Examination of its formula shows that it possesses, like each of its parents, a carboxyl group and an amino group and belongs to the group of substances called ampholytes (p. 48). Moreover it also possesses, again like its parents, the power of combining with another amino-acid, *e.g.*,



The new compound, containing three amino-acid residues, is the **tripeptide,** glycyl-alanyl-tyrosine. Again, it can be seen that the new compound can be linked, in virtue of its carboxyl or its amino group, with yet another amino-acid, and so on. Fischer actually synthesised such compounds and carried on the process of lengthening the chain until he arrived at one containing the residues of some eighteen amino-acids. This synthetic **polypeptide** resembled natural proteins in many respects, and there can be little doubt that such chains play an exceedingly important part in the structure of these compounds. There were, however, as Fischer was careful to emphasise, some essential differences between the synthetic and natural substances, and these led him to the conception that in addition to these polypeptide chains there are certain other groupings of amino-acids present as ring structures.

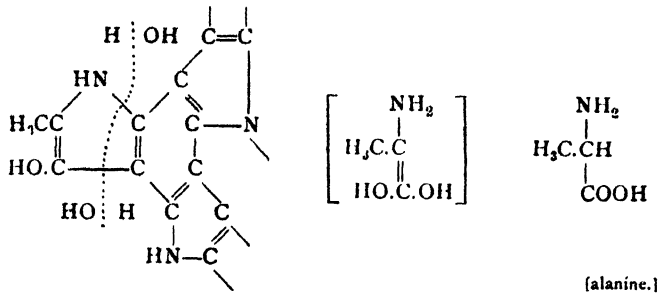
Later workers, especially Abderhalden, taking up the problem at this point, have, by careful search of the products of the hydrolysis of proteins, found certain such ringed structures. Examination of the constitution of these compounds shows them to be **diketo-piperazines**, the structure of which can best be illustrated in the following way. Writing the formula of a dipeptide, say aspartyl-alanine, not as was done above, but so—



it will be seen that the free amino and carboxyl groups are brought very closely together, and a condensation similar to that by which the dipeptide itself was formed can be visualised as being possible. As a matter of fact such a condensation takes place in actual practice simply by boiling an aqueous solution of such a dipeptide with the formation of a diketopiperazine. Further consideration will show that these six-membered diketopiperazine rings may have, as in the case mentioned, side chains containing either a carboxyl or an amino group or both, and by their presence these diketo-piperazines can be linked up with other amino-acids or even polypeptide chains. Hence we may visualise one or more poly-peptide chains radiating from a central diketopiperazine nucleus much as knitting needles may be made to stick out from a ball of wool.

Yet another conception of the cyclic structure present in the protein molecule is that advanced by Troensegaard. This worker regards

the main body of the protein as being made up of a number of fused benzene and pyrrole rings, and that these,



[alanine.]

whilst usually fairly stable, easily split up, as shown by the dotted line, during the hydrolysis of the protein to yield the constituent amino-acids.

At the very beginning of this chapter, in describing the broad features of proteins, reference was made to the fact that one of their special characteristics was the large size of their molecules. Their actual sizes are so large, in fact, that the determination of the molecular weight has provided many difficulties for the investigator. Of recent years, however, much progress has been made in the solution of this problem, and, although detailed descriptions of the methods employed are beyond the scope of this book, yet the student should have a broad knowledge of them and the results obtained by their use. It may be said at once that the ordinary methods of determining molecular weights of organic compounds, *e.g.*, by the usual freezing- and boiling-point determinations, are inapplicable owing to the lack of suitable solvents for protein, to the fact that proteins are so easily denatured, and because the actual rise or fall of temperature is so small. Briefly, the methods employed have involved the use of (*a*) data obtained by analysing proteins and their derivatives, (*b*) osmotic pressure determinations, (*c*) ultra-filtration and dialysis experiments, (*d*) sedimentation rates. Of these we shall refer to the first and the last. The analytical methods take various forms. For example, certain amino-acids, *e.g.*, glutamic acid, arginine, histidine, tyrosine, can now be separated quantitatively from protein hydrolysates. If we assume the presence of only one molecule of any one of these amino-acids in the protein molecule, then we have at once a method of determining the **minimum** molecular weight of the protein. On the other hand, the molecular weight of a protein such as hæmoglobin can be calculated from its content (1) of certain

amino-acids, (2) of iron, or (3) of oxygen. In this particular case there is yet another method available. Hæmoglobin and carbon monoxide are known to give a definite compound (*cf.* p. 201), analysis of which indicates that 16·721 grams of hæmoglobin combines with 1 gram-molecule of the gas. Hence 16,721 is taken to be the minimum molecular weight of hæmoglobin.

A method of which much use has recently been made is that of studying the rates of sedimentation of various proteins. This method has been perfected by Svedberg and his colleagues, who have employed a special ultra-centrifuge running at very high speeds. The figures which they have so obtained are very interesting and they would make it appear that the molecular weight of proteins are multiples of the figure 34,500. Whether this means that proteins are simply aggregates of units possessing a molecular weight of this order does not appear definitely proved yet. Some of the results so far obtained by these workers are as follows:—

Egg-albumin . . .	34,500
Serum-albumin . . .	67,500
Serum-globulin . . .	104,000
Hæmoglobin . . .	68,000
Hæmocyanin . . .	5,000,000
Edestin	208,000

TESTS FOR PROTEINS

Proteins are differentiated from carbohydrates and fats by a number of tests involving either the production of a marked colour or of the formation of a precipitate. The colour tests are not specific for proteins themselves but for certain amino-acids common to the majority of them. The precipitation tests are reactions involving amphoteric colloids.

Solubilities. — No fixed rule can be laid down regarding the solubilities of proteins as a group. A few are soluble in water, though the majority of them form colloidal solutions with this reagent. Others are soluble only in aqueous sodium chloride solution (5 to 10 per cent.) or in faintly acid or alkaline solutions. A few, chiefly vegetable proteins, are soluble in alcohol.

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 will be seen later.

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 fall mainly into two classes: the *albumins* and the *globulins*. These differ in solubility; the albumins being soluble in distilled water, while 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 such 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) immersed in distilled water the salts gradually pass into the water through the membrane and are 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 dialyse away. In this connection the terms "diffusion," "dialysis," and "osmosis" should be distinguished from one another (see p. 105).

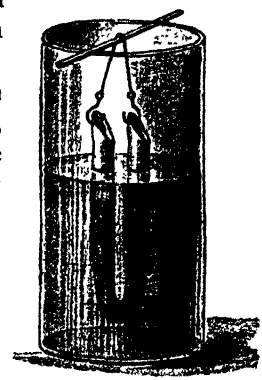


FIG. 7.—In this form of dialyser the substance to be dialysed is placed within the piece of parchment tubing suspended in the vessel of water.

Crystallisation.—Hæmoglobin, the red pigment of the blood, is a protein and is crystallisable (for further details, see p. 201). 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 and serum-albumin have been crystallised.

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

Colour Reactions.—These tests are of the highest importance, because by their use not only can the presence of a protein be detected but valuable information may also be gained regarding its amino-acid content.

1. **Biuret Test.**—Shiff has shown that all substances which contain two —NH.CO— groups linked to a carbon or to a nitrogen atom give the characteristic biuret [1] reaction when treated with copper sulphate and sodium hydroxide. Since native proteins and most of their split products (*cf.* the formula for the tripeptide, glycyl-alanyl-tyrosine on p. 60) contain such groups they give a positive reaction. The colour obtained varies, however, from a violet to a rose-red, the shorter the chain the more closely does the colour approach the latter. In all cases when carrying out the test, a blank test should be carried out to provide a standard colour for comparison.

2. The **xanthoproteic** reaction depends on the conversion of the aromatic acids of the protein molecule into nitro-derivatives by the action of the nitric acid.

3. **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) attached to a nuclear carbon.

4. The **formaldehyde** reaction (and the Adamkiewicz reaction) is due to the presence of the tryptophan radical (β-indolyl-α-amino-propionic acid).

5. **Sakaguchi's** reaction depends upon the presence of arginine in the protein molecule

6. **The Ninhydrin Reaction.** — This reaction was discovered by Ruhemann, who found that all acids containing a free amino group in the α position react with triketohydrindene hydrate (ninhydrin) with the production of an intense blue colour. The reaction is very delicate and will detect, for instance, 1 part of glycine in 65,000 parts of water. The reaction is characteristic only of amino-acids in the absence of ammonium salts and aliphatic amines.

Precipitants of Proteins.—Proteins are precipitated by a large number of reagents; the petones and proteoses are exceptions in many cases, and will be considered separately afterwards (see p. 147). The precipitation of proteins from biological fluids, *e.g.*, blood, is an essential preliminary to the quantitative estimation of certain constituents therein.

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

$$\underset{\text{[urea]}}{2CON_2H_4} \longrightarrow \underset{\text{[ammonia]}}{NH_3} + \underset{\text{[biuret]}}{NH_2CONHCOHN_2}$$

Proteins are precipitated from solution by the addition of:—

1. Strong acids, such as nitric acid.
2. Tungstic acid.
3. Picric acid.
4. Acetic acid and potassium ferrocyanide.
5. Acetic acid and excess of neutral salts, such as sodium sulphate.
6. Trichloracetic acid and sulphosalicylic acid.
7. Heavy metals, such as copper sulphate, mercuric chloride, lead acetate, silver nitrate, zinc hydroxide, etc.
8. Tannin.
9. Alcohol.
10. Saturation with certain neutral salts, such as ammonium sulphate.

It is necessary in this connection that the terms *coagulation* and *precipitation* should be carefully distinguished.

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 thrombin—*enzyme coagulation.*

There are, however, 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, *e.g.*, 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, 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. The protein is said to undergo *denaturation.* The change appears to be structural, involving a rearrangement of the linkages in the molecule rather than actual degradation. Albumins and globulins are most readily rendered insoluble by this method; proteoses and peptones are never rendered completely insoluble by the action of

alcohol. This property may be used in the separation of these proteins from others.

CLASSIFICATION OF PROTEINS

The knowledge of the chemistry of the proteins which is rapidly progressing will ultimately enable us to classify 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 our new knowledge. The classes of animal proteins, then, beginning with the simplest, are as follows :—

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 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. They give such typical protein reactions as the biuret test. They are strong bases and absorb carbon dioxide from the air. They form salts with acids. Among such salts, the sulphate is the principal one. On hydrolysis they first yield substances of smaller molecular weight analogous to the peptones and called *protones*, and then they split up into amino-acids. The number of resulting amino-acids is small compared with that from other proteins. 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, which is present to the extent of 87 per cent. (*cf.* table on p. 55), 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 example. 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. 48 to 55.

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° to 73° C. Serum-albumin, egg-albumin, and lact-albumin are examples.

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 and 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; hence they can be precipitated by removing the salt which keeps them in solution. This may be accomplished by dialysis (see p. 105).

The temperature of heat coagulation of globulins 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, such as fibrin (see Blood) and myosin (see Muscle), which are the result of enzyme coagulation on globulins.

5. The Sclero-proteins

These substances form a heterogeneous group of substances, which were formerly termed *albuminoids*. The prefix *sclero-* indicates the skeletal origin and often insoluble nature of the members of the group. The principal proteins under this head 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 the 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 lacks three of the essential amino-acids—tyrosine, tryptophan, and cystine. Animals which receive in their food gelatin to which tyrosine and tryptophan 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 tissues 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 hair, nail, hoof, and horn. It is very insoluble, and chiefly differs from most other proteins in its high percentage of the sulphur-containing amino-acid 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 ectoderm.

6. The Phospho-proteins

Vitellin (from egg-yoke), *caseinogen*, the principal protein of milk, and *casein*, the result of the action of rennet on caseinogen (see Enzymes, p. 145), are the principal members of this group. Among their decomposition products is a considerable quantity of phosphoric acid. They were formerly 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 is the case for 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 contains a metal, usually iron. These complex proteins have a respiratory function, and typical examples of them are hæmoglobin, hæmocyanin, and their allies.

(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 and by mucous glands and goblet cells. The mucins obtained from different sources vary in composition and reactions, but they all agree in the following points:—

(*a*) Physical character. They are viscous and tenacious.

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

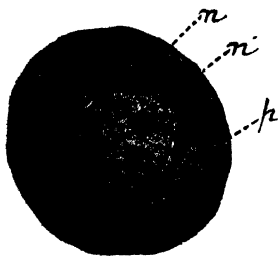


FIG. 8.—Diagram of a cell; *h*, protoplasm composed of spongioplasm and hyaloplasm; *n*, nucleus with intranuclear network of chromatin or nuclein; and *n'*, nucleolus. (Schafer.)

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 mucoids are possibly due to the nature of the protein part of the molecule, and also to the nature of the conjugated sulphuric acids which they contain. Those from cartilage, tendon, and aorta furnish chondroitin sulphuric acid which on further hydrolysis yields chondrosamine and glycuronic acid. The mucoids from cornea, vitreous humor, gastric mucosa, serum mucoid, and ovo-mucoid on the other hand contain mucoitin sulphuric acid, which yields *d*-glucosamine (chitosamine), and glycuronic acid.

(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 properties they resemble mucin.

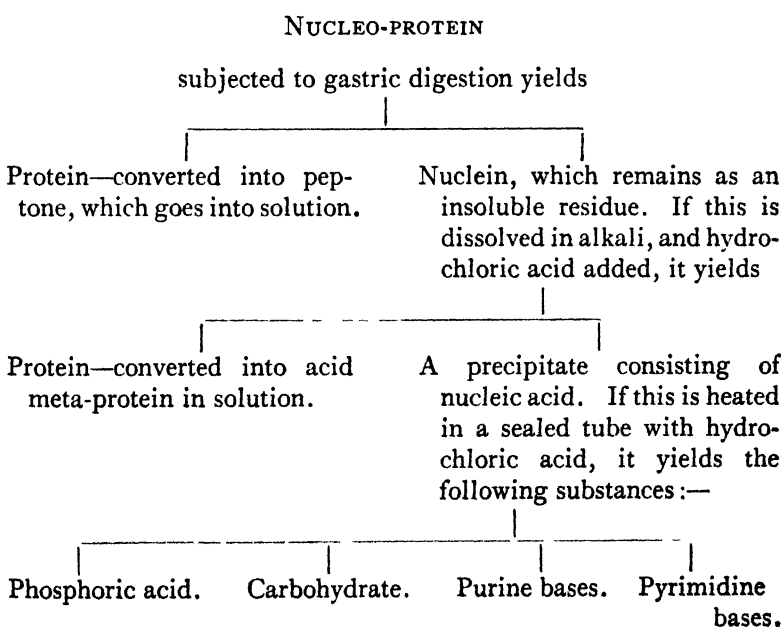
Nuclein is the name given to the chief constituent of cell-nuclei. It is identical with the chromatin of histologists (see Fig. 8).

On decomposition it yields the organic acid nucleic acid, together with a variable but usually small amount of protein. It contains a high percentage (10 to 11) of phosphorus. The nuclein obtained from the nuclei or from 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.

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 :—



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. In thymus nucleic acid this is 2-desoxy-aldo-pentose; in yeast nucleic acid, *d*-ribose.
   (*c*) Two members of the purine group in the same proportion, namely, adenine and guanine.
   (*d*) Two pyrimidine bases, namely, cytosine and thymine. In yeast nucleic acid, uracil takes the place of thymine (see p. 55).

The *purine bases* are specially interesting because of their close relationship to uric acid (which see, p. 300). 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 those which contain the amino group. If xanthine and hypoxanthine are obtained, they are the secondary products of oxidative and de-aminising 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.

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-aminised (*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 when acted upon by certain enzymes of 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. Metaproteins.
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 more complex 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 metaproteins, 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 by the action of dilute acids or alkalis on either albumins or globulins. The general properties of the *acid* and *alkali metaproteins* 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, like globulins, by saturation with such neutral salts as sodium chloride or magnesium sulphate. They are not coagulated by heat if in solution. In alkali metaprotein some of the sulphur in the original protein is removed. Sulphur removed in this way is probably from cystine.

A variety of metaprotein (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.

PROTEINS OF VEGETABLE ORIGIN

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 of 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, the other (glutenin) insoluble in alcohol. It is to the former that the gluten of dough owes its cohesiveness; hence grains such as rice, which contains no gliadin, cannot in consequence be employed for making bread.

CHAPTER IV

FOODS

The food-stuffs provided are milk, flour, bread, meat.

A. MILK.—1. Put a drop of milk on a microscope slide, cover it with a cover-slip and examine with the high power of a microscope. Make a sketch in your note-book of what you see.

2. Determine 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 fat.

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

4. *Formation of Surface Film.*—Heat some milk in an open vessel and note the formation of a solid film on the surface. Remove this with a glass rod and examine it to prove its protein nature.

5. To another portion of warm milk diluted with water add 20 per cent. acetic acid drop by drop until maximum precipitation occurs. A lumpy precipitate of caseinogen entangling the fat is formed. Filter the resulting mixture, wash the solid thus collected with distilled water and combine the filtrate and washings. Carry out the tests on the solid and liquid fractions as described in experiments 6 and 7 respectively.

6. According to the directions test the solid for

(a) *Protein:* dissolve a part of it in 2 per cent. sodium hydroxide and on it perform the biuret, xanthoproteic, Millon's, and glyoxylic tests (pp. 43-44).

(b) *Organic phosphorus:* boil a portion with dilute nitric acid and test for the presence of phosphorus (p. 42).

(c) *Fat:* (i) Extract a portion with ether. Carefully evaporate off most of the ether and pour the concentrated solution on a piece of paper. Note the greasy stain when the ether evaporates. (ii) To another portion apply the acrolein test (p. 30). (iii) To another portion apply the osmic acid test (p. 30).

7. (a) To a portion of the fluid add an equal volume of saturated ammonium sulphate. *Lactoglobulin* (p. 85) separates. After a few minutes filter the mixture, and saturate the filtrate with solid ammonium sulphate. *Lact-albumin* separates.

(b) Concentrate the remainder of the fluid (experiment 5) to one-third of its original bulk. During the process the albumin and globulin are coagulated and separate as a scum. Cool the concentrate and filter. Test portions of the filtrate for (i) lactose (milk-sugar), (ii) inorganic phosphates, (iii) chlorides. Note—the simple addition of ammonia to the concentrate produces a precipitate of earthy (*i.e.*, Ca and Mg) phosphates indicating the presence of these elements in milk.

8. 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 alkali before adding the ether and then shake. The fluid which lies beneath the ethereal solution of fat becomes translucent. As ether dissolves the fat without the addition of alkali, the opacity of milk is 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 the complete solution of the fat following the action of the alkali on the caseinogen.

9. *Caseinogen*, like globulin, is precipitated by saturating milk with sodium chloride or magnesium sulphate, or 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 it after the mixture has stood an hour or two.

10. *Guaiac Test.*—To about 3 c.c. water add $\frac{1}{2}$ c.c. of milk and sufficient tincture of guaiac (or an alcoholic solution of guaiaconic acid) to give a permanent cloudiness. Note any colour change. Add hydrogen peroxide to form a layer on the top of the previous solution and note the formation of a blue ring at the junction. Repeat this with boiled milk after cooling. *In no case conduct the test at a temperature greater than* 60° *C.*

B. FLOUR.—1. Mix some wheat flour with a little water into a stiff dough. Knead the dough in a beaker or dish of water, and notice that the water becomes opalescent. Show the presence of *starch* in the water by carrying out the iodine test. Repeatedly knead the remaining solid with fresh water until a positive iodine reaction is no longer obtained. The elastic sticky mass which remains is a mixture of proteins

called *gluten* (p. 75). On this carry out the series of experiments described in the next paragraph.

2. Gluten.—(a) Suspend a fragment of the gluten in water, add nitric acid, and boil. It turns yellow. Cool and add ammonia. It turns orange (xanthoproteic reaction).

(b) Boil another fragment with Millon's reagent. It turns brick-red.

(c) Place the remainder of the gluten in an evaporating basin, add some 70 per cent. alcohol, and heat gently on a water-bath until all the alcohol has evaporated. Repeat the procedure twice. On doing it a third time do not evaporate off the alcohol, but as soon as it is boiling carefully pour off the liquid portion into a second basin and concentrate it to one-quarter of its bulk. Pour the concentrate into a test-tube of water and notice the formation of a precipitate. The alcohol soluble fraction is *gliadin;* the insoluble fraction *glutenin* (p. 75).

C. BREAD contains the same constituents as flour, except that some of the starch has been converted into dextrins and glucose during baking (most flours, however, contain a small quantity of sugar). Extract bread crust with cold water, and test the extract for dextrin (iodine test) and for glucose (Benedict'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 divide into two portions. Grind (a) the first portion with distilled water, filter, and test the filtrate for *chlorides*, (b) the second portion with salt solution, filter, and test the filtrate for *proteins, phosphates, and reducing substances.*

E. VITAMIN A.—To 2 c.c. of a 30 per cent. solution of antimony chloride in chloroform in a dry test-tube add 0·1 c.c. (one drop) of a chloroform solution of the oil or fat. Note the production of a gentian-blue colour which changes in five to ten minutes to a red colour.

F. The ESTIMATION of LACTOSE in MILK.—As has already been shown (p. 10), the estimation of a carbohydrate, *e.g.*, glucose, lactose, etc., involves the use of an oxidising reagent, *e.g.*, Benedict's copper solution, etc. Since such reagents would oxidise any protein (or their degradation products) which may be present in a biological fluid (milk, blood, etc.), the removal of these substances is an essential preliminary before the estimation of the carbohydrate is carried out. (*Cf.* the estimation of glucose in blood, p. 188.)

To 10 c.c. of fresh milk first add 50 c.c. of distilled water and then, drop by drop, with constant stirring, 7 c.c. of colloidal iron. Finally

33 c.c. of distilled water are added, the mixture again stirred, and the whole filtered through coarse filter paper.

To 10 c.c. of the filtrate in a conical flask add 10 c.c. of 0·1N iodine solution and then 15 c.c. of 0·1N sodium hydroxide. Stir the mixture and allow to stand for 15 mins. Then add 15 c.c. of 0·1N sulphuric acid and titrate the solution with 0·1N sodium thiosulphate.

Finally, 10 c.c. of 0·1N iodine solution are titrated with the given $\frac{N}{10}$ thiosulphate sulphate.

Let the difference between the two titration figures, *i.e.*, the amount of iodine solution used to oxidise the lactose in the milk $= x$ c.c.; then $x \times 9 =$ the number of grams of lactose in 100 c.c. of milk.

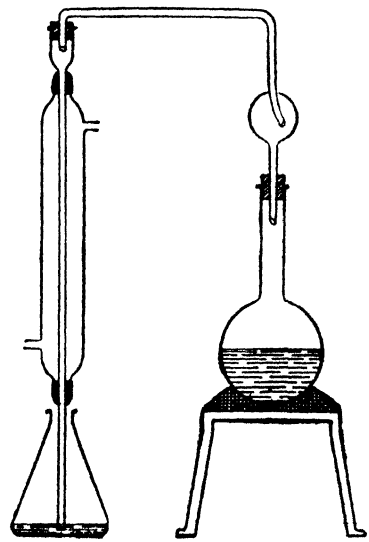


Fig. 9.

G. The ESTIMATION of the PROTEIN in MILK. — Into a Kjeldahl flask accurately pipette 5 c.c. of milk, add 20 c.c. of concentrated (nitrogen-free) sulphuric acid, 5 grams of potassium sulphate, 0·5 gram of copper sulphate, and heat the mixture over a Bunsen flame in a fume-cupboard. After the mixture has become water-clear the heating should be continued for a further half-hour. The flask is then cooled.

When cold, the contents are carefully washed into a litre Erlenmeyer flask and water is added until the total volume of the fluid is about 400 c.c. Then run in excess sodium hydroxide solution (40 c.c. of 40 per cent. for every 10 c.c. of concentrated sulphuric acid used in the digestion process) so that the two solutions do not mix. Drop in a few pieces of granulated zinc to avoid bumping in the subsequent distillation, and immediately fit a glass tube into the neck of the flask by means of a well-fitting rubber stopper. The other end of the tube leads into a second flask which contains a measured amount (50 c.c.) of standard sulphuric acid (0·1N acid is a convenient strength to use), the end of the tube dipping just below the surface of the acid. The mixture in the flask

is now boiled for about half an hour, when all the ammonia will have distilled over. The free acid remaining is then estimated by titrating it with 0·1N sodium hydroxide, a few drops of the methylene blue, methyl red indicator[1] being added. This indicator is purple in acid solutions and green in alkaline solution.

The difference between the amount of alkali required for neutralisation and the amount of acid originally taken is a direct measure of the ammonia evolved. Suppose this is equivalent to 17 c.c. 0·1N NH_3.

Then 5 c.c. of milk contains 17 c.c. 0·1N $NH_3 \equiv$ 17 c.c 0·1N N_2
$\equiv 17 \times 0{\cdot}0014$ grams N_2.

$\therefore$ 100 „ „ „ $17 \times 0{\cdot}0014 \times \frac{100}{5}$ grams N_2.

$\therefore$ 100 „ „ „ $17 \times 0{\cdot}0014 \times \frac{100}{5} \times 6{\cdot}38$ grams protein.

NOTE.—The amount of nitrogen multiplied by the factor 6·25 gives the amount of protein; in the case of milk the factor is 6·38; for vegetable proteins it is 5·68.

[1] This indicator, originally suggested by de Wesselow, is a mixture of 100 c c. of 0·02 per cent. methyl red and 30 c.c. of 0·1 per cent methylene blue. It is not affected by the presence of carbon dioxide.

THE PRINCIPAL FOOD-STUFFS

Having learnt the chief characteristics of the compounds called carbohydrates, fats, and proteins, and the reactions whereby they may be identified, the student is now in a position to investigate the chemical nature of some of the more important food-stuffs used by man.

The chief chemical substances in food are :—

1. Proteins
2. Carbohydrates } organic.
3. Fats
4. Vitamins
5. Water
6. Salts } inorganic.

In milk and in eggs, which form the exclusive foods of young animals, all varieties of these constituents 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 must be present in proper proportions, which vary for herbivorous and carnivorous animals.

When we remember the reason for the necessity of food is to replace worn-out body materials, to build up fresh tissues, and to supply heat to maintain our temperature usually above that of our surroundings, we see that a suitable diet must possess the following characters :—

1. It must contain the various chemical substances in proper amount and proportion and, moreover, in forms easily digestible. For example, 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 away unused in the fæces.

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

The nutritive value of a diet, particularly from the energy standpoint, depends mainly on the amount of carbon and nitrogen it contains in a really digestible form. A man doing a moderate amount of work will eliminate per diem some 250 to 280 grams of carbon, chiefly from the lungs in the form of carbon dioxide, and some 15 to 18 grams of nitrogen, chiefly by the way of the kidneys in the form of urea. The substances giving rise to these waste products are derived from the food or from the tissues of the body. During muscular exercise, while the output of carbon greatly increases, the excretion

of nitrogen shows insignificant variations. It is necessary then that the wastage of tissues taking place during exercise should be replaced by fresh material in the form of food and that 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, approximately 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 grams.
Fat	100 ,,
Carbohydrate	333 ,,

Ranke's diet closely resembles Voit's; it contains

Protein	100 grams.
Fat	100 ,,
Carbohydrate	250 ,,

The following peace-time dietary will be seen to be rather more liberal, but it 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.		Grams of					
			Nitrogen.	Carbon.	Proteins.	Fats.	Carbohydrates.	Salts.
	Metric System.	English System.						
Lean meat .	250 grams	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

Despite the large number of investigations on this question of what constitutes an adequate diet and of what substances it should be constituted, there is even yet no measure of agreed opinion. For example, Chittenden urged that the normal diet should contain only about half the customary quantity of protein, whilst Hindhede goes so far as to claim that young vigorous men can maintain nitrogen-balance on 22 grams of digestible bread-protein. The body certainly

does not assimilate the larger amount of protein usually taken, for the greater part of the nitrogenous constituents is converted into amino-acids, which are rapidly transformed by the liver chiefly into urea which is excreted, leaving the non-nitrogenous remainder to be utilised in the same way as are fats and carbohydrates for the production of heat and energy.

One hesitates, however, before accepting Chittenden's conclusions in their entirety, 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 (tyrosine, tryptophan, cystine, lysine, and histidine) are unusually precious for protein synthesis or special metabolic processes in the body, and that, in order to get an adequate supply of these, a comparatively large amount of protein must be ingested.

Further research has shown that there is something else in an adequate diet which is necessary, not only for growth but for maintenance. These factors (vitamins) 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 in most cases. For adults, however, 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. It also contains too little iron; hence children weaned late become anæmic. It is deficient, too, in vitamin C, certain organic substances, and material for conversion into hæmoglobin.

Microscopically it consists of two parts: a clear fluid and a number of minute fat globules floating in it.

The milk secreted during the first few days before parturition and of lactation is called *colostrum*. It contains very little caseinogen, but large quantities of globulin instead. This globulin is probably similar to, if not identical with, one of the proteins of the blood-plasma of the mother, and, moreover, one in which the newborn is relatively lacking. Certain observations, moreover, appear to show that with this fraction of blood-plasma are associated many of the antibodies so essential to the effective warding off of disease. Micro-

scopically, 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 neutral towards litmus. This is due to the presence of both acid and alkaline salts, particularly phosphates. Milk readily turns acid or sour as the result of fermentative change,

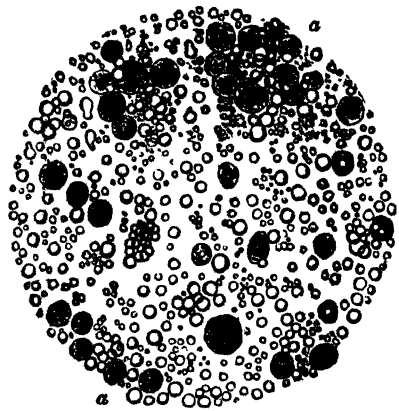


FIG 10.—Microscopic appearance of milk in the early stage of lactation, showing colostrum corpuscles (*a*) in addition to fat globules. (Yeo)

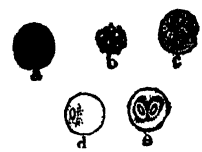


FIG. 11.—*a*, *b*, colostrum corpuscles with fine and coarse fat globules respectively; *c*, *d*, *e*, pale cells devoid of fat (Heidenhain)

part of its lactose being transformed into lactic acid (see p. 20). The specific gravity of cow's milk, measured with the hydrometer, varies from 1028 to 1034. When the milk is skimmed the specific gravity rises to 1038, owing to the removal of the light constituent, the fat. In all cases the specific gravity of water, with which other substances are compared, is taken as 1000.

Composition and Constituents of Milk

The following table contrasts the milk of woman and cow:—

	Woman.	Cow.
	Per cent.	Per cent.
Water	87·5	87·0
Total solids	12·5	13·0
Proteins (chiefly caseinogen)	1·7	3·5
Fat (butter)	3·4	3·7
Carbohydrates (lactose)	6·2	4·9
Salts	0·2	0·7

The individual constituents of milk may be placed in one of two groups showing the physical state in which they exist in the milk.

(*a*) *In true solution*:—Lactose, potassium, sodium, and calcium chlorides, potassium and sodium phosphates, vitamins B and C.

(*b*) *In colloidal solution or in suspension*:—Albumin, globulin, caseinogen, calcium and magnesium phosphates, fat, vitamin A, and certain enzymes.

The Proteins of Milk.—The principal protein in milk is called *caseinogen*. It belongs to the group of phospho-proteins (p. 69), and is of particular importance because it forms one source of supply of phosphorus for the animal body. Cheese consists of casein with the entangled fat. The other proteins in milk, present in small amounts, are *lact-albumin*, *lacto-globulin*, and traces of a protein which is soluble in alcohol. Lacto-globulin closely resembles serum-globulin, but lact-albumin differs from serum-albumin.

Caseinogen, although it is present in the surface film when milk is boiled, is not coagulable by heat. It is, as was originally pointed out by Hammarsten, a protein with acid properties, quite insoluble in water, but which forms soluble salts with such bases as potassium, sodium, and calcium hydroxides. It is precipitated from a solution of such salts by the addition of hydrochloric or acetic acids, as shown in the practical section. The caseinogen as it exists in cow's milk is combined with calcium as neutral calcium caseinogenate, though in human milk the caseinogen is said to be united with potassium. 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. This latter reaction forms the

basis of the clotting of milk, which will be studied in a later chapter (p. 131).

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, olein, and, in smaller amount, stearin. There are, however, smaller quantities of fats, especially butyrin and caproin, derived from fatty acids lower in the series. The statement that each fat globule is surrounded by a film of protein is, according to Ramsden's work, correct. Milk also contains small quantities of lipoids, namely, phosphatides and cholesterol, and yellow fatty pigments now regarded as composed of carotin and xanthophylls. The pigment of adipose tissue is probably carotin.

Milk Sugar or Lactose.—This is a disaccharide ($C_{12}H_{22}O_{11}$). Its properties have already been described in Chapter I., p. 19.

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 sterile. Equations showing the change produced are given on p. 20. When souring occurs, the acid which is formed precipitates a portion of the caseinogen.

Alcoholic Fermentation in Milk.—When yeast is added to milk, the sugar does not readily undergo alcoholic fermentation. Other lower organisms are, however, able to produce alcohol, as in the preparation of koumiss; the milk sugar is first inverted, that is, glucose and galactose are formed from it (see p. 20), and it is from these sugars that alcohol and carbon dioxide originate.

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

Differences between Different Milks.—The milk provided by nature for the growing offspring is different in the various classes of the animal kingdom. The quantitative variations are often marked, 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, cream, and vitamins 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 merely one of quantity only. In particular, the pendulum of

scientific opinion has swung backwards and forwards in relation to the question whether the principal protein, caseinogen, 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, lime water, or citric acid, 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.

It may be that human and cows' milk have unknown substances present in differing amount.

EGGS

In this country the eggs of hens and ducks are those particularly selected as foods. The *shell* is composed 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 *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 pigment), the other, smaller, transparent, and almost colourless: these are protein in nature, consisting of the phospho-protein called *vitellin* (p. 69).

Of the total weight 73 per cent. is water. The solids consist of protein (albumin, globulin, ovo-mucoid) 12·3 per cent., fat (lecithin and other phosphatides including cholesterol) 11·3 per cent., carbohydrate 1·6 per cent., and mineral matter 1·1 per cent.

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, and the more difficult of digestion, thus affording a reason for the clinical observation of the value of a " lightly boiled " egg in invalid diet.

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 and forms our chief source of nitrogen. Its chief solid constituent is protein, the principal protein being myosin. In addition to the extractives (mentioned below) 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 extractives include lactic acid, creatine, creatinine, xanthine, hypoxanthine, uric acid, urea, etc. Of the carbohydrates glycogen is the most important, the amount, however, varying with the condition of the animal at death and on the freshness of the meat.

The large percentage of water in meat should be particularly noted. If a man wished to take his daily quantity of 100 grams of protein entirely in the form of meat, it would be necessary for him to consume about 500 grams (1·1 lb.) of meat *per diem*.

FLOUR

White flour is usually 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 embryo 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 (see also Vitamins, p. 92). Brown flour contains a certain amount of bran in addition; it is still less digestible, but is useful as a mild laxative, the indigestible cellulose mechanically irritating the intestinal canal as it passes along.

Flour should contain 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 proceed.

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.

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	20·9
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.

3. Protein, except in potatoes, is fairly abundant, and especially so in the pulses (lentils, peas, etc.). The protein in the pulses is not gluten, but consists mainly of globulins, and is deficient in the cyclic amino-acids. For this reason, despite their high protein content, vegetables are not by themselves suitable as the sole source of protein in the diet.

4. In the mineral matter of 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 at or a little over that of the body, and forms dextrin and sugar from the starch; then alcoholic fermentation, due to the action of the yeast, begins. The bubbles of carbon dioxide, 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 7 to 10 per cent. of protein, 55 per cent. of carbohydrate, 1 per cent. of fat, 2 per cent. 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 the starch proper.

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, while game is frequently kept sufficiently long to undergo a certain amount of decomposition, with the formation of highly flavoured and strongly smelling substances so appreciated by some.

Beef Tea.—In making beef tea and similar extracts of meat the meat is placed in cold water, and this is gradually warmed.

An extremely important point in this connection is that beef tea and similar meat extracts should not be regarded as important 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, besides water, being the salts and extractives mentioned on p. 88.

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.

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.

ADJUNCTS TO FOOD

Among these must be placed *alcohol*, the value of which within moderate limits is not as a food, but as an apparent stimulant; *condiments* (mustard, pepper, ginger, curry powder, etc.) are stomachic stimulants, the abuse of which is followed by dyspeptic troubles; to these must be added *tea*, *coffee*, *cocoa*, and similar drinks which 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 the presence of the alkaloid *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. Coffee differs from tea in being rich in aromatic substances; tea, in addition, contains a bitter principle, tannin. To avoid the injurious solution of too much tannin, tea should be allowed to infuse (draw) for a few minutes only. Cocoa is not only a stimulant, but has a higher nutritive value than either tea or coffee; 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 an adjunct to other foods for two dietetic reasons:—(1) their vitamin content; (2) the fact that they confer bulk to the intestinal contents and thus provide the necessary

"roughage." Their potassium salts are, however, abundant. Cabbage, turnips, and asparagus contain 80 to 92 per cent. water, 1 to 2 per cent. protein, 2 to 4 per cent. carbohydrates, and 1 to 1·5 per cent. cellulose. 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.

VITAMINS

Even if an adult animal is fed on a mixture containing the correct quantities of pure protein, fat, and carbohydrate, to which is added a due admixture of salts and water, it does not thrive, and sooner or later shows evidences of malnutrition, pathological changes occurring which may result in the death of the animal.

That there is something missing from such a dietary which is apparently essential for the well-being of the animal has been recognised for a long time (Captain Cook, in his voyages to Australia, appreciated the fact that fresh fruit juices helped to keep his crews healthy), but it was not until the closing decades of last century that scientists began to interest themselves in such ideas. Out of the mass of evidence which investigators have since produced proving the existence of such substances, probably the most simple that we can put before the student is that presented by Hopkins in one of his earliest studies in this field. The curves shown in Fig. 12 record the results of feeding experiments carried out by this worker on rats. The lower curve traces the average daily weight for eighteen days (up to the point indicated by the vertical broken line) of eight male rats fed on a diet containing pure starch, lard, casein, inorganic salts, and water; the upper curve, the average weight of eight similar rats fed on the same diet to which, however, was added 3 c.c. of milk daily. It will be at once noticed that the rats receiving the milk showed a much more rapid rate of growth than those not receiving milk. On the eighteenth day the ration of milk was switched from one set of rats to the other, and two results almost immediately followed. The rats now receiving the milk commenced to grow rapidly, while those, now deprived of milk, after some days, during which they appear to maintain their weight, began to lose it. The experiment definitely proved that milk possesses a constituent necessary for the growth of the animal.

By experiments such as these, it has been proved that there is a number of such substances each having a specific function in the

animal economy. At the present time the number of these recognised is six, and the existence of others is suspected.

At an early stage of their investigation Funk, who was one of the first workers to attempt their isolation and purification, considered that they were nitrogenous substances of the nature of amines, and, because they were essential for life, he named them **vitamines**. Later work showed the absence of nitrogen from some of them and accordingly, to prevent confusion, the " e " was dropped. Hence **vitamins**.

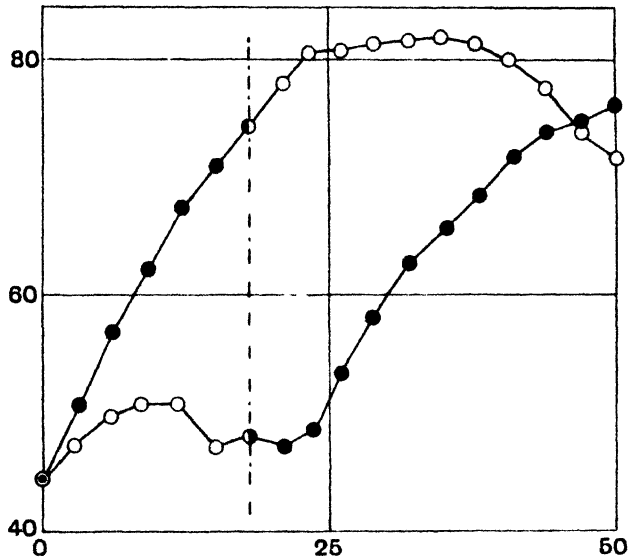


FIG. 12.

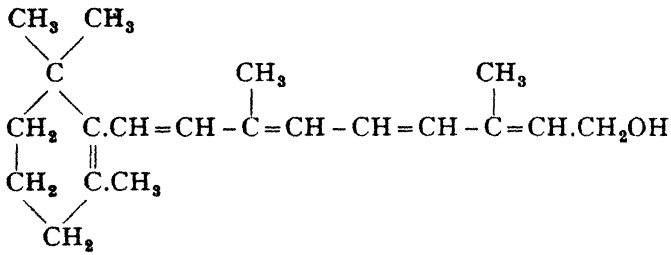
Vitamins were originally divided by M'Collum into two groups, the fat-soluble and the water-soluble vitamins, a classification which is still retained:—

1. Those soluble in fats and fat solvents, viz., A, D, and E.
2. ,, ,, water ,, ,, B_1, B_2, and C.

Space available only permits the briefest of summaries of our knowledge of these vitamins.

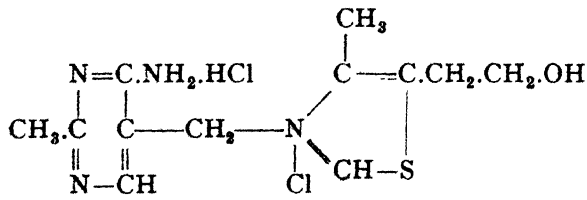
Vitamin A.—Deficiency of vitamin A causes disordered health, chronic conjunctivitis often accompanied by night blindness, xerophthalmia. Its richest source was originally thought to be cod-liver oil, but more is present in halibut oil. In both it is present in the non-saponifiable fraction. Its presence can be detected by the Rosenheim-

Drummond technique involving treatment of a chloroform solution of the fat with antimony trichloride, when, if the vitamin is present, a gentian-blue colour develops. More recently it has been shown that carotene, $C_{40}H_{56}$, can replace vitamin A in the diet, the tissues of the animal apparently being able to transform the carotene into the vitamin. Research has resulted in the following provisional formula being put forward for vitamin A :—



Vitamin B.—By vitamin B is meant the complex which includes vitamins B_1 and B_2 and possibly other related vitamins.

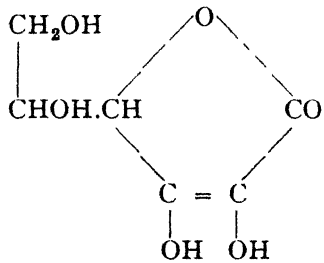
Vitamin B_1 (U.S.A. = F) —This is the " antineuritic " vitamin of the early workers on beri-beri. It is necessary for growth, whilst a diet deficient in it leads to a disordered nervous system. It has, moreover, a positive influence on appetite and metabolism. This was the vitamin which Funk claimed some twenty years ago to have obtained crystalline from a preparation of yeast. Its richest source is brewer's yeast, though it is present in vegetables and fruits (lettuce, carrot, spinach, orange). Milk contains only very small amounts of it. Chemically it is stated to be heat labile, although it seems reasonably stable to the modern processes of cooking and preserving foods. Williams has shown that the constitution of this vitamin in all probability is :—



Vitamin B_2 (U.S.A. = G).—This vitamin is a requisite for growth, deficiency leading generally to skin lesions superficially resembling those seen in pellagra. Nevertheless there is still much doubt whether human pellagra can properly be described as a vitamin deficiency disorder. It forms the heat-stable factor of vitamin B. Kuhn has

shown that, despite the fact that it does not possess marked acidic or basic properties, it can be adsorbed from acid solution on fuller's earth and removed therefrom by dilute pyridine or ammonia. Solutions of the vitamin so prepared possess a strong yellow-green fluorescence. The pigments to which this is due are highly sensitive to light and to chemical treatment, and loss of colour in either of these ways is nearly always accompanied by loss of biological activity. They are termed flavins. Lactoflavin, the pigment of milk, has been crystallised, the crystals showing strong B_2 activity. Analyses of such material suggest that it possesses the formula $C_{17}H_{20}O_6N_4$.

Vitamin C (the anti-scorbutic factor).—It occurs in fruits, especially of the citrus variety, and in vegetables. Amongst animal tissues, as was first shown by Szent-Gyorgyi, the suprarenal cortex is conspicuous for its high vitamin C content. Milk is relatively poor in it. It is soluble in water and in certain organic solvents, *e.g.*, methyl and ethyl alcohols and acetone, but not in fat solvents. It is readily oxidised, especially in alkaline solution, when its activity is destroyed. It is also destroyed in the process of drying and ageing of foods. Hurst *et al.* have proved by synthesis that its constitution is:—



Vitamin D.—The existence of this vitamin was proved in the following way. In 1919 Mellanby described the production of experimental rickets in puppies and at the same time suggested that rickets was due probably to the lack of fat-soluble vitamin A in the diet. Later M'Collum *et al.* showed that whilst cod-liver oil on oxidation lost the power of curing xerophthalmia it still retained, after such oxidation, the power of curing rickets. Hence the existence of the vitamin was established. Meantime Huldshinsky had demonstrated the curing of rickets in children by submitting them to ultra-violet radiations. Then came the observation of Steenbock and Nelson that a ration which ordinarily induced rickets in rats could be made anti-rachitic by simply exposing it to ultra-violet light. It was afterwards shown that the actual ingredient of the diet which was acted on by the radia-

tions was ergosterol (p. 38). Finally, from among the products of irradiation of ergosterol was isolated a crystalline substance (**calciferol**) which, however, despite its antirachitic properties, is probably not identical with the natural vitamin.

Vitamin E is present in the non-saponifiable fraction of the oil from wheat embryo. The seeds of cereals and plants contain an abundance of this vitamin, though certain oils (linseed, coco-nut, palm) are relatively deficient in it. The green leaves of lettuce, spinach, watercress are among the best-known sources of it. It is regarded as the reproductive factor in nutrition. One very remarkable fact concerning it can be mentioned. If a male rat be rendered sterile by withdrawing this vitamin from its diet, it remains in this condition throughout life irrespective of subsequent absence or presence of this vitamin in its diet, whereas the female is sterile only during the period when its diet is deficient, and recovers fertility when vitamin E is added once more to the diet.

CHAPTER V

PHYSICAL CHEMISTRY IN ITS PHYSIOLOGICAL APPLICATIONS

1. DIFFUSION.—(a) The rate of diffusion of a dissolved substance into solidified gelatin is approximately, although not absolutely, identical with that in pure water. Hence we may compare the rates of diffusion of different substances in the following way: A series of test-tubes is filled to the depth of about 10 cm. with a warm 10 per cent. solution of gelatin provided for the purpose. When the gelatin has solidified, add to different tubes one of the following solutions:—

Copper sulphate (10 per cent.).
Eosin (1 in 1000).
Methylene blue (1 in 1000).
Congo red (1 in 1000).
Laked blood.
Soluble Prussian blue.

Allow the tubes to stand for forty-eight hours and then measure the depth to which each substance has penetrated. What connection, if any, can be traced between the depth observed and the molecular weight of the substance?

(b) A similar experiment, slightly different in its results, can be carried out as follows. Dissolve 4 grams of gelatin and 0·15 gram of potassium dichromate in 120 c.c. of warm water. Pour some of the warm mixture into a test-tube and allow it to solidify. Then above the solidified gelatin run very carefully a few c.c. of a half-normal solution of silver nitrate. After forty-eight hours note the brown layers (insoluble silver chromate) which have separated at intervals along the mass of gelatin. This experiment was originally carried out by Liesegang, who placed his gelatin mixture in a Petri dish. In this case the silver chromate was precipitated in the form of concentric rings—*Liesegang rings*—around the spot where the silver nitrate solution was originally placed.

2. DIALYSIS.—A suitable experiment illustrating the process of

dialysis may be carried out as follows:—To 40 c.c. of 2 per cent. starch paste add 4 c.c. of a saturated ammonium sulphate solution and mix thoroughly. Place some of the mixture in one of the collodion bags provided for the purpose, and then suspend it by a piece of thread in a narrow cylinder of distilled water. After an interval of an hour carry out tests on the fluid on the outside of the bag—the dialysate—for the presence of the sulphate ion ($HCl + BaCl_2$) and for starch (iodine, p. 8).

3. **OSMOTIC PRESSURE.**—(a) A careful study should be made of the apparatus, set up in the laboratory, demonstrating osmotic pressure.

(b) *Demonstration of Osmotic Pressure in Red Blood Cells.*—Add two drops of blood to 5 c.c. of each of the following solutions:—Water, 0·3, 0·9, and 5·0 per cent. sodium chloride. Examine a few drops of each suspension under the microscope and explain your findings. In which tubes does the solution become red? Why?

4. **COLLOIDS.**—The colloidal arsenious sulphide provided for the following experiments has been prepared thus:—1 gram of arsenic trioxide was boiled with 150 c.c. of distilled water and the solution cooled. Addition of a further 100 c.c. of water was followed by the saturation of the solution with hydrogen sulphide. The excess hydrogen sulphide was finally removed by blowing nitrogen through the solution.

(a) *Precipitation of Colloids by Electrolytes.*—Into a series of clean test-tubes measure 3 c.c. of the arsenious sulphide provided, and to each tube add 3 drops of one of the following solutions, 0·1M and 0·01M sodium chloride, potassium chloride, calcium chloride, aluminium chloride, potassium sulphate, and sodium citrate. To a second series of tubes containing 3 c.c. of the colloidal iron provided add a similar quantity of these same solutions. Mix the contents of each tube well and allow them to stand. Note in which tubes precipitation occurs. Can this precipitation be related in any way to the valency of the positive or negative ions present in the precipitating solution?

(b) *The Protective Action of Starch and Gelatin.*—To a series of test-tubes each containing 2 c.c. of arsenious sulphide and 0·2 c.c. of a 10 per cent. solution of sodium chloride add the following quantities of a 1 per cent. starch solution: 0·25, 0·5, 0·75 c.c., and so on, up to 3 c.c. Then make the volume of the liquid in each tube up to 6 c.c. Mix the contents of each tube thoroughly and allow the tubes to stand for a few hours. Note what concentration of starch is necessary to prevent precipitation taking place. Instead of a starch solution a 1·5 per cent. solution of gelatin may be used.

5. REACTION OF FLUIDS.—(a) *The Colorimetric Determination of the P_H of a Fluid.*—**Pour 5 c.c. of the $\frac{N}{10}$, $\frac{N}{100}$, $\frac{N}{1000}$, $\frac{N}{10000}$ solutions of hydrochloric acid, sulphuric acid, acetic acid, and sodium hydroxide provided into a series of test-tubes and carefully label them. To the contents of each tube add one to two drops of the Universal Indicator and thoroughly mix. From the data given on the label of the bottle of indicator, determine what is the approximate P_H of each solution. Make a note of your results in your note-book.**

(b) Having roughly determined the P_H of the solutions mentioned in section (a) make a slightly more accurate determination thus:—Run 0·5 c.c. of the solution whose P_H is required into one of the cavities of the "spot" plate provided for the purpose. Making use of the results obtained in (a) select an indicator from those named on p. 125, add one drop of it to the solution on the plate, mix with a clean glass rod, and compare the colour so obtained with those given on the indicator colour chart (Clark's) to be found in the laboratory.[1] Confirm your result by using a second indicator if this is practicable (note that the ranges of certain indicators overlap).

(c) A more accurate method for determining the P_H of a fluid, colorimetrically, is as follows:—Into a test-tube run 5 c.c. of one of the solutions used in section (a) and to it add 5 drops (*i.e.*, **one drop of indicator for each c.c. of fluid) of the appropriate indicator (found by the "spot" plate method). Mix the contents of the tube thoroughly and compare the colour obtained with those of a set of standard P_H tubes containing the selected indicator, made up as described on p. 126. When determining the P_H of a coloured or cloudy fluid** (*e.g.*, **urine) this technique must be slightly modified** (*cf.* **p. 264).**

6. Using the technique which you have acquired, determine the P_H of tap water, distilled water, and freshly boiled distilled water. Why should the P_H of the second and third solutions differ?

7. (a) Take some distilled water and by means of a glass tube blow your expired air through it. Determine the P_H of a portion of the solution at intervals. What is the lowest value the P_H of the solution attains?

(b) Repeat the last experiment, substituting for distilled water the solution of sodium bicarbonate provided. Note the minimum P_H obtained in this case.

[1] Reprints of Clark's Colour Chart of Indicators may be obtained from Messrs Baillière, Tindall & Cox, 8 Henrietta Street, W.C.2.

8. *Titration Curves* obtained by addition of $\frac{N}{10}$ sodium hydroxide to :

(a) $\frac{N}{10}$ hydrochloric acid.

(b) $\frac{M}{10}$ phosphoric acid.

(c) $\frac{M}{10}$ glycine hydrochloride.

Carry out this exercise as follows : Into a test-tube accurately pipette 5 c.c. of $\frac{N}{10}$ hydrochloric acid, the P_H of which has already been determined (5 (*c*)). To it add 5 drops of thymol blue. Then from a burette run in, accurately, 0·5 c.c. $\frac{N}{10}$ sodium hydroxide. Mix the contents of the test-tube and determine, by comparison with the standard P_H indicator tubes provided, the P_H of the solution. Then to the same test-tube add a second 0·5 c.c. of $\frac{N}{10}$ sodium hydroxide and an extra drop of indicator. Again, after mixing the contents of the tube thoroughly, determine the P_H against the standard. Continue the addition of the sodium hydroxide, determining the P_H after each addition. Care must be exercised in noting when the P_H of the solution can no longer be determined by reference to the thymol blue standard. When such a stage is reached take a second test-tube, pipette into it 5 c.c. of $\frac{N}{10}$ hydrochloric acid, add a volume of $\frac{N}{10}$ sodium hydroxide equivalent to the sum-total (x c.c.) of the previous additions, then $(5 + x)$ drops of brom-phenol blue and determine the P_H of the solution. To the mixture add 0·5 c.c. of $\frac{N}{10}$ sodium hydroxide, mix the contents and again determine the P_H. Continue the addition of the $\frac{N}{10}$ sodium hydroxide until you have added 5 c.c. in all, taking care to use the appropriate indicators (*cf.* table on p. 125). Having completed the determinations plot points on graph paper showing the relation between the amount of $\frac{N}{10}$ alkali added and the P_H of the solution obtained. Connect the points and obtain the titration curve.

Using the same technique construct titration curves on the same graph paper from data obtained by the addition of $\frac{N}{10}$ sodium hydroxide

to 4 c.c. $\frac{M}{10}$ phosphoric acid and $\frac{M}{10}$ glycine hydrochloride solutions. In both these cases carry your readings as near as you can to P_H 10.

The curves which you obtain do not intersect exactly at P_H 7, but starting from that point compare the extent to which the addition of equal amounts of acid and alkali affects the P_H of (a) water, (b) a solution containing equal amounts of sodium dihydrogen phosphate and disodium hydrogen phosphate.

As an exercise try to determine from the appropriate curve what is the P_H of an $\frac{M}{10}$ solution of sodium dihydrogen phosphate and of an $\frac{M}{10}$ solution of disodium hydrogen phosphate. Check your results by actually determining by the above method the P_H of each of these solutions.

9. TO DETERMINE THE ISO-ELECTRIC POINT OF CASEIN.—Prepare a series of solutions in test-tubes according to the following data :—

Number of tube .	1	2	3	4	5	6	7	8	9
Distilled water, c.c. .	8·38	7·75	8·75	8·5	8·0	7·0	5·0	1·0	7·4
0·01N acetic acid, c.c.	0·62	1·25	...	...	...	...	...	...	...
0·1N acetic acid, c.c.	...	...	0·25	0·5	1·0	2·0	4·0	8·0	...
N acetic acid, c.c. .	...	...	...	...	...	...	...	...	1·6

Into each tube pipette 1 c.c. of the casein solution provided [1] and mix immediately. Note the presence or absence of a turbidity on mixing and after intervals of five and ten minutes. Record your results thus :—

No change	0
Opalescence	+
Slight turbidity	×
Slight precipitation	× ×
Heavy precipitation	× × ×

Determine the P_H of all the solutions, previously filtering where necessary. The P_H of the solution in which the heaviest precipitation occurs corresponds to the *iso-electric point of casein.*

[1] The solution of casein required is prepared thus :—To 0·25 g. of pure casein in a 50 c.c. volumetric flask add 20 c.c. of water and 5 c.c. N sodium hydroxide. On solution of the casein add 5 c.c. N acetic acid and make up to volume. Mix well.

SOLUTIONS

The importance of water for the living organism requires no emphasis. It constitutes on the average two-thirds of the animal body, and on its presence depends the successful performance of the numerous processes involved in the phenomena called life. In this connection it may well be recalled that while the organism may continue to exist without solid food, in some instances for so long a period as 40 to 50 days, yet total abstinence from water leads to a very early cessation of life.

Water, at ordinary temperatures, is a fluid. Its molecules are in constant motion, the rate of which increases with rise of temperature. Perfectly pure water consists of molecules, the formula of which is represented by $(H_2O)_n$. These molecules undergo practically no dissociation into their constituent atoms, and, for this reason, pure water is not a conductor of electricity.

Substances which are soluble in water may be divided into two classes according to their behaviour when dissolved in water.

For example, if a substance such as sodium chloride is dissolved in water, the solution is then capable of conducting electrical currents, and the same is true for most acids, bases, and salts. These substances 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 becomes 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 the sulphate ion SO_4. The term ion is thus not equivalent to atom, for an ion may be a group of atoms, such as SO_4 in the example just given.

Further, in the case of hydrochloric acid, the negative charge on the chlorine ion is equal to the positive charge on 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 of an electrolytic cell; 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. Compounds which exhibit this property of dissociation into ions, when dissolved in water, are known as **electrolytes.**

All electrolytes, when dissolved in water, do not dissociate to the same extent. Hence we may make a further division of this class. One of these subdivisions includes such compounds as hydrochloric acid, sulphuric acid, and sodium hydroxide—substances which are almost completely dissociated in dilute solution; the other, compounds like acetic acid and such substances as indicators which do not dissociate completely even with extreme dilution. Generally speaking, the former group contains **strong acids** and **strong bases**; the latter, **weak acids** and **weak bases.** Water, as will be seen later, is both a weak acid and a weak base.

On the other hand, there are certain substances which, when dissolved in pure water, do not become ionised and hence the resulting solutions remain incapable of conducting an electrical current. An example of such a substance is sugar. The sugar molecules in solution are still sugar molecules; they do not undergo dissociation. Such substances are called **non-electrolytes.**

How important a rôle this conception of electrolytic dissociation, which we owe to Arrhenius, plays in physiological chemistry will become evident when we come to study such subjects as osmotic pressure and the reaction of fluids.

The liquids of the body contain electrolytes in solution, and it is owing to this fact that the body is 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, ciliæ, 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 heart, the sinus, or venous end of the heart, is peculiarly susceptible to the stimulus of 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 argue that the results obtained are due to the action of ions. 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 the most favourable stimuli to them. Thus cardiac muscle, ciliæ, amœboid movement, karyokinesis, cell division are all alike in requiring a proper adjustment of the ions in their surroundings if they are to continue to function, but the proportions must be different in individual cases.

In 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 due to 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 such substances as 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 which 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.

Gram-molecular Solutions.—From the point of view of osmotic pressure a convenient unit is the gram-molecule, and before we proceed further it is necessary to define certain terms which we shall

constantly be using. A gram-molecule of any substance is the quantity in grams of that substance equal to its molecular weight. A gram-molecular solution is one which contains a gram-molecule of the substance per litre. Thus a gram-molecular solution of sodium chloride is one which contains 58·46 grams of sodium chloride (Na = 23·00; Cl = 35·46) in a litre. A gram-molecular solution of glucose ($C_6H_{12}O_6$) is one which contains 180 grams of glucose in a litre. A gram-molecule of hydrogen (H_2) is 2 grams by weight of hydrogen, and if this were compressed to the volume of a litre it would be comparable to a gram-molecular solution. It therefore follows that a litre containing 2 grams of hydrogen contains the same number of molecules as a litre which contains 58·46 grams of sodium chloride, or one which contains 180 grams of glucose. 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 gram-molecular solution.

DIFFUSION, DIALYSIS, AND 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 almost excluded in such experiments by placing the membrane (M) vertically as shown in the diagram (Fig. 13), and one of the two fluids A and B on each side of it. Diffusion through a membrane is not

makes no difference; it is only the number of molecules which causes the 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 dissociated 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 pressure 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 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.

One gram of hydrogen at atmospheric pressure and 0° C. occupies a volume of 11·2 litres; 2 grams of hydrogen will therefore occupy a volume of 22·4 litres. A gram-molecule of hydrogen—that is, 2 grams 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 gram-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 gram-molecular solution of sucrose ($C_{12}H_{22}O_{11}$) contains 342 grams of sucrose in a litre. A 1 per cent. solution of sucrose contains only 10 grams 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 up to which we have been leading, 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 the aqueous solution of any substance 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 gram-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 freezes at $-0{\cdot}052°$C.; its osmotic pressure is therefore $\frac{0{\cdot}052 \times 17{,}024}{1{\cdot}87} = 473$ 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 0·9 per cent. solution of common salt have the same osmotic pressure, or are **isotonic.** The osmotic pressure of blood serum is $\frac{0{\cdot}56 \times 17{,}024}{1{\cdot}87}$

=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, as was done in the practical section, 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 shrinkage of the protoplasm in the vegetable cell occurs; if red corpuscles are used they swell, ultimately breaking their walls and so liberating 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.

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. Theoretically it is difficult to imagine that a pure protein can exert more than a minimal osmotic pressure. Protein is made up of such huge molecules that, even when present to the extent of 7 or 8 per cent., as they are in blood-plasma, there can be 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

[1] Bayliss showed 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.

materials pass into the lymph, and increase its molecular concentration and its osmotic pressure; thus water is attracted (to employ the older view) 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.

But still there remains a difficulty with the proteins; they are 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. We can but state, however, that the exact mechanism of lymph-formation is one of the many physiological problems which await solution by the physiologists of the future.

Donnan's Theory of Membrane Equilibria.—In more recent times, many of the phenomena discussed above have been more easily understood in the light of a theory first put forward by Donnan regarding ionic equilibra. Briefly the theory defines the relations which will exist between the ions present in two solutions of electrolytes separated by a membrane which is impermeable to one of the ions. Donnan considered, in the first case, two electrolytes NaR (R being the non-diffusible ion) and NaCl on opposite sides of a membrane, such as has been pictured in Fig. 13 and represented below by a vertical line.

Na^+	Na^+
R^-	Cl^-
(1)	(2)

When the system reaches equilibrium, the following condition will exist:—

Na^+	Na^+
R^-	
Cl^-	Cl^-
(1)	(2)

It has been shown, both practically and theoretically, that in such circumstances the products of the concentrations of the diffusible ions on each side of the membrane are equal. To maintain electrical neutrality, however, the concentration of the kation Na^+ in compartment (I) must equal the sum of the concentrations of anions R^-

and Cl^-. In compartment (2), on the other hand, the concentrations of the kation Na^+ and the anion Cl^- are equal. But according to Donnan's equation

$$[Na^+]_1 \times [Cl^-]_1 = [Na^+]_2 \times [Cl^-]_2$$

hence $[Na^+]_1 > [Na^+]_2$

and $[Cl^-]_1 < [Cl^-]_2$

Such an inequality of the distribution of ions on opposite sides of a membrane is frequently met in biological systems, and helps us to understand the production of acid secretions by the kidney and gastric cells and of the differences between the concentrations of salts in blood and in cerebrospinal fluid and of those existing within and without the red cells of the blood.

Physiological Applications.—It will be seen how important all these considerations are from the physiological standpoint In the body, aqueous solutions of various substances are separated from one another by membranes. Thus the endothelial walls of the capillaries separate the blood from the lymph, the epithelial walls of the kidney tubules separate the blood and lymph from the urine; similar epithelium form an important part of all secreting glands; the wall of the alimentary canal separates 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 the 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. 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 at best a highly unsatisfactory and unscientific term. 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 we know it sometimes operates so as to neutralise the known forces of osmosis and filtration. (See also Permeability, p. 119.)

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, another battle-field of a similar kind, has been shown, by recent research, to be explicable on a physical basis (pp. 226 to 229). 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 since the readily diffusible substances will pass more easily through into the blood and lymph. It has been shown, however, that 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 should increase the facilities for osmosis and filtration.

COLLOID SUBSTANCES AND SOLUTIONS

The proteins and the polysaccharides 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, if they crystallise it is only with difficulty, 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 substance 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 it " sets " and then we get a gel. In the case of gelatin the condition is easily reversible, but this is not so with 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 yet another condition, 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 has been held responsible for the alteration in the physical (and possibly chemical) structure of the protein so that it becomes insoluble in the fluid in which it was previously apparently dissolved. (See Milk Curdling, p. 131; and Blood Clotting, p. 181.)

There are numerous analogies between these organic and inorganic colloids. Thus many 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, some 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.

Considering now the case of a colloid in the condition of a sol, as for instance the proteins 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 under the highest magnification, reveals no visible particles. The particles, 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 possesses too large pores to keep back any such fine particles from these 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 Sir 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 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.

If fresh serum or egg-white is placed on the outside of such a

filter, a filtrate, which is clear, colourless, and absolutely free from protein, is obtained.

Proteoses and crystalloids pass through the membrane easily; metaproteins slightly; caramel, biliverdin, and dextrins partially; while carbohydrates such as starch and glycogen and proteins such as egg-albumin, serum-albumin, egg-globulin, serum-globulin, fibrinogen, caseinogen, nucleo-proteins, and hæmoglobin do not pass through.

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. Some workers are inclined to regard the large size of the molecule as the reason for 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 not 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 has been shown with 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 Faraday phenomenon of light scattering; 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, the precipitating ions being

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 still require elucidation.

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. It is possible, as research proceeds, that other exceptions to the general rule may be found, and that 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. In its interior the arrangement of matter is symmetrical around any point, whereas on its surface the surroundings consist of liquid on one side only, while on the other side is solid or gas, or even 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. This layer constitutes a stretched elastic skin, and the power exerted by it 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 surface is smallest; *i.e.*, a sphere.

Animal cells are liquid, and when they are at rest, other forces being

absent, they also are spherical, and although 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. At points where the surface tension is lowered, pseudopodia are 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—the membrane 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. The latter process comes into play specially 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. On such considerations is based the Meyer-Overton theory of the narcotic effect which these volatile anæsthetics exercise on cells.

On p. 158 mention is made of the rôle played by bile salts in lowering the surface tension of the liquid in which they are dissolved. It is sometimes necessary to estimate the surface tension of a physiological liquid. This may be done in several ways: (1) By noting the rise or fall of the liquid in a capillary tube. (2) By suspending from

one arm of a balance a glass slide with one edge just in the surface film of the liquid; the liquid is removed and weights are added to counterpoise the slide in air; the weight added gives, by simple calculation, the surface tension of the liquid. (3) By counting the number of drops which compose a given volume of liquid when allowed to flow from a tube. As the number of drops is inversely proportional to their size, and as the drops will increase in size until their weights are equal to the tension of their surface layers, the size of the drop is proportional to the surface tension. Thus by counting the number of drops and by knowing the specific gravity of the liquid the surface tension can be determined. The apparatus used in this method is termed a " stalagmometer," and consists essentially of a glass pipette with a plane circular tip. The drops which escape when the pipette is allowed to deliver a constant volume are counted and compared with the number in an equal volume of water at the same temperature.

VISCOSITY

The particles of a liquid are not free to move one upon the other in the liquid without resistance. This " internal friction " is termed *viscosity*, and is of importance in the flow of liquids such as blood through small tubes. Viscosity in fact gives rise to the resistance which is offered to the passage of blood in the capillary system. A convenient method of determining the viscosity of a liquid is that introduced by Ostwald. This consists in comparing the time taken by a certain volume of the liquid under examination to flow between two marks on a capillary tube with that taken by an equal volume of water, both observations being made at the same temperature. The usual form of the apparatus is a U tube, one limb of which is narrow and has at its distal portion a bulb above and below which are the two marks referred to. Colloidal solutions such as blood-plasma, gum arabic, gelatin, etc., have a high viscosity, *i.e.*, they flow slowly, whereas dilute salt solutions have viscosities little different from water. It is, in part at least, the viscosity of a solution of gum in 0·9 per cent. sodium chloride which makes it so much better than the salt solution alone as a solution to be injected intravenously to replace blood lost, for example, in severe hæmorrhage. Owing to this physical property it is not removed from the circulation very rapidly by the kidneys.

PERMEABILITY

The usual way of explaining the action of a secreting cell is to say that the cell has selective power. On one side it is bathed by a nutrient fluid originating from the blood; on the other it pours out a new fluid, the secretion. The statement that the cell selects from the lymph certain materials to make saliva or gastric juice, and rejects others, is merely a handy way of explaining in everyday language the final result. It does not mean that physiologists really think that the cell possesses something akin to consciousness or choice. The passage of substances through cells and their membranes cannot be due entirely to the forces of diffusion, osmosis, and filtration; but another factor, the *permeability* of the cell-membrane and of the protoplasmic surface, comes also into play. The cell is permeable to certain substances and not to others. It has no real choice as to what shall pass through it and what it keeps back. It has been found that different ions modify the normal permeability in various directions. The electrical charge of the ions must be a determining factor in the passage of substances through the cells and its plasmatic membrane. An upset of the normal ionic balance leads to altered permeability; hence cellular activity becomes abnormal in disease. Their considerations may be exemplified by what is known in relation to glucose. This sugar is always present in the blood in health, when it is found wholly in the plasma; the corpuscles are impermeable to it, but in diabetes they become permeable. That this is not due to the mere size of the glucose molecule is seen when we go to the kidney, for in health the renal cells are practically impermeable to glucose, and it is not until normal conditions are upset in diabetes that the kidney cells allow this sugar to pass into the urine in detectable amounts. Nevertheless, sugars with still heavier molecules such as sucrose or lactose if introduced into the blood-stream find their way out into the urine even in health. No mere " sieve " theory will account for this, and the " lock and key " simile gives a better representation. The chemical configuration of the sucrose molecule is such that it has the key to unlock the protoplasmic door and get through: glucose has a different configuration and so cannot pass the barrier so long as the latter is in perfect health.

THE REACTION OF FLUIDS; HYDROGEN-ION CONCENTRATIONS

By the phrase " the reaction of a fluid " we mean that the fluid is acid, alkaline, or neutral with reference to some indicator. Litmus,

it is true, is the indicator which has been, and is, largely used in making such determinations, but, as we shall see later, its use is limited and resource has often to be made to other indicators. Hence the necessity of stating the indicator used in any estimation.

The mere statement that a fluid is acid or alkaline (say to litmus) gives us no clue as to the magnitude of its acidity or alkalinity. For example, two fluids might both turn red litmus blue; yet this reaction cannot possibly show us whether the two fluids have the same alkalinity, or if one is more strongly alkaline than the other. Such a comparison is sometimes one of considerable importance. Moreover, it is often necessary to make a solution of a certain degree of acidity, and the question at once arises what is a measure of the acidity we wish to attain. For, as will become evident later, the acidity produced by adding 10 c.c. of normal hydrochloric acid to 90 c.c. of water is not necessarily and, indeed, is seldom the same as that produced by mixing like volumes of acid and of a physiological solution, *e.g.*, gastric juice. Before we can deal with such differences, however, we must define and explain certain nomenclatures.

In the time of Lavoisier, acidity was attributable to the presence of oxygen. This was ultimately shown to be incorrect since certain substances, which do not contain oxygen, nevertheless gave an acid reaction when dissolved in water. Soon it was shown that an acid solution owed its typical properties to the presence therein of an excess of *hydrogen* ions, and an alkaline solution to an excess of *hydroxyl* ions. Once this stage was reached, our knowledge of the reaction of fluids became much more definite.

Pure water, as was stated early in this chapter, is practically un-ionised. Yet is it not absolutely free from hydrogen and therefore hydroxyl ions. These owe their existence to the electrolytic dissociation of a small number of water molecules. The process of ionisation of water molecules is not static, reaching a certain stage after a fixed period, but one which must be regarded as continuous, the number of hydrogen and hydroxyl ions present at any one moment being limited by the reverse process of re-combination of these ions to form molecules of water. Such a process as the ionisation of water is therefore reversible and may be represented by the following equation:—

$$H_2O \rightleftarrows H^{\cdot} \times OH'.$$

For all such conditions of unstable chemical equilibrium as here exists between the hydrogen and hydroxyl ions on the one hand, and

the molecules of water on the other, the law of mass action states that at a fixed temperature the product of the concentrations of all the reactants on one side of the equation bears a constant ratio to the product of the concentrations of the reactants on the other. In other words, in the case of the equilibrium arising from the ionisation of water, the product of concentration of hydrogen ions and the concentration of hydroxyl ions will bear a constant ratio (say k) to the concentration of water molecules. Now, as we have seen on p. 104, the concentration of each reactant may be measured in gram-molecules (or moles) per litre, such concentration being indicated by enclosing the chemical symbol in brackets. For example, [H] means a concentration of 1 gram of hydrogen per litre. So that, applying the law of mass action, we may represent the ionisation of water by the equation :—

$$[H^{\cdot}] \times [OH'] = k[H_2O],$$

or

$$\frac{[H^{\cdot}] \times [OH']}{[H_2O]} = k \text{ (dissociation constant of water).}$$

Remembering that the amount of water thus ionised is very small, we may regard the concentration of undissociated water molecules as remaining constant. Hence we may write

$$[H^{\cdot}] \times [OH'] = k_w.$$

In other words, at any given temperature the product of the concentrations of hydrogen and hydroxyl ions is always the same. *This holds true for any aqueous solution.* In the case of pure water $[H^{\cdot}] = [OH']$, so that we may write $[H^{\cdot}]^2 = k_w$. In other words, if we determine the concentration of hydrogen ions in pure water we can find a value for k_w. Such a determination can actually be made by a variety of methods, and it has been found that

$$k_w = \frac{1}{10^{14}} \text{ or } 10^{-14}.$$

Therefore $[H^{\cdot}]$ which equals $[OH']$ equals 10^{-7}; in other words, a litre of pure water contains one ten-millionth of a gram of hydrogen ions.

Now let us see what happens when we add some hydrochloric acid to pure water. The acid, as we have seen, almost completely dissociates yielding ions of hydrogen and chlorine. Hence the concentration of hydrogen ions present in the solution is considerably

increased. The presence of these additional hydrogen ions upsets the equilibrium depicted in the above equation representing the dissociation of water, and this results in the reaction moving in a direction right to left. In other words, re-combination of some of the hydrogen and hydroxyl ions takes place to form undissociated molecules of water. Thus two results follow when hydrochloric acid is added to water; firstly, there is an increase in the concentration of hydrogen ions; and, secondly, a decrease in the concentration of hydroxyl ions. Hence, if we determined the hydrogen-ion concentration of tenth-normal hydrochloric acid we would find it in the close neighbourhood of 10^{-1}.

We could arrive at much the same conclusion as that reached in the last paragraph in the following way. We have seen that the product of [H·] and [OH′] for *any aqueous* solution is constant, being equal to 10^{-14}. If therefore the hydrogen-ion concentration is increased by the addition of hydrochloric acid, and the product is to remain constant at 10^{-14}, then the hydroxyl-ion concentration must decrease.

Using much the same sort of argument, we might show, on the other hand, that by the addition of a base, like sodium hydroxide, to the water, the concentration of hydroxyl ions increases, the hydrogen-ion concentration decreasing. Thus the hydrogen-ion concentration of tenth-normal sodium hydroxide is near 10^{-13}.

Briefly we might summarise what we have learnt by saying :—

If [H] = [OH], the solution is neutral.
If [H] > [OH], the solution is acid.
If [H] < [OH], the solution is alkaline.

The value of the concentration of hydrogen ions, especially in biological fluids, is always very small, and to overcome the unwieldy sets of figures which the expression of such concentrations entails, it has become customary to deal with hydrogen-ion concentrations in logarithmic terms. This idea was originally introduced by Sörensen. According to this procedure, instead of saying that the hydrogen-ion concentration or *c*H of water is 10^{-7}, one says it has a hydrogen-ion exponent or P_H of 7. It will be noticed that the index alone is used, its negative sign being dropped. Thus a solution with a *c*H of $10^{-5\cdot6}$ has a P_H of 5·6. It follows that an acid solution, that is, one with a high hydrogen-ion concentration, will have a low P_H; an alkaline solution with a low hydrogen-ion concentration will have a high P_H.

It will be useful at this point to quote the P_H of some better-known solutions:—

Solutions.	P_H
$\frac{N}{10}$ hydrochloric acid	1
$\frac{N}{100}$ hydrochloric acid	2
$\frac{N}{1000}$ hydrochloric acid	3
$\frac{N}{10}$ acetic acid	2·87
$\frac{N}{10}$ sodium hydroxide	13
Adult gastric juice	1·2
Urine	5·5 to 8·0
Saliva	6·2 to 7·6
Blood	7·28 (average)
Pancreatic juice	8·2

BUFFER SOLUTIONS

Suppose one takes a litre of pure water (P_H7) and adds to it 1 c.c. of a normal solution of hydrochloric acid, a solution is obtained whose normality is approximately one-thousandth normal, and whose P_H is 3. A similar sort of shift of the P_H of water could also be produced by saturating it with carbon dioxide. Addition of alkali on the other hand would produce a shift from P_H7 towards P_H14. Luckily all solutions, and especially physiological ones, do not exhibit such large shifts of the P_H when small amounts of acid or base are added to them. Such solutions are called *buffer solutions*. The principle on which they work is very briefly as follows. Carbon dioxide, dissolved in water, yields carbonic acid, which, being a *weak* acid, only partially dissociates thus:—

$$H_2CO_3 \rightleftarrows H^{\cdot} + HCO'_3.$$

This is a reversible reaction and, at any one temperature, only a certain concentration of hydrogen and bicarbonate ions may exist in the solution. Hence if we increase the concentration of bicarbonate ions in such a solution, the equilibrium will be temporarily upset and the reaction will move from right to left, *i.e.*, a re-combination of hydrogen and bicarbonate ions will take place with the formation of undissociated molecules of carbonic acid. The hydrogen-ion concentration

will thus be lowered. In other words, the P_H would be increased. Such an increase of bicarbonate ions may be obtained by dissolving some sodium bicarbonate in the water. This salt is highly dissociated according to the equation:—

$$NaHCO_3 \rightleftarrows Na' + HCO'_3.$$

Thus the presence of sodium bicarbonate in the solution would depress the ionisation of the carbonic acid. It follows, therefore, that if we saturate water and a solution of sodium bicarbonate with carbon dioxide, the pure carbonic acid solution will be more acid than that containing sodium bicarbonate. Or, to use the P_H formula, its P_H will be lower. Such a substance as sodium bicarbonate is a buffer; its solution in water constitutes a buffer solution.

Other buffer solutions of great value in physiological work are those containing a mixture of acetic acid and sodium acetate, sodium dihydrogen phosphate and disodium hydrogen phosphate, potassium hydrogen phthalate and phthalic acid.

The importance of such substances in the animal organism will readily be grasped when a study of the blood is made. Here we can only refer to the fact that despite the presence in the blood, in different parts of the circulation, of varying quantities of carbon dioxide, the P_H of the blood itself varies only within very narrow limits, *e.g.*, 7·25 to 7·35.

Buffers are of great use, also, in bacteriological work where it is often necessary to maintain a medium or a solution at a constant P_H.

THE DETERMINATION OF P_H BY INDICATORS

The determination of the H-ion concentration of a solution is a constantly increasing practice in physiological and pathological work. There are various methods of carrying out such determinations. Some of these involve the use of expensive electrical apparatus, a description of which is outside the scope of an elementary book such as this. For most purposes, moreover, the much simpler methods involving the use of indicators give rapid and accurate information.

An *indicator* is a very weak acid or weak base which on dissociation yields ions, one of which has a colour different from that of the undissociated molecule. For example, the weak acid indicator HX, whose undissociated molecule is yellow, may dissociate, giving an ion X′ which is red; thus

$$\underset{\text{[yellow]}}{HX'} \rightleftarrows H' + \underset{\text{[red]}}{X'}.$$

The colour of the solution to which the indicator HX has been added will therefore depend on the extent of the dissociation of the indicator in that particular solution. If no dissociation occurs, the solution will be pure yellow; if partial dissociation takes place, the colour will be a mixture of yellow and red; if, finally, dissociation is complete, then the colour will be a pure red. Let us see how these different degrees of dissociation of the indicator may come about. The above equation represents a reversible reaction, and an equilibrium will arise according to the concentration of hydrogen ions or hydroxyl ions (from the dissociation of other compounds) in the solution in which the indicator is placed. If, for instance, the hydrogen-ion concentration is high owing to the presence of a highly ionised acid like hydrochloric acid, some of the hydrogen ions will recombine with the X ions to form undissociated (yellow) molecules of the weak acid indicator. This process might continue until there were present in the solution no undissociated molecules of indicator, when, of course, the solution would be a pure yellow. On the other hand, should the concentration of the hydrogen ions in the solution be lower, *i.e.*, the concentration of hydroxyl ions be higher, such hydroxyl ions will combine with the hydrogen ions (arising from the dissociation of the indicator) to form undissociated molecules of the much weaker acid, water. Such a dissociation may continue until all the indicator is ionised. The solution would then be coloured pure red. The dissociation of the weak basic indicators in solutions of different hydrogen-ion concentrations might be explained in this same very elementary way.

Indicator.	Range of P_H.	Colour Change.
Thymol blue (acid range)	1·2 to 2·8	Red to yellow.
Brom phenol blue	3·0 ,, 4·6	Yellow to blue.
Brom cresol green	3·8 ,, 5·4	Yellow to blue.
Methyl red	4·4 ,, 6·0	Red to yellow.
Brom cresol purple	5·4 ,, 7·0	Yellow to purple.
Brom thymol blue	6·0 ,, 7·6	Yellow to blue.
Phenol red	6·6 ,, 8·2	Yellow to red.
Cresol red	7·2 ,, 8·8	Yellow to red.
Thymol blue (alkaline range)	8·0 ,, 9·6	Yellow to blue.
Phenolphthalein	8·3 ,, 10·0	Colourless to red.

It will thus be realised that for any one indicator there exists a particular P_H at which no dissociation of its molecules occurs, and a second where complete dissociation takes place; between these

extreme ranges the indicator will be only partially dissociated. Now if such a series of solutions of varying P_H is made up, and to each solution is added a fixed amount (1 drop per c.c. of solution is the usual amount) of an appropriate indicator (one selected from the list of indicators given above), then there will be obtained a range of colours varying from, say, pure yellow at one extreme to pure red at the other, and consisting, between, of varying mixtures of yellow and red. It will be at once evident that in such a series of tubes we have a series of standards against which can be compared the colour obtained by adding the same indicator to a solution whose P_H is required. In other words we have a ready method of determining the P_H of unknown solutions. Standard tubes of known P_H are an essential part of the method, and for this purpose Clark and Dubs' buffer solutions made up according to data to be found in any standard text-book on the subject are often utilised.

One of the chief disadvantages attached to such a procedure is the amount of preparation involved in making up the buffer solutions, especially since these are not absolutely stable, and must be remade at intervals. This objection to the method is largely overcome by the adoption of a procedure, devised by Shore in this laboratory, in which use is made of the Universal Buffer mixture of Prideaux and Ward. This mixture dissolved in a given amount of water yields a solution whose $P_H = 3{\cdot}1$. Since the titration curve of the solution is a straight line throughout the range P_H 2·7 to 11·4, the addition of each cubic centimetre of 0·2N sodium hydroxide or hydrochloric acid to the solution will change its P_H by a fixed amount, *i.e.*, $\pm 0{\cdot}1185$. Shore, using the Universal Buffer solution as her starting-point, first makes two solutions, each of known P_H, one constituting the most acid solution required, the other the most alkaline. By mixing these two solutions in varying proportions, the volume of the mixtures being kept constant throughout, solutions of intermediate P_H values are obtained, *e.g.*,

P_H required	3·1	3·3	3·5	3·7	3·9	4·1
Cubic centimetres of acid constituent (3·1)	10	8	6	4	2	0
Cubic centimetres of alkaline constituent (4·1)	0	2	4	6	8	10

Longer ranges can be obtained simply by using an alkaline constituent of higher P_H. Solutions of P_H values below 3·1 can be made of sufficient accuracy for class purposes by mixing solutions of

$\frac{N}{10}$ hydrochloric acid ($P_H = 1$) and of $\frac{N}{1000}$ hydrochloric acid ($P_H = 3$) in a similar way to that described above.

It may be added that more accurate methods of determining the P_H of fluids by the calorimetric method have been devised by different workers, *e.g.*, Michaelis, Dale and Evans, Hastings, Sendroy and Robson. The results obtainable by such methods compare very favourably with those obtained by the more expensive hydrogen electrode.

Finally, where only an approximate determination of the P_H is required, use can be made of Clark's admirable colour chart mentioned in the practical section of this chapter.

AMPHOLYTES; ISO-ELECTRIC POINT

Ampholytes or amphoteric substances are those which possess the function both of an acid and of a base. Thus they can form salts either with bases or with acids. In solutions of high hydrogen-ion concentration they act as bases; in low hydrogen-ion concentrations as acids. For each ampholyte, however, there is one particular hydrogen-ion concentration where its ability to function either as an acid or as a base is reduced to a minimum. Such a hydrogen-ion concentration is called its *iso-electric point*.

It has already been shown that proteins and most amino-acids are ampholytes.

Space does not permit a full description of the behaviour of ampholytes at their iso-electric point. Nevertheless, it is very important to note that at its iso-electric point an ampholyte

1. Is generally least soluble.
2. Exhibits minimum swelling.
3. Migrates neither to the kathode nor to the anode in an electrolytic cell.

CHAPTER VI

THE DIGESTIVE JUICES

SALIVARY DIGESTION

1. The reaction of human saliva [1] usually ranges from P_H 6·2 to 7·6.

2. Apply colour tests for protein (p. 43) to some of the saliva collected.

3. To a few c.c. of saliva add 0·2 per cent. acetic acid drop by drop until the mucin is completely precipitated. Heat to boiling to complete the precipitation of the protein. Filter and test the filtrate for chlorides, sulphates, phosphates, and calcium.

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. *Action of Saliva on Starch.*—Place 20 c.c. of 0·5 per cent. starch solution into a large boiling tube immersed in a water-bath at 40° C. To this add 10 drops of saliva, with thorough mixing. Note the time. At intervals of one minute examine for the presence of starch by testing a drop on a procelain tile with dilute iodine. Simultaneously add 1 c.c. of the digestion liquid to 5 c.c. of Benedict's solution and test for reducing sugar (p. 6). Note (1) the time taken for the original opalescence of the starch to disappear; (2) the time taken before reducing sugar can be demonstrated; (3) the time elapsing before the starch-iodine reaction is no longer positive. This last time is the Achromic Period, and at the end of this period the Achromic Point is reached. When the Achromic Point is reached from the remainder of the digestion mixture prepare the phenyl osazone of the sugar present (p. 8). Examine microscopically and identify the osazone.

6. EFFECT OF H-ION CONCENTRATION ON ACTIVITY OF PTYALIN.—Using solutions of $\frac{M}{3}NaH_2PO_4$(A) and $\frac{M}{3}Na_2HPO_4$(B)

[1] To collect fresh saliva, first rinse out the mouth with distilled water and then chew a piece of paraffin wax to induce a flow of saliva.

(p. 126), make up a series of solutions of P_H values ranging from 4·5 to 9·5 in the following way :—Take eleven test-tubes and add to each the above phosphate solutions in the quantities given.

Number of Tube . .	1	2	3	4	5	6	7	8	9	10	11
Cubic centimetres of A .	10	9	8	7	6	5	4	3	2	1	0
Cubic centimetres of B .	0	1	2	3	4	5	6	7	8	9	10

Mix each thoroughly and by means of the indicator method (p. 99) determine the P_H of each solution. To each tube add 1 c.c. of starch solution (5 per cent.) and 1 c.c. of saliva diluted 1-200. Mix each rapidly and keep at 40° C. in a water-bath.

Follow the course of the digestion in each tube by means of the Iodine Reaction. Plot the Achromic Periods of each tube against its P_H in the form of a graph and determine the P_H at which ptyalin acts most rapidly, *i.e.*, its Optimum Reaction. Why is the curve not so steep on one side of the optimum point as on the other ?

7. EFFECT OF TEMPERATURE ON ACTIVITY OF PTYALIN.—To each of four test-tubes add 5 c.c. of 5 per cent. starch solution. Place tube 1 in a boiling-water bath, 2 in a water-bath at 40° C., 3 at room temperature, and 4 in a mixture of ice and salt. When each is at the temperature of its surroundings add to each 1 c.c. of undiluted saliva. Follow the digestion by the iodine test. What is the most favourable temperature for the action of ptyalin ?

GASTRIC DIGESTION

1. Prepare five test-tubes as under, and keep in water-bath at 40° C.

A.	B.	C.
3 c.c. water.	3 c.c. 0·2 % HCl.	3 c.c. 0·2 % HCl.
1 c.c. pepsin solution.[1]	1 c.c. pepsin solution	1 c.c. boiled pepsin solution.
Shred of fibrin.	Shred of fibrin.	Shred of fibrin.

D.	E.
3 c.c. 0·2 % HCl.	3 c.c. 0·2 % HCl.
1 c.c. water.	1 c.c. pepsin solution.
Shred of fibrin.	1 c.c. egg-white.

[1] The pepsin solution may be prepared by extracting the gastric mucosa of pig stomach with 150 c.c. glycerol for twenty-four hours and filtering. Commercial specimens of pepsin diluted with 50 volumes of water may be employed.

Observe the tubes, and note the following :—

In A the fibrin is unaltered.
In B the fibrin swells and dissolves.
In C and D the fibrin swells 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 in the following way :—

(a) Neutralise some of it with dilute alkali. Note the precipitation of acid metaprotein.

(b) Carry out the biuret reaction (p. 43) on 2 c.c. of it. Compare the colour (1) with that obtained with undigested protein and (2) that obtained in a blank experiment (2 c.c. water, one drop copper sulphate, and excess of caustic soda).

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

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

3. 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.

4. COLLECTION AND ANALYSIS OF GASTRIC JUICE.—Gastric juice for analysis should be obtained from a fasting individual. If, therefore, the exercise is to be performed in the morning, the subject should have no breakfast ; if to be carried out in the afternoon, he should have no lunch.

(a) *Passage of the Reyl Tube.*—Lubricate the tube with glycerol or liquid paraffin and place the olive-shaped tip on the tongue. Push the tip back to the posterior part of the tongue. By breathing deeply through the nose and by persistent swallowing the tube will pass into the œsophagus and into the stomach. There may be some slight initial discomfort, but this ceases when the tip is past the cardio-œsophageal sphincter.

(b) *Withdrawal of Resting Juice.*—By means of a syringe aspirate ALL the contents of the stomach. If it is coloured, test for bile (p. 157).

(c) *The Test Meal.*—When the stomach is emptied completely swallow a pint of water or oatmeal gruel. Immediately after this and at

fifteen-minute intervals withdraw 10 c.c. samples of the gastric contents. Before each sample is collected mix the contents of the stomach thoroughly by filling the syringe with gastric contents and then expelling them into the stomach again.

Each 10 c.c. sample is placed in a small beaker, and a small plug of cotton-wool placed in the syringe and the sample forced through this to remove most of the solid matter. The filtered fluid is then examined for (1) P_H, (2) free hydrochloric acid, (3) total acidity as follows :—

(1) P_H. By means of the spot-plate and Clark's colorimetric technique (p. 99) determine the P_H of the fluid.

(2) Free HCl. Pipette exactly 1 c.c. of the sample into a porcelain crucible, add one drop of the Töpfer-methylene blue reagent[1] and titrate with 0·01N sodium hydroxide. The end-point is a grey-green and is sharp. Confirm your result with a second titration.

(3) Total Acidity. Titrate 1 c.c. of sample with 0·01N sodium hydroxide, using phenolphthalein, the end-point is pink. Confirm this with a second titration.

Samples should be obtained over a period of from one and a half to two hours. When all the data on the samples have been obtained, express your results in the form of graphs.

5. CLOTTING OF MILK.—Prepare four test-tubes as under, and keep in a water-bath at 40° C.

A.	B.
3 c.c. milk.	3 c.c. milk.
2 c.c. water.	1 c.c. water.
1 c.c. rennin.	1 c.c. ammonium oxalate (saturated).
	1 c.c. rennin.
C.	**D.**
3 c.c. milk.	3 c.c. milk.
1 c.c. ammonium oxalate (saturated).	2 c.c. water.
1 c.c. calcium chloride (2 %).	1 c.c. rennin (boiled).
1 c.c. rennin.	

Note what occurs in each case. Why does coagulation not take place in B and D ?

[1] Töpfer-methylene blue reagent:—0·5 gram dimethylaminoazo benzene, 0·25 gram methylene blue, alcohol 100 c.c., at P_H 3·1 the colour is magenta, at P_H 3·2 the colour is green.

ENZYME ACTION

One of the first things which the student will notice regarding the chemical reactions which occur in the animal is the extraordinary ease and rapidity with which they are carried out, when compared with the same reactions performed in the laboratory. For example, to oxidise glucose completely to carbon dioxide and water *in vitro* it is necessary to employ such drastic reagents as cupric oxide at red heat or boiling chromic acid. On the other hand, the animal performs this oxidation smoothly and quickly at 37° C. at a reaction not far removed from neutrality. To give another example, the animal can hydrolyse in one hour at body temperature an amount of lactose which at the same temperature in the laboratory would require five weeks' exposure to the action of 2N hydrochloric acid for complete conversion to glucose and galactose. These types of reaction are reminiscent of those familiar to all as **Catalytic Reactions,** such as the oxidising action of hydrogen peroxide, which is greatly exalted by the presence of small amounts of certain inorganic substances such as iron or manganese. A **Catalyst** may be defined as that which by its presence alters the rate of a reaction already in progress, which does not combine with the final products of the reaction and which can be recovered at the end of the reaction. Catalysts may be of two main types—those which facilitate a reaction, known as *positive catalysts*, and are commoner than those which inhibit or delay a reaction, and are known as *negative catalysts*; these latter are rare.

The following schematic diagram may be given to illustrate some of the phenomena attending the action of catalysts.

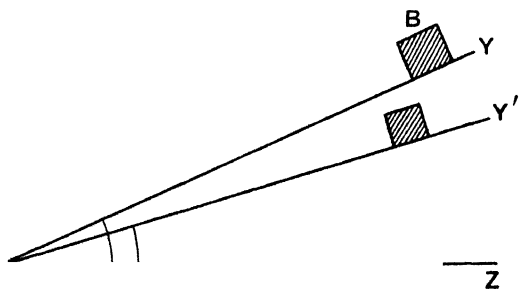


FIG. 14.

B is a weight resting on an inclined plane XY whose angle with the horizontal XZ is variable.

Let the angle YXZ be the angle at which the weight will just slide to the bottom of the incline. If now the plane be oiled, then

the weight will slide at, say, the angle Y'XZ. In each case the weight moves down the plane, but in the second instance this is facilitated by the catalyst (oil). Furthermore, if the lubricant (catalyst) be increased within limits, the rate of movement (rate of reaction) is also accelerated. Furthermore, imagine a trigger holding the weight. When this is released then work is performed, but this work bears no relation in any way to the trigger action. The model also shows (1) that there may be a loss of lubricant (catalyst) by its adherence to the weight; (2) that the form of energy with and without catalyst is different; in sliding down plane YX [without oil (catalyst)] the movement is SLOW and kinetic energy is small, friction is great and HEAT is produced; in sliding down plane Y'X [with oil (catalyst)] movement is rapid, friction is low, little heat is evolved, and the kinetic energy is great.

As examples of true catalytic action we may instance the union of hydrogen and oxygen. At N.T.P. this union *does* take place, but to form a fraction of a milligram of water would take centuries, *i.e.*, the rate is infinitely slow. At a temperature of 500° C. there is just measurable combination. In the presence of metallic platinum (a catalyst) the formation of water is practically instantaneous. Or take what is perhaps the most common catalytic action of all, viz., the catalytic action of water. Ammonia and hydrogen chloride do not combine when perfectly dry; in the presence of a trace of water they form ammonium chloride with explosive violence.

Criteria of Catalysts.—(1) Catalysts do not combine chemically with the FINAL products (*cf.* action of platinum on hydrogen and oxygen). (2) Catalysts are unchanged at the end of the reaction unless destroyed by, for instance, products of the reaction. (3) Small quantities of a catalyst are as efficient as large if time be sufficient. This is usually impracticable owing to paralysis of catalyst by end-products of the reaction.

Mode of Action of Catalysts.—Catalysts are substances, not properties, and may act by surface condensation on particles in the colloidal state, *i.e.*, in heterogeneous systems of at least two phases. At the same time there may possibly be formed certain intermediate products, which subsequently are converted into the end-products. **Enzymes** are colloidal organic catalysts produced by living organisms, both plant and animal. The term enzyme was first introduced by Kühne in 1878. Enzymes are named as a rule after the material (*substrate*) upon which they act, and employing the suffix, ASE. For example, an enzyme hydrolysing MALTOSE is termed MALTASE. AMYLASE will act on starch (AMYLOSE). At the same time it should

be noted that a few, the earliest discovered, have received specific names which tradition compels one to use, pepsin, for instance, or trypsin.

Catalytic Properties of Enzymes.—(1) The final extent of the enzyme action is independent of the amount of the enzyme, provided time is available. (2) Minute amounts of enzyme are sufficient. (3) Enzymes can be inactivated or destroyed by various agencies, *e.g.*, heat, or certain poisons, or even slight changes in hydrogen-ion concentration.

Physical and Chemical Properties of Enzymes.—Enzymes are **colloids,** exist in **colloidal solution,** and being colloidal particles have certain properties peculiar to this physical state. To take a purely inorganic example, platinum or gold can be made to " dissolve " in water under certain conditions. This is not a true solution but a colloidal solution. This solution gives the Faraday-Tyndall test, and the particles show Brownian movement under the ultramiscroscope. A true solution does neither of these. Being particles, colloids in solution have a surface area. For example, colloidal gold particles have a diameter of about one millionth of a centimetre, that is, if a sphere of gold 1 mm. in diameter were dispersed in colloidal solution, the total surface area of the particles would be about 100 square metres. Colloidal particles, therefore, show surface properties. Molecules do not, and phenomena attending the possession of a surface are characteristic of colloids in " solution." One of these phenomena is surface tension, that is, the film at the surface behaves as if it were under tension or were stretched.

Suppose a substance capable of lowering surface tension is present in a colloidal solution, and suppose it to collect at the surface film of the particle, then the surface tension at the film is lowered, *i.e.*, FREE ENERGY is lost. The second Law of Thermodynamics states that if this CAN occur then it WILL occur. Thus it will be realised that there can be a concentration at the surface of a colloidal particle. This collecting or concentration at the surface of a colloidal particle is known as ADSORPTION. Such a process is seen when a piece of fibrin is placed in a solution of congo-red. The fibrin will be found to be coloured red and the union is a firm one. It cannot be broken by solution of the dye in water.

Diffusibility. — Colloids diffuse through membranes either not at all or very slowly. Enzymes being colloids conform to this.

Hysteresis.—A solution of a crystalloid, say, sodium chloride, is the same after boiling or violent shaking as it was before. A colloid is not, and a solution of a colloid is liable to change. The change, obvious and irreversible, is well seen in boiling an egg when the

white (albumen) is coagulated, or in "beating" the white of an egg when a stiff white froth is formed which is permanent. This change is known as HYSTERESIS.

Electrical Charge. — A typical crystalloid in aqueous solution such as sodium chloride can be represented as being formed of a kation [Na] with a positive charge, and the anion [Cl] with a negative charge, thus $Na^+ + Cl'$. These charges remain unaltered under all conditions. Colloidal particles in solution also have an electrical charge, but this *may* vary under different conditions. The following may be cited as examples:—

	Reaction		
Colloid.	Acid.	Neutral.	Alkaline.
Globulin . .	+ve	No change	−ve
Albumen . .	−ve ($\epsilon H = 10^{-6}$)	−ve	−ve
Diastase . .	+ve	...	−ve

On the other hand arsenious sulphide and ferric hydroxide are always negatively and positively charged respectively.

This possession of an electrical charge in part explains **Mutual Precipitation of Colloids.** Here one colloid in solution may unite with another to form colloidal complexes with perhaps precipitation. For example, arsenious sulphide (As_2S) being negative will react with the positively charged ferric hydroxide [$Fe(OH)_3$] and cause precipitation. Use is made of this property in removal of protein from blood prior to quantitative experiments (see p. 188) or in selective precipitation of enzymes. Colloids have a certain **osmotic pressure.** According to Perrin, colloidal particles, molecules, and ions are equivalent as to osmotic pressure, *i.e.*, they act in proportion to their number in unit volume. It will be seen that, as the number of colloidal particles is invariably small in unit volume, the osmotic pressure will likewise be low.

Constitution of Enzymes. — On this question there is still some diversity of opinion. Northrop, who has investigated the nature of crystalline urease, pepsin, and trypsin, believes that enzymes are simply proteins. Waldschmidt-Leitz, on the other hand, will only admit the protein to be the carrier of the real prosthetic group (p. 70). Some of these preparations withstand tryptic digestion, but enzymic activity is lost when they are submitted to the combined actions of pepsin and trypsin.

Preparation of Enzymes.—Some enzymes can be obtained by simple extraction from the cell by water, glycerol, or alcohol either with or without preliminary destruction of the cell membrane. Other methods include rapid cooling of the tissue, extraction of all fat by

cold toluol, and grinding the defatted tissue in a mill to a fine powder, or the more refined methods of Waldschmidt-Leitz and others in which selective adsorption on alumina is effected. The enzyme is subsequently recovered from its combination with the hydroxide.

The remaining properties of enzymes will be given under specific isolated headings.

Combination of Enzyme with Products.—As a rule such combination tends to delay or inhibit the reaction. If to a solution containing the enzyme invertase and cane sugar there be added either or both the final products, viz., glucose or fructose, it will be found that the rate of hydrolysis of the uninverted sucrose is decreased. If instead of either glucose or fructose, galactose be added, no effect on the rate is demonstrable. Galactose is not a product produced by the action of invertase from its substrate sucrose.

Effect of Temperature.—Each enzyme is most active at a special and individual temperature, the OPTIMUM, usually at or a little above body temperature. Below this, activity diminishes till at 0° C. it is nil. On heating above the optimum temperature activity similarly diminishes, and in the region of 70° to 100° C. varying with the enzyme it is permanently destroyed. Cooling does not destroy the enzyme. It only inhibits its action, and recovery takes place on warming.

Effect of Reaction.—As in the case of temperature, each enzyme of the body is most efficient at a special individual hydrogen-ion concentration. This is the OPTIMUM reaction of the solution. Departures from this inhibit the enzyme; in certain cases the enzyme may be destroyed. For example, pepsin is permanently and completely destroyed in alkaline solution.

Effect of Concentration of Enzyme and of Substrate.—In general terms the rate of action is proportional to the concentration of either enzyme or substrate. The relationship, however, is not linear.

Specificity of Enzymes.—Most enzymes act upon one type of substrate and one type only. Urease, for example, will hydrolyse no other substance than urea. Maltase will hydrolyse α-glucosides but not β-glucosides. Emulsin, on the contrary, will hydrolyse β-glucosides but will leave the α-glucosides completely unattacked. Such specificity has been compared to each lock having its own key, or each hand its own glove, and is so precise that use is frequently made of it for analytical purposes as, *e.g.*, the identification and estimation of urea by urease (p. 259).

Reversibility of Enzyme Action.—It is agreed that in addition to the easily demonstrable analytical action of enzymes such as the

hydrolysis of lactose to glucose and galactose by lactase, there is also the power to synthesise, in the case cited, lactose from the two monosaccharides. It would appear, therefore, that under one set of conditions easy to produce *in vitro* enzymes are analytical, while under other conditions, difficult to reproduce *in vitro*, but apparently common *in vivo*, the synthetic action is evident.

A simple chemical analogy to this is seen in the ordinary reaction between an ester and water, *e.g.*,

$$\underset{\text{Ethyl acetate}}{C_2H_5.OC.O.CH_3} + \underset{+\text{ water}}{H_2O} \rightleftarrows \underset{\text{ethyl alcohol}}{C_2H_5.OH} + \underset{\text{acetic acid}}{CH_3.CO.OH}$$

The student will remember that this is a reversible reaction and can be controlled at will to proceed in either direction, *i.e.*, the ester can be broken down (analytical reaction) or built up (synthetic) according to the conditions. In the same way the cell can control enzyme reactions to cause reversibility of action.

Zymogens.—Most enzymes are found within certain cells of the body in the form of precursors termed zymogens. For example, in the peptic cells of the fundus of the stomach there is present not pepsin but its zymogen, *pepsinogen*. In the pancreas there is *trypsinogen*. These zymogens are inactive but are rendered active by combination with **co-enzymes.** As in the case of enzymes the constitution of co-enzymes is not known. It is possible that they are relatively simple, *e.g.*, phosphate. As opposed to the enzyme or its zymogen they are thermostable. Once the zymogen and its co-enzyme are brought into contact the enzyme is formed and is functional. The union of zymogen and co-enzyme cannot be broken once the enzyme is formed.

Anti-enzymes.—As the name explains, these are substances which inhibit the action of enzymes. They may be formed *in vivo* by injecting an enzyme into the blood-stream. They are found, for example, in intestinal parasites (worms) and these inhibit the enzymes of the digestive juices.

Theory of Enzyme Action.—"Enzyme action takes place at surfaces of colloid particles suspended in solutions of hydrolyte and NOT between substances in true solution."—Armstrong.

Classification of Enzymes

(Slightly Modified from Haldane)

1. *Enzymes hydrolysing Esters.*
   - (*a*) Phosphatase acting on phosphoric esters.
   - (*b*) Lipase acting on fats.
   - (*c*) Lecithase acting on lecithin.

2. *Enzymes hydrolysing Carbohydrates.*
   - (*a*) Amylase acting on starch.
   - (*b*) Maltase acting on α-glucosides.
   - (*c*) Emulsin acting on β-glucosides.
   - (*d*) Invertase (sucrase) acting on cane sugar.
   - (*e*) Lactase acting on lactose.
3. *Enzymes hydrolysing the C – N Linkage.*
   - (*a*) Urease acting on urea.
   - (*b*) Arginase acting on arginine.
   - (*c*) Asparaginase acting on asparagine.
   - (*d*) Pepsin acting on protein.
   - (*e*) Trypsin acting on peptone (usually).
   - (*f*) Erepsin acting on peptone and polypeptides.
   - (*g*) Rennin acting on caseinogen.
   - (*h*) Thrombin acting on fibrinogen.
4. *Enzymes hydrolysing the C – N Linkage, probably with Oxidation.*
   - (*a*) Adenine deaminase acting on adenine.
   - (*b*) Guanine deaminase acting on guanine.
   - (*c*) Amino-acid deaminase acting on amino-acids.
5. *Enzymes inserting Water without Hydrolysis.*
   - (*a*) Glyoxalase acting on methyl-glyoxal.
6. *Enzymes activating Hydrogen Peroxide.*
   - (*a*) Catalase acting on hydrogen peroxide.
   - (*b*) Peroxidase acting on other peroxides.

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 a serous or albuminous gland; before secretion the cells of the acini are swollen out with granules; after secretion has occurred the cells shrink, owing to the granules having been converted into the secretion.

The submaxillary and sublingual glands are in part mucous glands: their secretion contains mucin which is absent from parotid saliva. The granules in the cells of the two glands are larger

than those of the parotid gland: they are composed of mucinogen, the precursor of mucin (see Fig. 16)

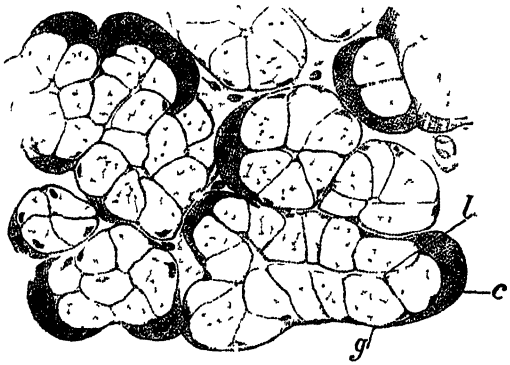


FIG. 15.—Submaxillary gland after a period of rest (Ranvier)
l, lumen of alveolus, *g*, mucus secreting cells *c* crescent of albuminous cells

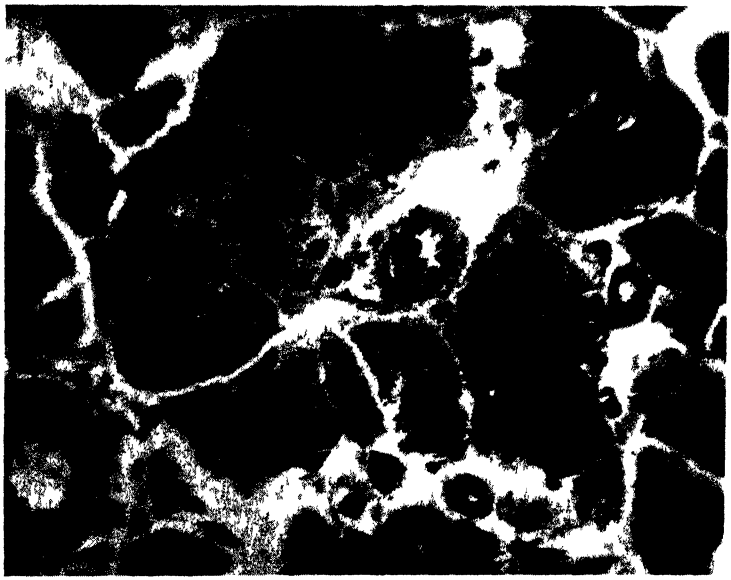


FIG. 16.—Submaxillary gland showing granules (Hewitt, Bird, and Fulton)
Untouched photograph

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. 15).

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 carbon dioxide 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 nervous stimulation 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. In man mixed saliva contains an average of about 0·5 per cent. of solids, and due to the salts in it the reaction is slightly alkaline; the specific gravity is between 1002 and 1006. These figures are in general terms only and are subject to wide variations in differing conditions of age and health. The nature of the food plays a large part in determining the composition of saliva. The secretion of saliva is also affected by such emotional conditions as mental stress, excitement, or fear.

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

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

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 successively split into soluble starch, erythro-dextrin, achroö-dextrin, and maltose.

Ptyalin acts similarly, 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° to 40°), and in a neutral or faintly acid medium; a small amount of alkali makes but little difference; a very small amount of additional acid stops its activity. The conversion of starch into sugar by saliva in the stomach goes on for a variable time, for the food swallowed into the fundus of the stomach is not subjected to peristalsis and consequent admixture with gastric juice until a later stage in digestion. It has been shown that, in man, salivary digestion may continue longer than is usually conjectured, especially under any condition which diminishes the formation of gastric juice, such as exercise or ingestion of fat. Instinctive realisation of this is found in the common practice of taking fat with any starchy food, *e.g.*, butter with bread, or cream with porridge. This popular custom is, as is often the case, supported by scientific observation. The hydrochloric acid which is poured out by the gastric glands first neutralises the saliva and combines with the proteins in the food, and ultimately the ptyalin is destroyed, so that it does not resume its action 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 proteoclastic enzyme (pepsin) in a solution of inorganic salts which also contains a little free hydrochloric acid.

Gastric juice can be obtained during the life of an animal by means of a gastric fistula. Gastric fistulæ have also been made in man, 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 were made on him by Dr Beaumont. Recently Carlson has observed a similar case. By the introduction of the " Fractional Test Meal," as described in the practical section, the composition of gastric juice can be observed for long periods in man.

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.

(*a*) The *cardiac glands* are simple tubular glands quite close to the cardiac orifice.

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.

(*b*) The *fundus glands* are those situated in the remainder of the cardiac half and fundus 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 certain aniline dyes. They are called *parietal* or *oxyntic* (*i.e.*, acid-forming) cells.

(*c*) The *pyloric glands*, in the pyloric canal, have long ducts and short tubules lined with cubical cells. There are no parietal cells, but other cells are found for which the term mucoid has been suggested.

The **central cells** of the fundus glands, and to a less degree the cells of the pyloric glands, are loaded with granules. During secretion they discharge their granules, those which remain being chiefly situated near the lumen, leaving in each cell a clear outer zone (see Fig. 17). These are the cells which 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 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 have seen a similar enzyme precursor in the salivary cells (p. 139),

and shall find others in the pancreas. The zymogen in the gastric cells is called *pepsinogen*. The rennet 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 a puzzling but important 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 least unsatisfactory. He considers that the acid originates by the interaction of sodium chloride and sodium dihydrogen phosphate, as is shown in the following equation:—

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

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

$$Na_2HPO_4 + CO_2 + H_2O \longrightarrow NaHCO_3 + NaH_2PO_4$$

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 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 the free acidity is lowered by the food and saliva, and also by intestinal 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 in the body by acting in an acid medium. A mixture of two substances, *pepsin* and *hydrochloric acid*, is really the 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 produced by fermentative processes from ingested food.

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

By an ingenious surgical technique he separated 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 protein reactions. It contains from 0·4 to 0·6 per cent. of hydrochloric acid. It is strongly proteoclastic, 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 diminishing quantity as long as any food remains in the stomach. When there is no food given there is no juice. Sham feeding with meat, however, 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.

There is also, however, a chemical mechanism. Administration of meat extract, or dextrin, or even water, produces a copious flow of gastric juice. Thus the products of gastric and salivary digestion are themselves stimulants of gastric secretion.

THE ACTIONS OF GASTRIC JUICE

Gastric juice has the following five characteristics:—

1. It is **antiseptic,** owing to the hydrochloric acid present; putrefactive processes do not normally occur in the stomach, and many of the micro-organisms producing such processes, which are swallowed with the food, are destroyed.

2. It hydrolyses sucrose into glucose and fructose. This also is due to the acid of the juice, and is frequently assisted by inverting enzymes contained in the vegetable food swallowed. The juice has no appreciable action on starch other than the action of dilute acid.

3. It contains **lipase,** or a fat-splitting enzyme. 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 to a small extent into their constituents, glycerol and fatty acids. This action is due mainly to lipase which has regurgitated from the duodenum, 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. Administration of fat in the food increases the regurgitation from the duodenum by modifying the action of the pyloro-duodenal sphincter.

4. It **curdles milk.** This is due to the action of the enzyme rennin. The conditions of this action we have already studied (p. 131). It is still a matter of discussion whether pepsin and rennin are two enzymes or not. Whichever view is adopted, the curd of casein formed from the caseinogen is subsequently digested as are other proteins.

5. It is **proteoclastic**: this is the most important action of all. The proteins of the food are converted by the pepsin-hydrochloric acid complex 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 hydrolytic, 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 metaprotein; 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 metaprotein.
2. Proteoses.
3. Peptones.
4. Polypeptides.

1. **Acid Metaprotein.** — 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. 73. We shall find later that, in pancreatic digestion, an alkali metaprotein is formed instead of the acid modification.

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 through a semipermeable membrane.

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 table below will give 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.

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

It will be noted that proteoses and peptones are classified mainly on physical differences such as solubility and "salting out." It is, however, convenient to retain the various names for the present until more is known of their true chemical nature. They are doubtless mixtures of complex polypeptides, and the peptide chains become shorter as digestive cleavage progresses.

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 have furnished us with the key to the problem; just as toxins introduced directly into the blood-stream stimulate the cells to produce antitoxins, so harmful substances produced within the body are provided with anti-substances capable of neutralising their effects. Weinland was one of the earliest to suggest that the gastric epithelium forms an anti-pepsin, the intestinal epithelium an anti-trypsin, and so on. The bodies of parasitic worms that live in the intestine are particularly rich in these antibodies.

NEUTRALISATION OF GASTRIC JUICE

This question is of sufficient importance to merit a paragraph to itself in view of its relation to certain pathological processes.

It will be realised that, despite its being secreted at a strength of about 0·5 per cent., the HCl in the stomach is not normally in direct contact with the lining of that organ at that concentration. Almost as soon as it is poured out it encounters ingested food, and its effective strength is thereby reduced by neutralisation to between 0·1 and 0·2 per cent. Furthermore, on the food passing out of the stomach as chyme, the pylorus permits regurgitation of the alkaline contents of the duodenum, thus preventing the acidity from rising till more food is taken.

Regurgitation is normal and is controlled by the pyloric sphincter. The action of the sphincter may be summarised thus:—

1. Solid objects (food) in pyloric antrum cause TONE.
2. Hydrochloric acid in pyloric antrum causes RELAXATION.
3. Hydrochloric acid in duodenum causes TONE.
4. Vagal stimulation causes TONE.
5. Sympathetic stimulation causes RELAXATION.

Derangements of control of the pyloric sphincter may be of clinical importance.

MEASUREMENT OF THE PROTEOCLASTIC ACTIVITY OF DIGESTIVE JUICES

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. When the fibrin is dissolved the carmine is set free, and from the depth of colour of the solution the amount of fibrin digested can be estimated. The disadvantage of the method is that it can be used only 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 grams 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 glycerol 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 and to each add an equal volume of one 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 proteoclastic 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 coagulated by heating to 95° C. They are then placed in the digesting fluid at 36° C., and the coagulated egg-white is digested. After a given time the tubes are removed; and if the process of digestion has not gone too far, only a part of the 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 is proportional to the square root of the amount of pepsin. In most other cases of enzyme activity the rapidity of action is directly proportional to the amount of enzyme present (p. 136).

3. **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 on both peptic and tryptic enzymes. In 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 gram of caseinogen (commercial 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 remain 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.

[1] Hamburger has used the same method in investigating the digestive action of juices on gelatin. The tubes are filled with warm gelatin solution, which 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 temperature (36° to 40° C.) at which artificial digestion is usually carried out would melt the gelatin. He has also used the same method for estimating amyloclastic activity, by using starch as substrate.

(*b*) *Caseinogen Method for Trypsin Estimations.*—One gram 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.

4. **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:—

$$\begin{array}{l} R.CH.NH_2 + O \quad CH_2 \\ \quad | \\ \quad COOH \end{array} \longrightarrow \begin{array}{l} R.CH.N : CH_2 + H_2O \\ \quad | \\ \quad COOH \end{array}$$

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 (p. 266).

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—

$$R.NH_2 + HNO_2 = R.OH + H_2O + N_2$$

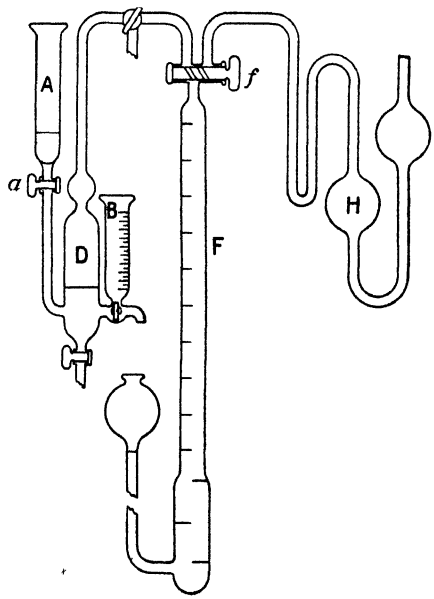


FIG. 18.—Van Slyke's apparatus.

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. 18 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 a 1 per cent. sulphuric acid solution, the excess of nitric oxide being 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 acidified 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 grams 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 indicating the 20 c.c. point. *a* is then closed, and *e* and *f* turned so that D and F are connected.

(2) *Decomposition of the Amino-substance.*—Ten c.c. (or less) are measured off in B. A known amount of this is run into D, and D is shaken for three to five minutes. With most amino-acids, proteins, or partially or completely hydrolysed proteins, five minutes' vigorous shaking is sufficient. (A small motor may be used 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 grams potassium permanganate

and 25 grams caustic potash per litre), is then shaken for a minute, whereby 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.

THE ACID OF GASTRIC JUICE

The digestive powers of the juices are proportional to the dissociation of the acids therein. 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 (achlorhydria) or diminished (hypochlorhydria) in some diseases. 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. Charring must be avoided. Red crystals form, or, if much peptone is present, there will result a red paste. The residue is of a bright red colour, even when only 1 part of hydrochloric acid in 10,000 is present. Organic acids do not give the reaction.

(*b*) Tropæolin test. Drops of a saturated solution of tropæolin-OO in 94 per cent. methylated spirit are allowed to dry on a porcelain slab at 40° C. A drop of the fluid to be tested is placed on a tropæolin drop, still at 40° C., when, 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) gives with this in the cold a bright red colour.

Tropæolin and Töpfer's reagent are only 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 deep 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. The reaction is due to the production of formaldehyde and acetaldehyde by the oxidising agent used, the thiophene interacting with the aldehydes so produced.

CHAPTER VII

THE DIGESTIVE JUICES (*continued*)

PANCREATIC DIGESTION

1. Prepare four test-tubes as under :—

A.	B.
3 c.c. water.	3 c.c. 1 % Na_2CO_3.
1 c.c. neutral trypsin.	1 c.c. neutral trypsin.
Shred of fibrin.	Shred of fibrin.

C.	D.
3 c.c. 1 % Na_2CO_3.	3 c.c. 1 % Na_2CO_3.
1 c.c. boiled trypsin.	1 c.c. neutral trypsin.
Shred of fibrin.	2 c.c. egg-white.

Employing the method described (p. 99), determine the P_H of each of the tubes A, B, C, and D.

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

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.

In C no digestion occurs as the trypsin has been destroyed by heating to 100° C.

2. Into a 250 c.c. flask place 100 c.c. of 4 per cent. caseinogen in 0·4 per cent. sodium carbonate solution. Add 5 c.c. of trypsin. Plug the neck of the flask with cotton-wool and place in the incubator for 8 to 10 days at 37° C. Note the odour due to putrefactive processes. Which amino-acids give rise to these products? Note also the chalky deposit. Collect this, wash with water and recrystallise from boiling water. Examine microscopically and compare their appearance with those in illustration on p. 50.

3. ESTIMATION OF TRYPTIC ACTIVITY (*Sörensen's Method*).— **Into a 250 c.c. flask place 150 c.c. 4 per cent. solution of caseinogen**

dissolved in 0·4 per cent. sodium carbonate. Add 5 c.c. of trypsin. Treat 20 c.c. of the mixture at once by the method described immediately below, and place the remainder in the incubator at 37° to 40° C. and take 20 c.c. samples at fifteen-minute intervals. Treat each of these as follows :—Place the 20 c.c. (accurately measured with a pipette) in a conical flask, add a few drops of phenolphthalein and make neutral by adding 0·1N HCl. Take approximately 20 c.c. of formalin and make it neutral to phenolphthalein by adding 0·1N sodium hydroxide. Add the neutral formalin to the neutral tryptic digest and note the development of acidity.

The essential reaction involved is :—

$$R.CH.NH_2.COOH + H.CHO \longrightarrow R.CH.N.CH_2.COOH + H_2O$$

and it will be seen that the basic group $-NH_2$ is methylated and its basic properties destroyed. See p. 151.

Titrate with 0·1N sodium hydroxide and record the number of c.c. required to neutralise the solution. Record your results in the form of a graph, plotting volumes of 0·1N acid against time and so obtain a graph showing the course of the hydrolysis.

4. ACTION OF PANCREATIC JUICE ON STARCH.—Prepare four test-tubes as under :—

E.	F.
3 c.c. starch solution.	3 c.c. starch solution.
1 c.c. neutral pancreatic juice.	1 c.c. neutral pancreatic juice.
3 c.c. water.	3 c.c. 2 % Na_2CO_3.
G.	**H.**
3 c.c. starch solution.	3 c.c. starch solution.
1 c.c. neutral pancreatic juice.	3 c.c. 2 % Na_2CO_3.
3 c.c. 2 % Na_2CO_3.	½ c.c. bile.
½ c.c. bile.	

Put these into the water-bath, and test small portions of each, after neutralising, every half-minute by the iodine reaction. The Achromic Point is reached first in G; then in F; while E and H undergo no change. Test G and F for the presence of reducing substances with Benedict's solution.

This experiment shows the favourable influence which bile exerts on pancreatic digestion. It is, however, more marked still in the case of fats. (See 6 below.)

5. 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.

6. Boil 10 c.c. of fresh milk; cool, and add 3 drops of an alcoholic solution of phenolphthalein together with dilute sodium carbonate until the solution is a distinct pink colour. Divide this into three test-tubes, and treat as under :—

A.	B.	C.
3 c.c. milk.	3 c.c. milk.	3 c.c. milk.
1 c.c. lipase.	1 c.c. boiled lipase.	1 c.c. lipase.
½ c.c. water.	½ c.c. bile.	½ c.c. bile.

Place all in the water-bath at 40° C. and note any changes. In A and C the pink colour disappears owing to neutralisation of the alkali by the fatty acids liberated by the lipase. The action is most rapid in the presence of bile, *i.e.*, in C. In B no action results, and it serves as a control.

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

BILE

1. Perform the following experiments with ox-bile. Observe its colour, taste, smell, and reaction to litmus paper.

2. Acidify 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 on p. 45, 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 porcelain basin add a drop of a 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 passing through the froth.

5. GMELIN'S TEST FOR BILE PIGMENTS.—Place 3 c.c. or so

of fuming nitric acid in a test-tube, and pour a few c.c. of bile carefully down the side so as to form a layer on the surface. Notice the succession of colours—red, green, blue, and yellow—at the junction of the two liquids. This test may also be performed on a flat porcelain dish; 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. This test is not applicable to urine directly. For this purpose Huppert's test and the reaction with iodine are usually employed. The former is described under pathological urines (p. 263).

6. HAY'S TEST FOR BILE SALTS.—Take two beakers or test-tubes full of water; to one add a few drops of bile, or a 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.

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. 19).

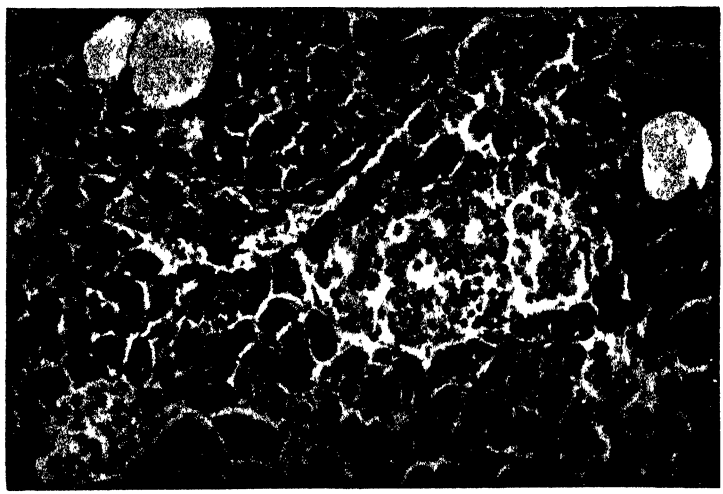


FIG. 19.—Pancreas showing islet of Langerhans.

As in 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 solids	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 proteoclastic enzyme. In the fresh juice, however, this is present in the form of trypsinogen.
(ii) Amylase, an amyloclastic enzyme.
(iii) Lipase, a lipoclastic enzyme.
(iv) A milk-curdling enzyme.

(*b*) A small amount of coagulable protein.
(*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 those 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. This flow still occurs when all the nerves supplying the duodenum and pancreas are cut. 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 pass into the blood from 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 extracted which, when injected into the blood-stream in minimal doses, produces a copious secretion of pancreatic juice. The substance is termed *secretin*. It is associated with another substance which lowers arterial blood-pressure but with which it is not identical.

The old view that secretin existed in the duodenal mucosa as a precursor, " pro-secretin," which by the action of the hydrochloric

acid is converted into secretin, must be abandoned. One of the reasons against it is the clinical observation that pancreatic digestion can proceed satisfactorily in the absence of this acid in the gastric juice.

It seems that there is a relationship between pancreatic secretion and the passage of bile into the duodenum. The mechanism is as follows :— Peristaltic waves pass from the stomach to the small intestine preceded by waves of inhibition, which at the ampulla of Vater release the sphincter of the common bile duct and permit the entrance of bile into the duodenum. Aided by the specific bile salts, the preformed secretin is absorbed into the blood-stream, and is carried to the pancreas to stimulate it into activity.

J. Mellanby has prepared a very active secretin of which 0·03 mg. will induce profuse secretion in a cat. Secretin is soluble in water and alkali, is insoluble in dilute acid, is possibly a polypeptide, and probably is the same substance in different species of the higher animals.

The question arises whether there are any secretory nerves controlling the production of pancreatic juice. Pavloff discovered them in the vagus, an observation subsequently confirmed. Their action, however, is small—the chief mechanism for secretion of pancreatic juice is that described above. Secretin is one of a large number of chemical messengers or hormones. These substances are usually produced in one part of the body and are carried by the blood to another part, there to exert their action. Hormones are not enzymes and have no enzyme-like properties. For example, they are thermostable and probably relatively simple in chemical constitution.

The pancreatic juice does not act alone on the food in the intestines. In addition, bile, succus entericus and bacterial action also play a part.

The **succus entericus** or intestinal juice has no action on native proteins. It is stated to possess a slight lipoclastic 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 (p. 19). There are two other hydrolysing enzymes in the succus entericus, one of which, *maltase*, acts on maltose, and the other, *lactase*, on lactose.

Some years ago, however, Pavloff showed that succus entericus has a still more important action, namely, to activate the proteoclastic power of the pancreatic juice. Fresh pancreatic juice has very little action 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 proteoclastic mixture, though neither juice by itself has any proteoclastic activity. The substance that activates trypsinogen or, in other words, liberates trypsin from trypsinogen is the intestinal juice, and has been called by Pavloff an enzyme of enzymes, or *entero-kinase*.

Dixon and Hamill's work 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 proteoclastic enzyme which digests the protein moiety, and thus liberates the trypsin (J. Mellanby and Woolley).

Intestinal juice contains another enzyme called *erepsin*; this is a peptoclastic enzyme, and breaks up proteoses and peptones into their final cleavage products, the polypeptides and amino-acids, and so completes the action of the 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. Its optimum P_H is 8·1.

(*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 by gastric juice. It does not, however, digest collagen.

(*e*) It acts on solid proteins such as fibrin, and eats them away from the surface to the interior; as acid is absent, there is no preliminary swelling as in gastric digestion.

(*f*) Trypsin effects a more complete hydrolysis of protein than does pepsin. Acting in an alkaline medium in which pepsin is destroyed it continues the breakdown of protein which was initiated by pepsin in the acid medium of the stomach. Trypsin rapidly splits the proteose and peptone which have been discharged in the acid chyme into the duodenum. From these polypeptides are produced.

When once the peptone stage is passed the products of further cleavage no longer give the biuret reaction; hence they are frequently termed *abiuretic*.

By the action of erepsin the polypeptides in their turn are resolved in great part into their constituent amino-acids, such as leucine, tyrosine, alanine, aspartic acid, glutamic acid, arginine, tryptophan, and many others. The constitution and properties of these cleavage products are described on pp. 47 to 55. In addition to these there is a certain amount of ammonia. The red colour which is given to a tryptic digest by chlorine or bromine water is due to the presence of tryptophan.

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 the form of very short polypeptide linkages.

It will thus be seen that there are two important differences between the actions of 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, and, similarly, the action of erepsin is facilitated by the previous action of pepsin and trypsin.

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 uncooked starch. The absence of this enzyme in the pancreatic juice of infants is an indication that milk is their natural diet.

3. **Action on Fats.**—These are split by pancreatic lipase into glycerol and fatty acids. The fatty acids unite with the alkalis

present to form soaps (*saponification*, see p. 34). If a glycerol extract of pancreas is filtered through porcelain, 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 and is not destroyed by boiling. Bile salts also activate the inactive lipase, and this in part accounts for the adjuvant action of bile on lipase.

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 globule, thus preventing 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 gastric rennin) 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 possibly, as some believe, a function of trypsin.

BACTERIAL ACTION

The gastric juice is an antiseptic; the pancreatic juice is not. An alkaline fluid like pancreatic juice is the most suitable medium in which certain bacteria flourish. In an artificial digestion the fluid soon becomes putrid, unless special precautions are taken to exclude or kill bacteria. 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, and thus they can break down starch to sugar, proteins to peptones and amino-acids, and fats to glycerol and fatty acids. In addition the action of the bacterial flora of the intestine causes the formation of other important end-products; some, especially those arising from proteins, are toxic. The chief end-products formed in this way from:—

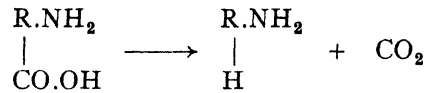
I. *Carbohydrates*, such as starch, are carbon dioxide, hydrogen, lactic acid, ethyl and higher alcohols, butyric acid. Cellulose is

broken down, in part only, to carbon dioxide and methane. This decomposition is the principal cause of intestinal gases, the amount of which will obviously be increased on a vegetarian diet.

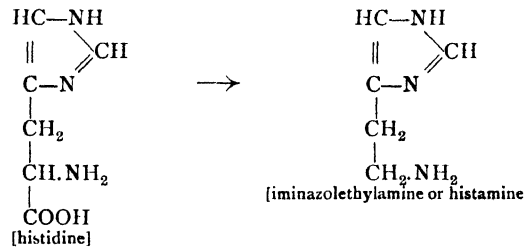
II. *Fats* are glycerol, palmitic, stearic, and other higher acids which in turn are broken down to valeric, butyric, and other lower fatty acids.

III. *Protein* are peptones and amino-acids. Other acids are produced from these latter, by deamination, *i.e.*, loss of ammonia. These in turn give rise to such products as phenol and cresol (from tyrosine), or indole and skatole (from tryptophan).

From these amino-acids amines can also arise by decarboxylation, *i.e.*, loss of carbon dioxide. This is one of the chief characteristics of putrefactive bacteria. The change may be represented thus :—



and specifically in the formation of histamine from histidine thus :—



In this way the well-known putrefactive bases, putrescine and cadaverine (whose clinical importance has been much overrated), are formed from ornithine and lysine (see p. 51).

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 the tract than was formerly supposed. These organic acids do not, however, hinder pancreatic digestion.

Ammonia-producing organisms flourish best in the lower regions of the small intestine; the ammonia neutralises the organic acids produced higher up, and in the large intestine the contents are consequently neutral or slightly alkaline.

THE BILE

The liver is an organ which plays a part in the metabolism of all three classes of food-stuffs. It is concerned in the storage of

carbohydrates as glycogen; it deaminises amino-acids derived from protein and converts the liberated ammonia into urea. It unsaturates fats brought to it by the blood-stream. Lastly, as a gland it produces a secretion with which we are concerned here, namely, the Bile.

Primarily, bile is a secretion which passes from the liver by way of the bile duct into the duodenum close to the pylorus. It, nevertheless, has other functions such as are described below. The excretion of bile is continuous even during prolonged fasting, and has been collected from living animals by means of a biliary fistula, an operation which is occasionally performed in human beings. It is difficult to determine accurately the amount of bile normally secreted during a given period; for man it has been variously estimated at from 500 to 1000 c.c. daily.

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 about three times that in the portal vein.

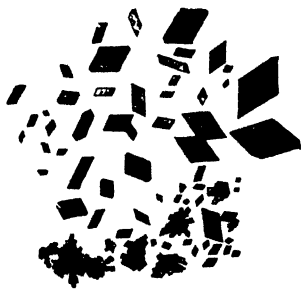


FIG. 20.—Hæmatoidin crystals.

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. 20). Moreover, bilirubin yields hæmopyrrol, a substance also obtained from blood pigment.

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 apparently does not

liberate hæmoglobin from the corpuscles since no free hæmoglobin is discoverable in the blood-plasma in the splenic vein.

THE USES OF BILE

In addition to bile being a secretion, it has also an excretory function in so far as it provides a channel whereby such substances as cholesterol, lecithin, and certain waste products of metabolism are removed from the organism and passed into the intestine to be ultimately eliminated in the fæces.

In some animals, moreover, 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 the practical exercises (pp. 154 to 157). 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 putrefaction 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 free fatty acids and soaps, and assists the absorption of fat in this way.

THE CONSTITUENTS OF BILE

The constituents of the bile are the bile salts proper (taurocholate and glycocholate of sodium), the bile pigments (bilirubin, biliverdin), a mucinoid substance, small quantities of fats, soaps, cholesterol, lecithin, urea and mineral salts, of which sodium choloride 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 intestine 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 analysis 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. Research indicates 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. 70 to 72.)

THE BILE ACIDS

These compounds which are present as salts of the alkali metals (chiefly sodium) may be divided broadly into two classes, *i.e.*, the

glycocholic acids and the *taurocholic* acids, the former predominating in the bile of man and of herbivora, the latter in carnivora. The most striking difference between the two groups of compounds is that only the taurocholic acids contain sulphur. Both types of compounds are " conjugated " amino-acids, and as such may be hydrolysed by hot dilute acids or alkalis.

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 :—

$$\underset{\text{[glycocholic acid]}}{C_{26}H_{43}NO_6} + H_2O \longrightarrow \underset{\text{[glycine]}}{CH_2.NH_2.COOH} + \underset{\text{[cholalic acid]}}{C_{24}H_{40}O_5}$$

Taurocholic acid ($C_{26}H_{45}NO_7S$) similarly splits into taurine or amino-ethyl-sulphonic acid and cholalic acid :—

$$\underset{\text{[taurocholic acid]}}{C_{26}H_{45}NO_7S} + H_2O \longrightarrow \underset{\text{[taurine]}}{C_2H_4.NH_2.HSO_3} + \underset{\text{[cholalic acid]}}{C_{24}H_{40}O_5}$$

Pettenkofer's reaction, described in the practical section, 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), which gives the purple colour with cholalic acid.

In recent years, the constitution of the bile acids have been investigated by an ever-growing number of workers. Not only have new bile acids been described but certain new chemical relationships have been traced between the various members of the group, not only with the so-called " sex " hormones, with the most actively known carcinogenic compounds, but also with substances having anti-rachitic action.

THE BILE PIGMENTS

The two chief bile pigments are bilirubin and biliverdin, which, like the others, are iron free. 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 ($C_{33}H_{36}N_4O_6$), perhaps the most important of the bile pigments, is derived from hæmoglobin, liberated during red-cell destruction, and is closely related to porphyrin. It is formed in the liver and most probably also in such tissues as spleen, bone-marrow,

and lymph glands. It is easily soluble in chloroform, somewhat less soluble in alcohol, and only slightly soluble in ether and benzene. The spontaneous evaporation of its solution in chloroform may yield the substance in reddish-yellow rhombic plates. It gives a characteristic colour reaction (red-violet) when subjected to the well-known diazo reaction of Erhlich (a mixture of sulphanilic acid, hydrochloric acid, and sodium nitrite). This coloration is the basis of VAN DEN BERGH'S REACTION for the detection and estimation of abnormal amounts of bile pigment in blood serum. Such an addition of the diazo reagent may result in the immediate development of a red-violet colour reaching a maximum intensity in thirty seconds. Clinically, this is known as the **direct reaction** and is indicative of *obstructive jaundice*. At the other extreme, the addition may yield no, or a very slow and incomplete, development of colour. It is then regarded as an **indirect reaction** and is associated with *non-obstructive or hæmolytic jaundice*. In addition, there is yet a third type of reaction —the **biphasic reaction.** In this case the colour development commences immediately or within thirty seconds (*cf.* direct reaction), but its maximum intensity is reached only after a variable period. These differences in the rate and intensity of the reaction have stimulated much investigation, but no definite explanation of them has yet been forthcoming. Working on the knowledge that sodium bilirubinate gives a direct reaction, Barron added this pigment to serum in increasing amounts and submitted the resulting solutions to the test. It was found that serum containing more than 12 mg. per cent. of the bilirubinate gave an indirect reaction; from 12 to 16 mg. per cent., a biphasic reaction; more than 16 mg. per cent., a direct reaction. The conclusion he drew from such results was that the serum contains some constituent possessing the power of adsorbing the bilirubin, such adsorption interfering with the normal coupling with the diazonium salt.

On oxidation (exposure to air or treatment by fuming nitric acid, as in Gmelin's test (p. 157)) bilirubin yields

Biliverdin ($C_{33}H_{36}N_4O_8$). This substance sometimes occurs in bile. In diarrhœa the fæces may possess a greenish colour due to the presence of biliverdin (the brown colour of normal fæces is due to stercobilin which, like the urinary pigment urobilin, is a reduction product of bilirubin). Biliverdin, on oxidation, yields a number of compounds including

Bilicyanin, a blue pigment. Though not present, normally, in bile, this substance together with its precursors, bilirubin and biliverdin, is found in gall-stones.

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.

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 taking place—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 term 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 it is once more synthesised into the bile salts. Some of the taurine is absorbed and excreted as tauro-carbamic acid ($C_2H_4NHCO.NH_2HSO_3$) 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 such, though being in urine, is usually known as 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 sterol (phytosterol) of the food. In some cases it is reduced to form a derivative termed *coprosterol*.

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.

In the mouth and œsophagus, owing to the type of epithelium (stratified), absorption is reduced to a minimum. In the stomach, where the epithelium is columnar and thus thinner, absorption occurs only to a limited extent. It is, however, in the small and large

intestines that absorption of soluble materials and water principally takes place.

It may be said that, with few exceptions, only substances capable of true solution in water can be absorbed from the alimentary canal. Materials such as water and certain soluble salts such as sodium chloride are absorbed unchanged. Similarly with some simple organic substances, for example, urea or glucose; these are not changed prior to leaving the intestine. More complex organic components of food such as carbohydrates, *e.g.*, starch, or proteins, *e.g.*, albumin, which are of high molecular weight and colloidal in nature, must first be converted respectively into the diffusible crystalloids sugar and amino-acids and other products of hydrolysis.

There are two channels of absorption from the intestine; the blood-vessels (portal capillaries) and the lymphatic vessels or lacteals.

Absorption, however, is no mere physical process of diffusion and filtration. The cells through which the absorbed substances pass are living, and in virtue of this not only select materials for absorption, but may and do also change those substances while in contact with them, if necessary regulating their concentration by selective absorption of solvent or solute. The cells concerned 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 these the columnar epithelium is the more important. When these cells are removed, or rendered inactive by sodium fluoride, absorption becomes a process of simple filtration or diffusion, and dependent on pressure differences of hydrostatic or osmotic nature.

Absorption of Carbohydrates.—Though the sugar formed from starch by ptyalin and amylase is maltose, that found in the blood is glucose. Under normal conditions little if any glucose is absorbed by the lacteals. The glucose is formed from the maltose by the maltase of the succus entericus. Sucrose and lactose are also converted into glucose and fructose and glucose and galactose respectively before absorption; but if these disaccharides are injected into the blood-stream direct, they are unaltered by the liver and, as neither sucrase nor maltase are normal constituents of blood, finally leave the body by the urine.

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. As glycogen, however, is also found in animals on a minimum carbohydrate diet, it must be concluded that it can be formed by the liver cells from protein. The carbohydrate store, when

required, leaves the liver in the blood of the hepatic vein as glucose once more.

The above is a brief statement of the glycogenic function of the liver as taught by Claude Bernard, and accepted by the majority of physiologists.

Where solutions of sugar stronger than isotonic enter the intestine, the first action of the bowel is to cause a dilution until the concentration has decreased to a point at which absorption is most effectively carried out. It has been shown that all sugars are not removed from the intestine at the same rate, and that each is absorbed at a rate which is constant for each individual sugar and which is independent of its initial concentration. It will be realised that on purely physical grounds the three hexose sugars—galactose, glucose, and fructose, should be absorbed at the same rate. To account for the different rates of absorption two conceptions are possible: first to postulate some selective physiological activity of the intestinal mucosa, and secondly, to postulate that each sugar undergoes some chemical reaction prior to absorption. As long ago as 1914 a synthesis of hexoses to glycogen was suggested and in part experimentally shown, but not to an extent sufficient to account for the wide differences in the rates of absorption. More recently Verzar has attempted to bring the behaviour of hexoses in the intestine into harmony with the known reactions of hexoses, particularly glucose in muscle. There it is agreed that one of the initial chemical reactions is esterification of glucose with phosphoric acid to form the so-called "lactacidogen" or "hexose-phosphate." Verzar postulates a similar phosphorylation of the hexoses in the gut by, presumably, enzymes derived from the columnar epithelial cells of the alimentary canal. He further postulates that it is only after such phosphorylation that carbohydrates are absorbed *into* the cell. Esterification thus increases absorption only by increasing the diffusion gradient. Once *in* the cell the ester is hydrolysed and the regenerated sugar passes as such into the portal blood. Many objections can be raised to this conception, and it must be realised that while it is an attractive hypothesis it cannot be taken as definitely proved.

Absorption of Proteins. — Under very abnormal conditions a certain amount of protein such as albumen or globulin can be absorbed unchanged. Under normal conditions, however, the food proteins, after preliminary gastric digestion, are, by the two enzymes—trypsin, of the pancreatic juice, and erepsin, of the succus entericus—broken down beyond the peptone stage into their final cleavage products, polypeptides and amino-acids, and these pass into the blood as such,

for the amount of non-protein nitrogen in that fluid is increased during absorption. Moreover if an animal is fed on the cleavage products from a pancreatic digest, nitrogenous equilibrium is still maintained. These amino-acids are partly utilised by the cells of the body to repair waste, but partly and to a still greater extent converted by the liver into the waste substances urea and ammonia, which are 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 been abandoned.

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 custom of speaking of the cleavage products of protein as "building stones." Each tissue has special architectural features in its protein molecules, and these molecules are reconstructed by using the building stones that previously had been used in the building of other protein molecules, either in another animal or in vegetable structures. The building stones which are in excess or are unsuitable are simply got rid of as waste substance.

A large number of them are never actually built into protoplasm, but are carried to the liver when the amino-group is removed. This process is *deamination* (p. 293). The nitrogenous portion is converted into urea and ammonia, the non-nitrogenous portion of the protein molecule being available for calorific processes (urea formation, p. 294). One can now definitely state which are the acids that on p. 83 we compared to diamonds, because they are unusually precious for the synthesis of protein by tissue cells; they are principally phenylalanine, tyrosine, and tryptophan, for if they are injected into the blood-stream they do not give rise to any appreciable increase in the urea formed. Lysine and histidine are also in the same category. Proteins destitute of these amino-acids are of inferior nutritive value.

Absorption of Fats.—The fats in the intestine undergo two changes: one, a physical change (emulsification), the other, a chemical change (hydrolysis or saponification). The lymphatic vessels are the principal

channels for fat-absorption, and their contents have a milk-like appearance 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 microscopic preparations of the villi stained in such a way that the fat is seen. Fig. 21 illustrates the appearances so observed.

The columnar epithelium cells first become filled with fatty

FIG 21.—Section of the villus during fat-absorption *ep*, epithelium, *c'*, goblet cells in the epithelium, *l*, central lacteal containing disintegrating lymph cells, *cap*, capillary vessels (Hewitt, Bird, and Fulton) Untouched photograph.

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 special cells largely in 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, in the opinion of most physiologists, from protein as well.

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.

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, but, as they stain black 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 soap into which it has been broken up in the intestine. These substances, being soluble, readily pass into the epithelial cells, which reconvert them into fat once more. This appears in the form of small globules, surrounding or becoming mixed with the protoplasmic granules that are ordinarily present. 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 shown on p. 157. It is also a solvent of fatty acids, particularly oleic acid, and soaps. 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. It may even be that the fatty acids combine with the paired bile acids to form complexes which are water soluble and diffusible.

As in the absorption of hexose sugars, a preliminary phosphorylation is postulated by Verzar as accompanying the passage of fats into the intestinal epithelium. In support of this it may be said that those conditions which inhibit esterification also inhibit absorption.

In cases of disease in which bile is absent from the intestine a large proportion of the fat in the food passes into the fæces as such, either hydrolysed or unhydrolysed or a mixture of both.

In conclusion it must be mentioned that the lymphocytes are greatly increased in the blood during absorption, not only of carbohydrates, fats, and proteins, but also during the absorption of water,

the condition then obtaining being part of the normal " post-prandial leucocytosis."

FÆCES

The contents of the small intestine on reaching the ileocæcal valve are still semi-fluid in consistency. The transformation into fæces takes place in the large bowel during their passage through it, especially by the absorption of water.

Normally fæces are slightly alkaline in reaction (P_H 7·0 to 7·5), dark brown in colour unless much fat is present, and with an odour due to products, *e.g.*, skatole, and in varying amount, methane, methyl mercaptan, and hydrogen sulphide, formed from protein by bacterial action. The normal pigment is stercobilin, but on the other hand pigmented fruits and berries or certain drugs may give characteristic colours to the stool. For example, calomel produces a green stool, bismuth a black stool. After ingestion of rhubarb or senna the stool is yellow, while the presence of blood in the intestine causes a very dark " tarry " stool and the absence of bile produces the typical grey-white acholic motion. It will thus be seen that the colour of the fæces is bound up with the diet.

The consistency, too, depends on the type of food taken and may vary from a thin pasty mass to a firmly formed stool. Exceedingly thin and watery motions are ordinarily of pathological significance.

Normal fæces contain :

Water	60 to 70 per cent.
Nitrogen	5 ,, 10 ,,
Fatty material	10 ,, 20 ,,
Ash	10 ,, 20 ,,

The chief constituents of fæces are: (1) Food residues comprising those fractions of the diet which cannot be digested, or, having been digested cannot be absorbed, or products capable of absorption but which have escaped being absorbed. (2) Remains of digestive secretions neither destroyed nor reabsorbed. (3) Bacterial flora of the intestinal tract. (4) Cellular elements desquamated from the mucosa of the alimentary canal.

Thus the fæces of an individual on an ordinary mixed diet will contain cellulose, fruit seeds and skins, muscle fibres, shreds of connective tissue, starch, fat, fatty acids, soaps, bile acids and pigments, cholesterol, coprosterol, mucin, and inorganic constituents such as calcium and iron. Of these substances cellulose is one of the most important. It is not digestible, and its addition to the diet

leads to an increased bulk of the fæces. It is sometimes termed "roughage." Moreover, it stimulates by its presence the glands of the mucous membrane to secrete intestinal juice (succus entericus), and finally it stimulates the smooth muscle of the alimentary canal and thus increases peristalsis. On an average from 20 to 30 per cent. of dried fæces is bacteria. About 128,000,000,000,000 are evacuated daily by a normal individual. The vast majority are dead.

The amount and composition of fæcal lipoids have been carefully studied by Bloor and others. The lipoids are largely independent of the fat intake. About one-third of the fæcal lipoid is unsaponifiable, *i.e.*, cholesterol, etc. No doubt a large proportion of this is derived from the bacteria. On the other hand, the presence of cellulose interferes with the digestion and absorption of protein, for 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 was 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, the indigestible cellulose stimulating peristalsis. Thus on an ordinary mixed diet 35 grams of dry substance and 100 grams of water are daily excreted in the fæces, whereas on a vegetable diet the quantities are 75 and 260 grams respectively.

CHAPTER VIII

THE BLOOD AND RESPIRATION

BLOOD

Carry out the following experiments on defibrinated blood.

1. *Reaction.*—**Moisten red and blue litmus paper with sodium chloride solution and add a drop of blood to each. After two or three seconds wash the blood off with salt solution and note the colours. Repeat with congo-red paper.**

2. *Microscopic Appearance.*—**Examine a drop of blood under low ($\frac{2}{3}$ in.) and moderate ($\frac{1}{6}$ in.) magnification. Note rouleaux formation. Identify the formed elements as far as possible.**

3. *Specific Gravity.*—**Determine this in the usual way with a hydrometer. A more accurate determination of the specific gravity of blood can be made as follows :—Prepare a mixture of benzene and chloroform of specific gravity approximately 1·050. Place this in a small cylinder or large test-tube and allow** *one small* **drop of blood from a pipette to fall into the centre of the liquid. Observe what happens to the drop. If it sinks it has a greater specific gravity than the liquid, if it floats a lesser. Accordingly add to the mixture either chloroform to increase the specific gravity, or benzene to reduce it, until the drop of blood remains suspended in the fluid. Care has to be taken after the addition of either substance to see that the liquid is thoroughly mixed by stirring, and that the blood does not adhere to the side of the glass.**

4. *Chemical Constituents.*—**Dilute 5 c.c. blood with 25 c.c. water, and heat to boiling. Note the coagulum. Add 25 c.c. water containing one drop glacial acetic acid, and heat once more to boiling. Permit the coagulum to settle, and then filter. Treat the** *filtrate* **and the** *coagulum* **separately as follows :—**

A. Evaporate the filtrate in a porcelain basin to 10 to 15 c.c. and test the concentrate for the presence of

(i) *Chlorides*, **by adding nitric acid and silver nitrate.**

(ii) *Phosphates*, **by adding nitric acid and ammonium molybdate and heating.**

(iii) *Glucose*, **by adding Benedict's sugar reagent and boiling.**

B. Incinerate the coagulum in a porcelain crucible and, after cooling, dissolve the residue in dilute hydrochloric acid. Test the solution obtained for the presence of

(iv) *Iron*, **by adding potassium ferrocyanide or thiocyanate.**

5. *Hæmolysis* **(The Laking of Blood).—Prepare 8 test-tubes as follows :—**

Number of Tube . . .	1	2	3	4	5	6	7	8
Cubic centimetres 1 % NaCl .	2	3	4	5	6	7	8	9
Cubic centimetres water .	8	7	6	5	4	3	2	1

To each test-tube add 3 drops of blood and mix gently. Set aside to stand for one hour. During this time, observe the rate of hæmolysis as indicated by the loss of opalescence and increasing depth of colour. Correlate with these findings the concentrations of sodium chloride in each tube. In those tubes where no hæmolysis has occurred at the end of the period, add (1) a few drops of ether or chloroform, or (2) 2 drops of a 5 per cent. solution of bile salts, or (3) 2 drops of a 5 per cent. solution of saponin. Notice what happens.

6. 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. Repeat with fresh and boiled milk** (*cf.* p. 77).

Benzidine Test.—**Take half a test-tube of blood diluted until practically colourless ; add a few drops of benzidine dissolved in glacial acetic acid, and 1 c.c. of 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** (*cf.* p. 264).

7. CATALYTIC ACTION OF BLOOD.—To 1 c.c. defibrinated blood add 2 to 3 c.c. hydrogen peroxide (20 vols.) and allow to stand. Note the evolution of gas (oxygen).

8. BLOOD CRYSTALS.—Mix a drop of rat's defibrinated 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. Five to ten minutes usually elapse before the crystals are seen.

Place a little blood, obtained by pricking the finger, on a slide, and allow it to dry; cover, run glacial acetic acid under the cover slip and boil: DO NOT CHAR; when cool repeat this with fresh acid and then examine microscopically for the dark brown crystals of hæmin.

9. BLOOD-PLASMA AND COAGULATION.—(a) Coagulation has been prevented in specimen A by the addition of an equal volume of saturated sodium sulphate solution, or of a quarter of its volume of saturated magnesium sulphate solution. The corpuscles have settled, and the supernatant salted plasma has been siphoned off.

(b) Coagulation 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.

(c) Put a small quantity of A into three test-tubes and dilute each with about ten times its volume of the following liquids, viz.:—

A1. With distilled water.

A2 and A3. With a solution of fibrin-ferment (thrombin) containing a little calcium chloride.

(d) Put A1 and A2 into the water-bath at 40° C.; leave A3 at the temperature of the air. A1 coagulates slowly or not at all; A2 coagulates rapidly; A3 coagulates less rapidly than A2.

(e) 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.

Collect some of the coagulum obtained in A2 for further investigation below (10 (b)).

10. BLOOD-SERUM AND FIBRIN.—(a) Blood-serum is the fluid residue of the blood after the separation of the clot; it is blood-plasma *minus* the fibrin and with thrombin added. 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 already studied in Chapter III.), extractives and salts in solution. The proteins are serum-albumin and serum-globulin.

(b) Wash some of the fibrin obtained in 9 (c) above, and show that

it is protein in nature by carrying out on it the Xanthoproteic, Millon's, and Rose's reactions (p. 43).

(c) **SEPARATION OF THE SERUM PROTEINS.**—Dilute serum with fifteen times its volume of *distilled* water. It becomes cloudy owing to the partial precipitation of serum-glubulin. Add a few drops of 2 per cent. acetic acid; the precipitate becomes more abundant, but dissolves in excess of the acid.

Pass a stream of carbon dioxide through serum diluted with twenty times its bulk of water. A partial precipitation of serum-globulin occurs.

Saturate some serum with magnesium sulphate by adding crystals of the salt and grinding the mass in a mortar. A precipitate of serum-globulin separates.

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. Filter the mixture and saturate the filtrate obtained by shaking it with solid ammonium sulphate. A precipitate of the albumin is produced.

11. **PREPARATION OF FIBRINOGEN.**—Add to oxalated plasma an equal volume of saturated sodium chloride, *i.e.*, half saturate the plasma with salt. Note the formation of a precipitate of fibrinogen.

12. **AGGLUTINATION.**—Many substances cause clumping of the corpuscles of blood. One such substance occurs in the scarlet-runner bean. Its presence therein can be shown by the following experiment:—Prepare a dilute (1 to 10) solution of defibrinated blood in isotonic sodium chloride. Prepare also an extract of two or three beans by grinding them in the same strength of salt for two or three minutes, and filtering.

Take four small narrow tubes, and to each add the same amount of the diluted blood, approximately 1 c.c. To the first add 5 drops of the bean extract, to the second 3 drops, to the third 1 drop, and to the fourth 3 drops of the salt solution. Mix each tube thoroughly, and observe.

Agglutination occurs rapidly and completely in the first, not so rapidly in the second, more slowly in the third, and not at all in the fourth or control tube.

The phenomenon of agglutination is the basis of certain clinical tests, as, for example, that for typhoid fever.

HÆMOGLOBIN AND ITS DERIVATIVES

13. **THE SPECTROSCOPE.**—Direct the spectroscope to the window and carefully focus the Fraunhofer lines. Note especially D in the yellow

and E, the next well-marked line, in the green (Fig. 29, spectrum 1, p. 208).

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.

14. 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 30 of water); then fill the second tube with the same mixture diluted with an equal bulk of water (1 in 60); half fill the third tube with this and fill up the tube with an

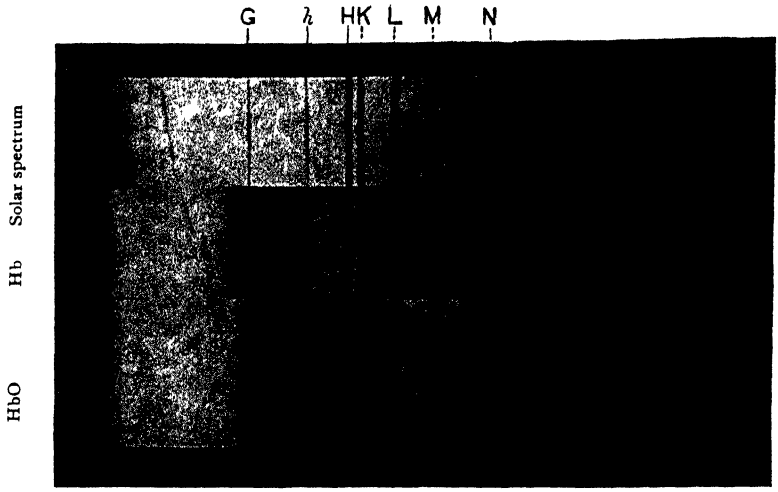


FIG. 22.—The photographic spectrum of reduced hæmoglobin and oxyhæmoglobin. (Gamgee.)

equal bulk of water (1 in 120), and so on. The sixth tube will contain 1 part of blood to about 1000 of water and will be nearly colourless.

(a) Into another series of six test-tubes pour some of the contents of each of the first series and add one drop of a freshly prepared 10 per cent. sodium hydrosulphite solution. Note the change of tint from red to purple. Stokes's reagent may be employed in this experiment instead. 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.

(b) 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 reduced hæmoglobin the bands are no longer seen, whereas those of oxyhæmoglobin in specimens similarly diluted are still visible.

(c) 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.

15. PREPARATION OF BLOOD PIGMENTS.—Defibrinated ox-blood suitably diluted may be used in the following experiments. Examine the preparations with the spectroscope and map out the spectra seen.

(a) *Carbon Monoxide 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 reducing agents. 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 (Fig. 29, spectrum 4).

(b) *Methæmoglobin.*—Add a few drops of potassium ferricyanide to dilute blood, and warm gently. The colour changes to mahogany-brown. Examine the preparation with the spectroscope. Note the characteristic band in the red (Fig. 29, spectrum 5). On dilution, other bands appear (Fig. 29, spectrum 6). Treat with ammonium sulphide and note that the band of hæmoglobin reappears.

(c) *Acid Oxyhæmatin.*—(i) 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 filtrate. Compare the position of the absorption band in the red with that of methæmoglobin; that of acid oxyhæmatin is further from the D line (Fig. 29, spectrum 7).

(ii) Add some glacial acetic acid to undiluted defibrinated blood. Extract this with ether by gently agitating it with that fluid. The ethereal extract should be then poured off and examined spectroscopically. The band in the red is seen, and on further diluting with ether, three additional bands appear.

(d) *Alkaline Oxyhæmatin.*—(i) 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. 29, spectrum 8).

(ii) 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, add to it a few drops of undiluted defibrinated blood. The fluid shows the spectrum of alkaline oxyhæmatin. This may then be used for the next experiment.

(e) *Reduced Hæmatin.*—Add a reducing agent to the solution of alkaline oxyhæmatin ; 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. 29, spectrum 9). This is reduced (alkaline) hæmatin or hæmochromogen. The spectrum of alkaline oxyhæmatin reappears in a short time after vigorous shaking with air.

(f) *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. 29, spectrum 10).

Milroy has described a stannous compound of hæmatoporphyrin which may be prepared as follows :—To half a test-tube of glacial acetic acid (5 c.c.) add 1 drop of blood and heat to boiling. Add a small amount of stannous chloride, a trace of fine granulated zinc, and 1 drop of concentrated hydrochloric acid. Boil for half a minute and add crystalline sodium acetate ; boil once more and filter. On spectroscopic examination two bands are seen which resemble those of oxyhæmoglobin. Milroy has applied this reaction to the identification of blood in urine and in fæces, and found it even more delicate than the test of obtaining reduced alkaline hæmatin, and examining spectroscopically.

(g) *Cytochrome.*—This pigment is a mixture of hæmochromogens, and occurs in vertebrate and invertebrate muscular tissue. It is considered to play an important rôle in tissue respiration.

16. CHEMICAL METHOD OF BLOOD GAS ANALYSES.—(a) *Estimation of the Oxygen Capacity of Blood.*—The blood is laked thoroughly, treated with potassium ferricyanide, oxygen liberated, collected over water, and measured. Thoroughly oxygenate about 30 c.c. of blood (defibrinated or oxalated) by placing in a 200 or 300 c.c. flask and rotating. In this way the blood is spread as a thin film on the side of the flask and is exposed to oxygen. Pipette 20 c.c. into the small bottle of the apparatus illustrated on p. 269. The last drops of blood should not be *blown* out from the pipette but should be expelled by closing the end of the pipette and warming the bulb with the hand. Note : a "20 c.c." pipette delivers only about 19·6 c.c. of blood. To

the blood add dilute ammonium hydroxide (1 : 250), which is free from carbon dioxide, till the blood is completely laked; about 30 c.c. are generally required. Laking is complete when a thin film of the blood is perfectly transparent. Place 5 c.c. of fresh saturated aqueous solution of potassium ferricyanide in the test-tube within the bottle, replace the rubber stopper, ensure that the apparatus is air-tight, and place the bottle in a water-bath at room temperature till the volume is constant. Bring water inside and outside the burette to the same level. Note the reading X. Invert bottle to mix the blood and ferricyanide. Replace in the water-bath till temperature is once more constant. Oxygen has been evolved and has displaced water in the burette. Equalise the levels once more by raising the burette. Note the reading Y. The difference between the readings, *i.e.*, Y – X, is the number of c.c. of oxygen liberated from 19·6 c.c. of blood at the temperature and pressure of the laboratory.

For more accurate work, means are taken to maintain the apparatus at strictly uniform temperature, to allow for the solubility of oxygen in, and for the vapour pressure of, water at the particular temperature employed.

(b) *Estimation of the Carbon Dioxide Content of Blood.*—This can be carried out on the same specimen as used above in the estimation of the oxygen capacity. Remove the rubber stopper and place in the bottle another small tube containing 4 c.c. of saturated aqueous solution of tartaric acid. Replace the stopper, and bring to room temperature and atmospheric pressure. The remainder of the estimation is carried out as described above for the oxygen content. Note that a certain amount of carbon dioxide is lost during the process of oxygenation, and that special methods have to be taken to avoid this (p. 220).

RESPIRATION

17. ESTIMATION OF OXYGEN AND OF CARBON DIOXIDE IN AIR.—The apparatus employed is a modified form of Haldane's, and consists essentially of—

(a) A gas burette to receive the sample of air for analysis.

(b) A glass bulb containing 10 per cent. caustic soda for the absorption of the carbon dioxide.

(c) A glass bulb containing 10 per cent. pyrogallic acid in 60 per cent. potash for the absorption of oxygen.

(d) A four-way tap which enables connection to be made between the gas burette and the soda or pyro bulbs or the outside air.

The stems of the soda and pyro bulbs are engraved so that the volume of gas may always be estimated from the same point.

Before reading the gas burette the fluid in the graduated tube and in the movable bulb must always be adjusted to the same level.

Method.—Adjust the level of the soda and pyro bulbs so that the solutions, at atmospheric pressure, stand at the engraved marks on the stems.

Before commencing an analysis it is necessary that all O_2 and CO_2 contained in the apparatus should be absorbed, and therefore the air is driven over into the soda and pyro bulbs a few times by manipulation of the four-way tap and raising and lowering the bulb of the gas burette.

This manœuvre having been completed, the apparatus may be regarded as containing only nitrogen, which will not affect the subsequent readings.

(a) Place the gas burette and the pyro bulb in communication, and level the pyro solution to the mark engraved on the stem. Shut off the pyro bulb and establish contact between the burette and the soda bulb.

(b) Level the soda to the engraved mark and put the gas burette into communication with the air.

(c) Raise the bulb and fill the gas burette with fluid, and then, lowering it, draw in the sample of air to be analysed, finally closing the tap.

(d) Put the soda bulb in contact with the burette, adjust the levels, and measure the volume of gas drawn in.

(e) Absorb the CO_2 by passing the gas over into the soda bulb a few times, and repeating until the volume is constant, at which point all the CO_2 has been absorbed.

Adjust the level of the fluid in the soda bulb and then read the volume in the gas burette. The difference between this and the first reading gives the amount of CO_2 absorbed.

(f) Drive the gas out of the CO_2 bulb by raising the reservoir. Turn the tap to shut off the bulb and place the burette in communication with the pyro bulb, and absorb the O_2 by passing the gas backwards and forwards into the bulb.

When there is no further diminution in volume bring the pyro to the mark on the stem. Then shut off the bulb and read the volume of gas remaining in the burette.

The readings obtained are :—

First reading = Total volume of air.
Second „ = Diminution due to absorption of CO_2.
Third „ = „ „ „ O_2.

From these calculate the percentages of oxygen and carbon dioxide in the sample taken.

The apparatus is obviously adapted for analysis of any sample of gas containing oxygen and carbon dioxide, such as alveolar air, a specimen of which is obtained as described on p. 227.

18. ESTIMATION OF BLOOD SUGAR.—Numerous methods have been devised for the estimation of sugar in blood. Some of the older methods, *e.g.*, those of Michaelis and Rona and of Bertrand, required 25 c.c. of blood or serum, and were unsuitable, therefore, for the purpose of carrying out continuous observations on the same person as is frequently essential in the study of certain pathological disturbances of metabolism like that of diabetes mellitus. Accurate micro-methods enabling this to be done are now available. Of these the following require mention : Hagedorn and Jensen, MacLean, and Folin and Wu ; the two former are volumetric methods, whilst the last involves the use of the colorimeter (p. 317).

METHOD OF HAGEDORN AND JENSEN.—*Principle of the Method.*—The proteins of the blood are precipitated with zinc hydroxide. The filtrate so obtained is heated with potassium ferricyanide solution, which is in part reduced by the glucose present. The unreduced ferricyanide is estimated by adding an iodide solution and titrating the iodine set free with sodium thiosulphate, according to the equation $2H_3Fe(CN)_6 + 2HI \longrightarrow 2H_4Fe(CN)_6 + I_2$. The reversal of the reduction reaction is prevented by precipitation of the ferrocyanide formed as a zinc salt.

The solutions required are :—

(i) Caustic soda, N/10.

(ii) Zinc sulphate, 0·45 per cent.

(iii) Potassium ferricyanide, 1·65 gram ; anhydrous sodium carbonate, 10·6 grams, dissolved in a litre of water. This solution is unstable to light, and should be kept in a dark bottle.

(iv) Potassium iodide, 25 grams ; zinc sulphate, 50 grams ; sodium chloride, 250 grams ; dissolved in a litre of water.

(v) Iron-free glacial acetic acid, 3 c.c., dissolved in 100 c.c. of water.

(vi) Soluble starch, 1 gram, dissolved in 100 c.c. of saturated sodium chloride solution.

(vii) Sodium thiosulphate solution, N/200.

(viii) Potassium iodate solution, N/200, prepared by dissolving 0·3566 gram of dry potassium iodate, accurately weighed out, in 2 litres of water.

The estimation is carried out as follows :—

Pipette into a test-tube (15 × 150 mm.) 1 c.c. of N/10 caustic soda and 5 c.c. of the 0·45 per cent. zinc sulphate solution. A gelatinous precipitate of zinc hydroxide forms. 0·1 c.c. of blood from a capillary pipette is introduced into the test-tube, the pipette being washed out twice with the mixture and blown empty. Now put the test-tube into a boiling water-bath for three minutes, and then filter the contents into a boiling tube through cotton-wool which has been previously washed. The filter is finally washed twice with 3 c.c. of water. 2 c.c. of the alkaline potassium ferricyanide solution is now added to the filtrate and the whole is heated on the boiling water-bath for fifteen minutes. After cooling, add 3 c.c. of the iodide-sulphate solution and 2 c.c. of the 3 per cent. acetic acid. Using two drops of the 1 per cent. solution of soluble starch as an indicator, titrate the resulting liquid with N/200 sodium thiosulphate. As a check, the whole process should be repeated without blood to obtain a blank determination on the reagents used. The thiosulphate, moreover, is standardised by titration of 2 c.c. of the N/200 potassium iodate, to which has been added 3 c.c. of the potassium iodide, zinc sulphate solution, and 2 c.c. of the acetic acid. Using the factor so obtained the titration figures for the blood and blank experiments are corrected. Let x be the difference in cubic centimetres between these corrected figures.

Then since 2 c.c. of the ferricyanide solution are reduced by 0·355 mg. glucose, the amount present in the blood under examination is :—

$$355 \times \frac{x}{2} \text{ mg. per 100 c.c. blood.}$$

BLOOD AND LYMPH

Blood is the medium by which oxygen and food materials are carried to various tissues of the body, and by which the various waste products are conveyed to the appropriate execretory organs in order to be eliminated in their several proper ways. While circulating in the body, and for a short time after shedding, blood is a slightly viscous, opaque, red fluid, alkaline to litmus, saltish in taste, of specific gravity 1·045 to 1·075, having a freezing-point (Δ) – 0·51° to 0·62° C., and with, in some cases, an odour peculiar to the animal.

Microscopic examination shows blood to be composed of two main fractions :—

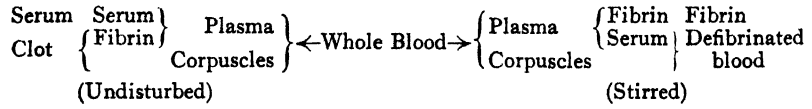
1. The formed elements, cells, or **corpuscles** which float in
2. The fluid portion or **plasma.**

The former (the cells) are of two types—firstly, those containing the pigment to which blood owes its colour, termed the **erythrocytes** or red cells, and secondly, the colourless white cells termed the **leucocytes.**

When allowed to stand undisturbed after shedding, the fluid, mobile blood becomes, in from five to ten minutes, more and more viscous, until it sets to a red jelly. This slowly shrinks to a firm clot which finally extrudes a pale straw-coloured liquid, the **serum.** In this floats the coagulum, red in colour owing to the entangled corpuscles.

On the other hand, if, during the process of clotting, the clot be removed as it is formed, by stirring with, *e.g.*, a feather, the solid separating matter is obtained as a white stringy substance adherent to the stirrer, while the corpuscles are left suspended in the serum. Such blood is termed **defibrinated,** as the fibrin which forms the clot is removed. In appearance it cannot be distinguished from whole uncoagulated blood, from which, however, it differs in chemical composition most profoundly.

We can summarise these changes thus :—



The formation of fibrin is the essential part of the process of coagulation. Under certain circumstances blood may be caused to clot very slowly or not at all, and in such a specimen the corpuscles have time to sink to the bottom. The red cells occupy the lowest layer surmounted in order by the white cells, usually with some fibrin, and finally the

fluid plasma. The layer of leucocytes constitutes the " buffy coat " of the older writers.

Total Volume of Blood.—This has been estimated in various ways; for example (1) by direct decapitation and collection of the blood which escapes and which remains in the body, or (2) by addition to circulating blood of known amounts of some preferably non-toxic substance, such as sodium chloride, vital-red (a dye which stains the plasma), dextrin, or carbon monoxide; by appropriate means the concentration of the added material can be calculated, thus enabling the volume of blood to be obtained.

The first method, apart from inherent inaccuracy, is of limited application in man, but use of the others gives a mean value of one-twelfth to one-fourteenth of the body weight.

Proportion of Corpuscles to Plasma.—By allowing the corpuscles to sink naturally, or by centrifuging whole blood in a graduated tube, the volume occupied by the corpuscles can be estimated. This is between 43 to 50 per cent. of the whole blood.

Before taking up the more detailed chemistry of the blood we shall discuss the phenomenon of coagulation and the various factors determining its occurrence, rate, and extent.

Microscopically the inception of clotting is seen to be the formation of a network of threads of a gelatinous nature radiating from, and running between, certain disintegrating white corpuscles.

Ultramicroscopically these filaments are seen to be composed of minute elongated granules which coalesce in their long axes to form the threads. This is the stage described above as a jelly.

The threads in turn shorten, their minute structure is lost, shrinkage takes place, and the serum is squeezed out.

It should be noted that the formed elements are not an essential component of the coagulation process. Plasma will, and does, clot perfectly normally in their absence, though their presence facilitates the process especially at room temperature or in blood obtained from a starving animal. On the other hand, to be an efficient protection against excessive loss of blood through hæmorrhage, either internal or external, the presence and participation of certain formed elements (the platelets, see later) is essential. The factors which modify coagulation are as follows:—

A. *Those which cause inception or acceleration of clotting.*

1. Temperature slightly above normal.

2. Contact with material which blood can wet, *e.g.*, a feather *in vitro* or damaged vessel walls *in vivo*.

3. Agitation, particularly in shed blood.

4. Addition of coagulants. These may be animal substances, such as thrombin (see below), which causes complete clotting *in vitro*, but only increases the rate, even in large doses, *in vivo*; or pure organic substances, such as salicylic acid or quinine, which cause local thrombosis *in vivo* and are used clinically for this purpose in the treatment of varicose veins.

5. Rapid injection of many tissue extracts. This produces thrombosis, and is termed in this case the " positive phase " of coagulation (see the " negative phase " later in B).

B. *Those which cause inhibition or retardation of clotting.*

1. Temperature at or near the freezing-point of blood.

2. Complete or partial contact with material which blood cannot wet, *e.g.*, oil or paraffin.

3. Addition of great excess of water (30 volumes).

4. Addition of neutral salts, such as sodium or magnesium sulphate, in large amounts.

5. Addition of soluble oxalates, fluorides, or citrates.

6. Addition of anticoagulants such as an extract of leech heads, or any substances (frequently autolytic products) which inhibit thrombin, and termed antithrombins, or addition of large amounts of certain commercial peptones.

7. Injection of small amounts of tissue extracts, or the slow injection of larger amounts. This is the " negative phase " referred to in A.

It is easy to enumerate the agencies which hasten or hinder coagulation of the blood; it is much more difficult to explain their action. No other subject has produced such a number of theories as blood-clotting, but none of these can be regarded as satisfactory.

It may be regarded as fairly certain that within the vessels one of the constituents of the plasma, a protein with many globulin characteristics called *fibrinogen*, exists in a soluble form. When the blood is shed the fibrinogen is altered in such a way as to give rise to the comparatively insoluble material *fibrin*. The majority of recent views on blood coagulation assume that this change is brought about by the activity of *thrombin*, which originates from the disintegration of platelets and colourless corpuscles when the blood leaves the blood-vessels or comes into contact with foreign matter. Howell explains the fact that the blood does not coagulate during life, by assuming the presence in the blood of an anticoagulant termed heparin, which is believed to be produced in the liver.

This simple view does not meet many difficulties, so the theory has been complicated by assuming that thrombin has a precursor

called *prothrombin*, which is converted by calcium and by disrupting white cells of the blood into thrombin.

It has long been recognised that ionisable calcium salts are necessary for clotting and that this can be hindered by removal of these salts as, *e.g.*, by adding a soluble oxalate, fluoride, or citrate to the blood. This is undoubted, as is the fact that blood so treated can again be made to clot by recalcification. How the calcium acts is unknown; most agree that fibrin is not a calcium compound of fibrinogen, and it is generally supposed that in some way calcium co-operates in the elaboration of thrombin.

The injection of certain tissue extracts causes intravascular clotting; and again it is an undoubted fact that blood clots more rapidly when it is shed if it is allowed to come in contact with the tissues of the wound than when it is received directly through a clean cannula into a clean vessel. Here we have the contact of blood with a surface and with a material that can be wetted by it.

To mention one more of the many views related to the foregoing, it has been (until recently) believed that peptone restrains coagulation because it induces the liver to produce an increased supply of antithrombin, so that the blood does not clot even when it is shed. This is supported by two statements, neither of which is true; the first statement is that peptone will not prevent or hinder the coagulation of shed blood; the incorrectness of this can be proved by anyone who tries the experiment. The second statement is that if the liver is shut off from circulation peptone no longer manifests its action. In this laboratory Pickering and Hewitt have shown that this also is not the case. Provided the precaution is taken that the blood is well oxygenated, peptone restrains coagulation equally well whether the liver is in the circulation or not. This precaution was neglected by the earlier experimenters, even though they knew another undoubted fact, viz., that carbon dioxide greatly favours coagulation and that peptone-plasma may be made to clot by simply passing a stream of carbon dioxide through it.

No doubt can be cast on the statement that intravascular conditions are favourable to non-coagulation, but difficulties exist to prevent Howell's view from being accepted in its entirety. One of these is the fact that even large amounts of heparin produce finally an increased coagulability after a temporary incoagulability. The conditions which restrain coagulation *in vivo* appear to be physical rather than chemical.

Many derivatives of organic materials restrain coagulation; an extract of leech heads does it, a decoction of yeast, certain preparations

of nucleic acid, and so forth. Such materials (breakdown products of varying origin) cannot be considered as definite substances concerned in a physiological process.

All the facts mentioned above point to the change of fibrinogen into fibrin being not a chemical but a physical change, that normally both fibrinogen and prothrombin are in intimate relation with the other proteins, albumin and globulin, in the blood-stream, and are thereby prevented from interacting together and with calcium. On contact with foreign substances, as, for example, tissue extracts, this protective action is lost, and thrombin is formed from the calcium and the prothrombin. The newly formed thrombin then reacts with the fibrinogen with the production of fibrin, first as ultra-microscopic particles which ultimately form the typical threads and the clot.

The main agent appears to be a disturbance of surface conditions around the colloidal protein particles; surface action and surface tension must be disturbing factors in such a complex colloidal mixture as the blood.

So long as the surface conditions remain normal, that is when the blood is in living, healthy blood-vessels, the blood remains fluid. If these normal conditions are imitated, *e.g.*, by enclosing the blood within a piece of surviving blood-vessel or an isolated heart, clotting is also much delayed. If the blood is received within an oiled vessel through an oiled cannula, there is again delay because the imitation of the normal surface conditions is more or less successful. Injury to the vessel walls or contact with foreign objects at once upsets the normal surface conditions, and clotting commences. Also the inhibitory effect of peptone on the coagulation of shed blood can be readily demonstrated only if care is taken to preserve the normal surface conditions by surrounding it with oil.

It is hard to be dogmatic in such a question as blood coagulation, but we can summarise the known facts of coagulation in a simple schema to show possible reactions of blood constituents after contact with foreign surfaces.

Prothrombin + Calcium + Platelets + Tissue Juices→Thrombin.
Thrombin + Fibrinogen→Fibrin.

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 centrifuge.

Lymph, 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 more than very slightly 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 1·026 to 1·029.

Its chief constituents may be enumerated as follows :—

1000 parts of plasma contain—

Water	902·90
Solids	97·10
Proteins: 1, albumin . . .	46 to 67
2, globulin . . .	12 ,, 23
3, fibrinogen . . .	3 ,, 6
Extractives (including fat) . . .	5·66
Inorganic salts	8·55

In round numbers, plasma contains 10 per cent. of solids, of which 8 per cent. is protein in nature.

Serum and lymph contain the same three classes of constituents —proteins, extractives, and salts. The specific gravity of serum is 1·026 to 1·032. 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.	(The substance called thrombin is possibly of protein nature.)

The gases of the plasma and serum are small quantities of oxygen, nitrogen, and carbon dioxide. The greater part of the oxygen of the blood is combined in the red corpuscles with hæmoglobin; the carbon dioxide 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 possesses many reactions in common with serum-globulin, but differs from it in that it may be separated from it by half saturation with sodium chloride. It is coagulated 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 (*cf.* table on p. 195). All mammalian fibrinogens have the same elementary composition.

Serum-globulin.—This substance, which is a typical globulin, is considered in the practical exercises at the head of this chapter. It consists of two individual proteins—englobulin and pseudo-globulin. The former is insoluble in water and in 33 per cent. ammonium sulphate; the latter is soluble in water, and is only precipitated by much stronger (45 per cent.) ammonium sulphate.

Serum-albumin.—This also is perhaps not a single substance. It is distinct from egg-albumin in certain physical properties such as optical rotatory power. These native proteins, englobulin, pseudo-globulin, and albumin, have specific immunological reactions, and are therefore chemically not identical.

Thrombin.—Schmidt's method of preparing it is to take serum and add excess of alcohol to precipitate all the proteins, including thrombin. After some weeks the alcohol is poured off; the insoluble serum-globulin and serum-albumin are extracted with water to obtain thrombin, which is not so easily coagulated by alcohol as are the others. A simpler method of preparing fibrin ferment in an impure but efficient form is to dilute 5 c.c. serum with one litre of water.

B. **Extractives.**—These are both non-nitrogenous and nitrogenous in composition. The non-nitrogenous are sugar (70 to 100 mg. per cent.), fats, serum lutein (p. 40), soaps, cholesterol, and cholesterol esters; the nitrogenous are urea (20 to 40 mg. per cent.) and still smaller quantities of uric acid (urates), creatine, creatinine, xanthine, hypoxanthine, and amino-acids. Acetone bodies, lactic acid, gases, and an unknown sulphur-containing substance are found in traces. Included in this group are the non-nitrogenous pigments carotin and xanthophyll.

C. **Salts.**—The most abundant salt is sodium chloride; it constitutes between 60 and 90 per cent. of the mineral matter. Potassium chloride is present in much smaller amount. It constitutes about 4 per cent. of the 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 nucleated animal cells. Their protoplasm yields proteins belonging to the nucleo-protein and globulin groups. They contain small quantities of fat, lipoids, and glycogen. Their number (excluding platelets) is about 10,000 per cubic millimetre and depends on the diet. Starvation causes a diminution, a meal an increase in their number. They are of several types, of which here we can only mention the platelets, which average 200,000 per cubic millimetre. So small as just to be distinguished, they appear to be nucleated, are said to be amœboid, and probably play, when present, a part in coagulation.

THE RED BLOOD CORPUSCLES

The red blood corpuscles are much more numerous than the white, averaging in man 5,500,000 per cubic millimetre, and in woman 4,500,000 or 400 to 500 red to each white corpuscle. The usual method of enumerating the corpuscles is by employing the Thoma-Zeiss hæmacytometer.

Thoma-Zeiss Hæmacytometer. — This instrument consists of a glass slide (*s*) seen in section in Fig. 23. 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. 24). 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 of different proportions, and is similarly employed for counting the leucocytes. In some cases a micrometer slide ruled in larger squares

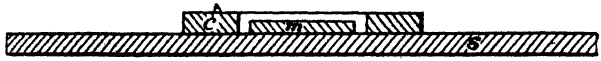


FIG. 23.—Thoma-Zeiss hæmacytometer.

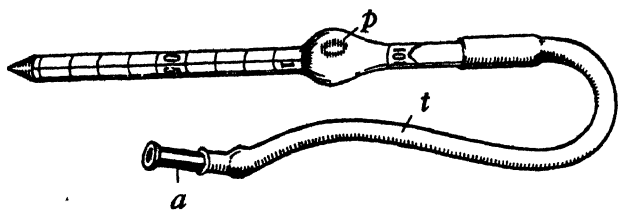


FIG. 24.—Pipette of Thoma-Zeiss hæmacytometer.

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. In counting the colourless corpuscles the addition of a dye, such as methyl violet, renders the operation easier.

Differential counts to show the relative proportions of the various types of white cells are made in appropriately stained blood films.

Red blood corpuscles 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 and certain anæmic conditions nucleated red corpuscles are, however, found.

Water causes *hæmolysis*, *i.e.*, the corpuscles swell up and rupture, liberating the red pigment (oxyhæmoglobin), and leaving a globular colourless membrane or stroma. This is termed laking the blood.

Hæmolysis is also produced by soaps, bile salts, and other substances, usually lipoid solvents. *Strong salt solution* causes the corpuscles to shrink: they become wrinkled or crenated. The action of water and salt solution is explained by the outer layer of the corpuscle acting as a semipermeable membrane 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, which is immediately altered and precipitated. It remains adherent to the stroma as a brown globule (see Fig. 25).

The divergence from the normal physiological concentration of 0·9 per cent. sodium chloride, which the corpuscle will endure without hæmolysis, is obviously determined by its fragility. This is estimated by subjecting the cell to gradually diminishing strengths of salt. Clinically this test is employed to detect one type of jaundice in which the resistance is reduced. The healthy corpuscle will withstand sodium chloride of 0·42 per cent. without loss of hæmoglobin, whereas in the particular pathological condition cited, this concentration causes complete hæmolysis.

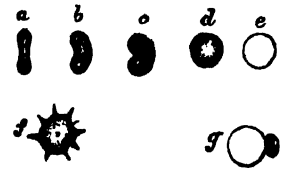


FIG. 25.—*a-e*, successive effects of water on a red blood corpuscle, *f*, a red corpuscle crenated by salt solution; *g*, action of tannin on a red corpuscle.

Composition.—1000 parts of red corpuscles contain—

Water	688	parts
Solids organic	303·88	,,
Solids 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 combined with hæmoglobin forming oxyhæmoglobin.

The corpuscles also contain a certain amount of carbon dioxide (see Respiration, at the end of this chapter).

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 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. 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 is a respiratory pigment, and its function is to carry molecular oxygen from the lungs to such other tissues as require it for oxidative purposes.

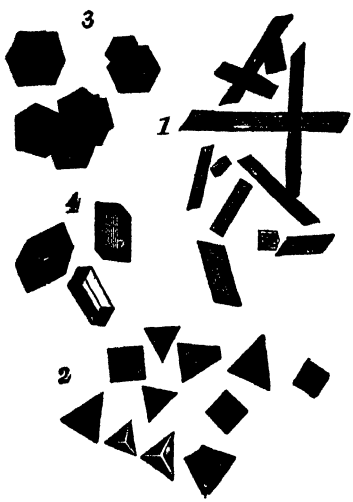


FIG. 26.—Oxyhæmoglobin crystals magnified: 1, from human blood; 2, from the guinea-pig; 3, squirrel; 4, hamster.

Crystals of oxyhæmoglobin have been obtained with varying degrees of ease from the blood of over one hundred species of animals. The following methods are used:—

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. 26) represent the form of the crystals so obtained.

In nearly all animals the crystals are rhombic prisms. 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 other methods which are described later.

THE CHEMISTRY OF HÆMOGLOBIN

The question at once arises as to the chemical identity of the hæmoglobin found in the various species of animals. The methods employed for solving this problem are mainly three :—

(*a*) Crystallographic and chemical examination of various specimens.

(*b*) Determination by accurate spectroscopic examination of the absorption bands produced by the pigment and its derivatives.

(*c*) Determination of the avidity with which various hæmoglobins unite with oxygen.

As a result of the application of these methods, it seems probable that there are many different hæmoglobins.

Perhaps the best way in which we can study hæmoglobin is to detail some of the derivatives which can be obtained from it, and afterwards to indicate schematically their interrelationships.

Hæmoglobin is a conjugated chromo-protein, easily oxidised and reduced, which, on treatment with acids or alkalis, is largely broken down into a protein (globin) and the oxygen-carrying, iron-containing substituted pyrrol derivative, *hæmatin*. Hæmoglobin as such exists only at or near neutrality.

In the presence of free molecular oxygen, hæmoglobin is converted into *oxyhæmoglobin* which, in turn, can be easily reduced to *reduced hæmoglobin*. On treatment with acid or alkali, reduced hæmoglobin gives rise to reduced *acid* or *alkaline hæmatin*.

Methæmoglobin is formed from oxyhæmoglobin by treatment with potassium ferricyanide, and is the oxide of hæmoglobin. It contains, therefore, the protein globin and an iron-containing pigment.

Hæmin is the hydrochloride of hæmatin, and is obtained by heating blood with glacial acetic acid at the boiling-point with the addition of sodium or potassium chloride.

Hæmochromogen is derived from oxyhæmoglobin, or reduced hæmoglobin, by first warming with alkali and then reducing the pigment produced.

It will thus be seen that hæmin and hæmochromogen have one

common feature in their preparation, viz., they are subjected to the action of a hydrolysing agent, acid or alkali. Bearing in mind the general action of such reagents on a conjugated protein, we should expect the changes to be represented thus :—

(*a*) Oxyhæmoglobin (in presence of acid) loses globin and gives hæmin.

(*b*) Oxyhæmoglobin (in presence of alkali) loses globin and gives hæmochromogen on reduction.

Hæmin and hæmochromogen should therefore be simply related as oxidation and reduction products of each other, and should be globin free. Until recently this was believed.

From the work of Barcroft and his school it is now believed that this is not the case, and that while hæmin is protein free, hæmochromogen is *not*, but still contains a globin moiety. If the salt hæmatin hydrochloride (hæmin) be decomposed by alkali, the base is thereby liberated. Reduction of this base and examination of the reduced product shows it not to be hæmochromogen, which, according to the above scheme, it should be, but a separate distinct pigment. For this the name *hæm* has been suggested.

Hæm is thus an iron-containing, protein-free pigment, capable of oxidation and reduction, and exists in two forms, *oxidised hæm* and *reduced hæm*, whose relation to hæmin is as shown :—

$$\text{Reduced hæm} \underset{\text{Acid oxidation}}{\overset{\text{Alkaline reduction}}{\rightleftharpoons}} \text{Hæmin} \underset{HCl}{\overset{NaOH}{\rightleftharpoons}} \text{Oxidised hæm}$$

The difference between hæm on the one hand, and hæmochromogen and reduced hæmoglobin on the other, lies in the absence of globin from the first, and its presence in the two latter.

The difference between hæmochromogen and reduced hæmoglobin is the nature of the protein. Experimentally it can be shown that in reduced hæmoglobin the protein is the natural globin, whereas in the hæmochromogen the globin is denatured. Or putting this in another way, if reduced hæm is taken, and to it is added globin, either hæmochromogen or reduced hæmoglobin is obtained. If the globin be denatured, the former is produced; if not denatured (*i.e.*, natural), the product is the reduced hæmoglobin.

Schematically, this may be shown thus :—

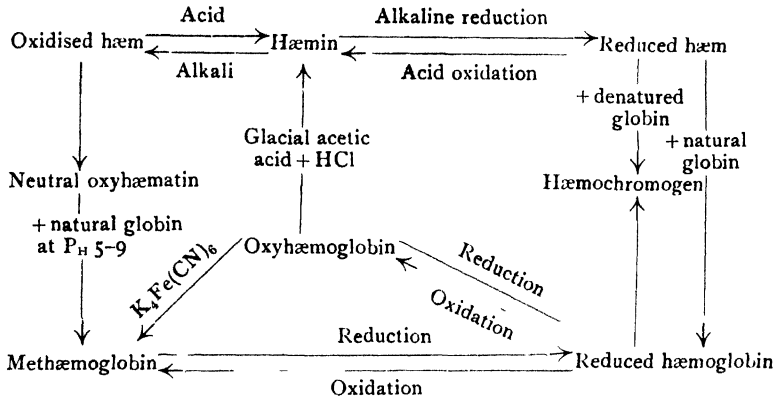
$$\text{Reduced hæm} \begin{cases} + \text{natural globin} \rightarrow \text{Reduced hæmoglobin.} \\ + \text{denatured globin} \rightarrow \text{Hæmochromogen.} \end{cases}$$

One other point must be mentioned, and that is the relation of hæm and methæmoglobin. Hill and Holden have succeeded in combining natural globin with neutral oxyhæmatin obtained from oxidised hæm. This union can be effected within a wide range of hydrogen-ion concentration.

Schematically, it may be represented as follows :—

1. Oxidised hæm→Neutral oxyhæmatin.
   [oxidised hæmatin]
2. Neutral oxyhæmatin + natural globin→methæmoglobin.

A complete scheme representing these various relations has been constructed by Pryde, and is here appended :—



In addition to the pigments detailed above, which, it will be remembered, all contain iron, others can be obtained which are free from this element. Hæmatoporphyrin is one of these. It is iron-free hæmatin : it may be prepared by mixing blood with strong sulphuric acid, the iron being taken out as ferrous sulphate. It has been obtained crystalline by Willstätter. 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 (p. 166).

Hæmopyrrol, which is formed by reduction from hæmatoporphyrin, has been proved to be a mixture of several pyrrol derivatives. It is also obtained from the derivative of chlorophyll called phyllopor-phyrin, 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. Carbon monoxide 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.

They differ in the colour they impart to a solution in water, and they differ in the position (constant for each compound) of the absorption bands as seen spectroscopically.

As spectroscopic examination of blood pigments is of very considerable importance, a short description of the instrument used may be given here.

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 luminous 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 is 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 it is termed, 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 larger forms of spectroscope are described in textbooks of physics. They are not in common routine use for clinical and ordinary biological work. For these purposes the smaller and more convenient type, termed the direct-vision spectroscope, is employed, in which, by an arrangement of alternating prisms of crown and flint glass (Fig. 27), the spectrum is observed in the axis of the whole instrument.

If now we interpose between the source of light and the slit of the spectroscope a vessel containing one of the blood pigments, there will be seen dark (absorption) bands corresponding to light absorbed by

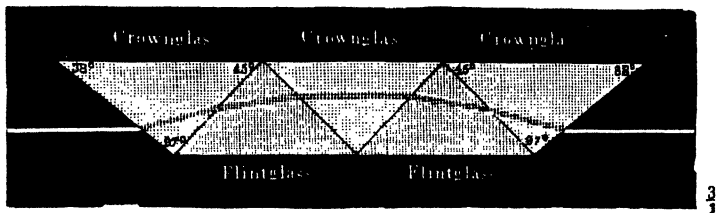


FIG. 27.—Arrangement of prisms in direct-vision spectroscope.

the coloured solution. Thus a solution of oxyhæmoglobin of a certain strength gives two bands between D and E lines; reduced hæmoglobin gives only one; and other red solutions, though to the naked eye similar to oxyhæmoglobin, will give characteristic bands in other positions.

In the examination of the spectrum of small objects, or of small amounts of material, a combination of microscope and spectroscope, termed the *micro-spectroscope*, is used.

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), or, best of all, sodium hydrosulphite. One gram 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.

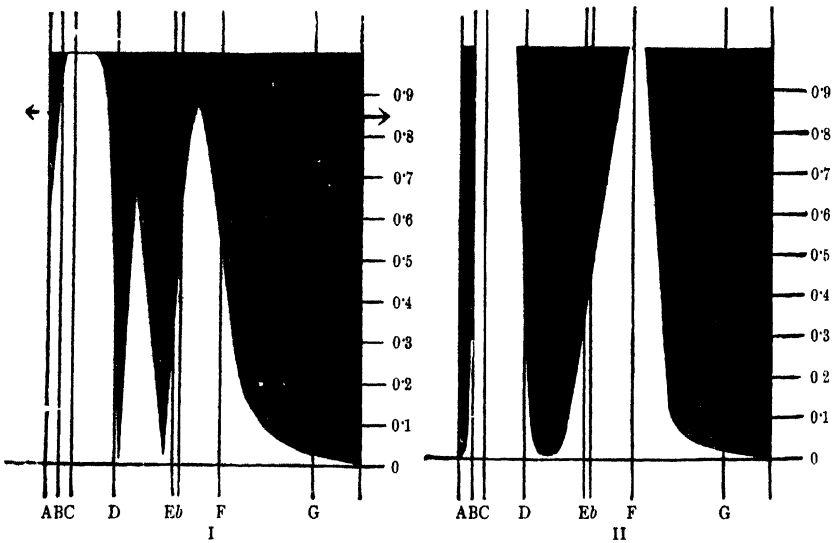


FIG. 28.—Graphic representations of the amount of absorption of light by solution (I) of oxyhæmoglobin; (II) of reduced hæmoglobin, of different strengths. The shading indicates the amount of absorption of the spectrum; the figures on the right border express percentages. (Rollett.)

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.

Fig. 28 illustrates a method of representing absorption spectra diagrammatically. The solution was examined in a layer 1 cm. 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 reduced hæmoglobin in II. The width of the shadings of 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. 29, 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. 28, 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 cm.) 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 single band (γ band) of hæmoglobin (Fig. 29, 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 reduced 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.

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 the urine in certain diseased conditions; it is therefore of considerable practical importance. It can be crystallised, and is usually stated to contain the same amount of oxygen as oxyhæmoglobin, but combined differently. Buckmaster's work, however, has shown that methæmoglobin contains only half as much oxygen as oxyhæmoglobin. This oxygen is not removable by the air-pump, nor by a stream of a neutral gas such as hydrogen. Methæmoglobin can, however, by reducing agents, such as ammonium sulphide, be made to yield hæmoglobin; it is of a brownish-red colour and gives a characteristic absorption band in the red between the C and D lines (Fig. 29, spectrum 5).

Potassium or sodium ferricyanide 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 carbon dioxide, 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 also contains oxygen. What occurs

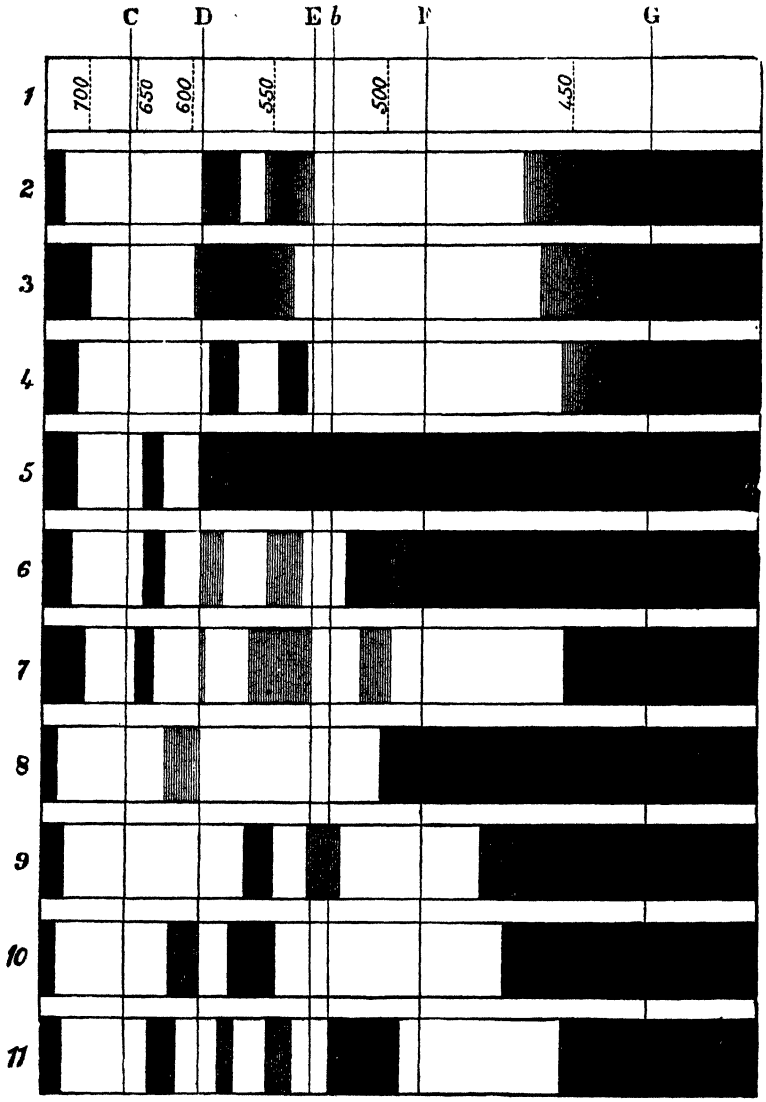


FIG. 29.—1, Solar spectrum. 2, Spectrum o oxyhæmoglobin (0·37 per cent. solution). First bands, λ 589-564; second band, λ 555-517. 3, Spectrum of reduced hæmoglobin. Band, λ 597-535. 4, Spectrum of CO-hæmoglobin. First band, λ 583-564; second band, λ 547-521. 5, Spectrum of methæmoglobin (concentrated 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 oxyhæmatin (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 oxyhæmatin. Band from λ 630-581. 9, Spectrum of reduced hæmatin. First band, λ 569-542; second band, λ 535-524. 10, Spectrum of acid hæmatoporphyrin. First band, λ 607-593; second band, λ 585-566. 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.

is that, after the oxygen is discharged from oxyhæmoglobin, it is replaced by oxygen derived from the reagents added.

As stated above, oxyhæmoglobin is *oxygenated* hæmoglobin, whereas methæmoglobin is *oxidised* hæmoglobin.

Carbon Monoxide Hæmoglobin may be readily prepared by passing a stream of carbon monoxide or coal gas through blood or through a solution of oxyhæmoglobin. It has a cherry-red colour. On dilution with water this fades to a pink, and so long as any colour is distinguishable this tint remains. Oxyhæmoglobin under similar circumstances fades to a straw-coloured liquid. Its absorption spectrum is very like that of oxyhæmoglobin, but the two bands are slightly nearer the violet end of the spectrum (Fig. 29, spectrum 4). Reducing agents, such as ammonium sulphide, do not change it; the gas is more firmly combined than the oxygen in oxyhæmoglobin, and thus when present, even in small amounts, it interferes with the normal respiratory processes.

Nitric Oxide Hæmoglobin. — This compound is formed when ammonia is added to blood, and then a stream of nitric oxide is passed through it. 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 is not of theoretical importance only, but some practical application in poisoning by gas liberated from high explosives.

TESTS FOR BLOOD

These may be summarised from preceding paragraphs. Briefly, they are microscopic, spectroscopic, and chemical. The best chemical test is the formation of hæmin crystals. These, sometimes called Teichmann's crystals, are prepared for microscopic examination by heating a trace of fresh or dried blood with a drop of glacial acetic acid on a slide; on cooling, dark brown plates and prisms belonging to the triclinic system separate out, often in star-shaped clusters and with rounded angles (Fig. 30).

FIG. 30.—Hæmin crystals magnified. (Preyer.)

In the case of an old blood-stain it is necessary to add a crystal of sodium chloride or potassium

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.

The production of such crystals is complete proof of the presence of one of the iron-containing derivatives of hæmoglobin. This test is of great value in medico-legal work, and is valuable in that, within wide limits, the age of the stain is of no account.

Regarding the other chemical tests, it should be noted that in the application of the guaiac test, excess of the alcoholic guaiacum reagent should not be used, nor should heat be applied. Furthermore, substances other than blood, such as pus, milk, saliva, when fresh, react positively. On boiling and cooling they do not give a positive reaction, whereas blood does. Certain inorganic medicinal substances are found in urine when a patient is taking them, *e.g.*, iodides, which also react positively.

Buckmaster claims that by using a solution of guaiaconic acid he can detect hæmolysed blood in a concentration of 1 in 5,000,000. The test is thus capable of great sensitiveness.

The benzidine test is more satisfactory than the guaiac in ordinary hands, as it is more delicate, the blue colour develops immediately, and gives an absorption spectrum. It is not so susceptible to the interfering substances given under the guaiac test. The colour given with pus is green.

The above tests, including spectroscopic, show only that a suspected material is or is not blood. They cannot differentiate between human blood and that of the common mammals. This 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, and an attack of vaccinia renders a person immune to smallpox for a certain number of years. Vaccination is an instance of what is called active immunity. The study of immunity has also rendered possible what may be called passive immunity, produced by the injection of antitoxic material as a cure for diphtheria, tetanus, snake-poisoning, etc.

The power the blood possesses of killing certain bacteria is not limited to the colourless corpuscles or *phagocytes*, but is also a property of the fluid part of the blood.

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*.

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"; every one 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, occurs 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 diphtheria toxin, much in the same way that yeast cells will produce alcohol when grown in a solution of sugar. If a certain small dose called the

"minimum 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 into the blood produces 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 injection into human beings suffering from diphtheria, and it rapidly cures the disease. The two actions of the blood, antitoxic and anti-bacterial, are frequently associated, but may be entirely distinct.

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.

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. Widal's reaction applied to the blood in typhoid fever depends on this fact. The substances that produce this effect are called *agglutinins*. They 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. 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.

Until quite recently little was known regarding the nature of the chemical processes underlying immunological reaction. Now, however, information is in our possession which opens up the way to a fuller understanding of their ultimate nature.

It will have been seen that immunity (or immunological reaction) depends on the introduction into an animal of a protein differing from those peculiar to it, such as living or dead micro-organisms or serum

proteins of another species of animal. Furthermore, such protein must not have been irreversibly coagulated, *e.g.*, by heat, and must not have been subjected to even mild *alkaline* hydrolysis, though equally mild *acid* hydrolysis does not inhibit the reaction.

Such a protein which causes immunological reactions is termed an **antigen.** The commoner properties of antigens can be summarised thus :—

(*a*) They are mainly protein in nature; some are polysaccharides or lipoids.

(*b*) Those of protein nature contain aromatic amino-acids in their molecule and are optically active, *i.e.*, not racemised.

(*c*) They are foreign to the animal into which they are injected.

(*d*) Many protein antigens in small doses cause an increased sensitiveness to the foreign protein, so that a second small dose injected after some weeks may produce death. This phenomenon is termed *anaphlyaxis*.

(*e*) They give rise on injection to the formation of *antibodies*.

Antibodies are of two main types, the first of which comprises the agglutinins, the precipitins, and the lysins; the second of which is the antitoxins.

One notes a difference in the actions of these. The first type act by changing the physical state of the substance agglutinated, precipitated, etc., whereas antitoxins act by neutralising the toxin by whose presence they are induced to form.

In general, it may be stated that—

(*a*) Agglutinins are formed by injection of bacteria.

(*b*) Precipitins are formed by injection of protein or some polysaccharides.

(*c*) Lysins are formed by injection of cells.

(*d*) Antitoxins are formed by injection of toxins.

The most remarkable of all the phenomena of these reactions is their specificity. On this specificity is based the " biological " test for blood mentioned above, and it is the most important of all the medico-legal tests for blood.

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. 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.

It is quite impossible to enter here into the question of the chemical basis of this remarkable specificity. All that can be done is to state that there are concerned in the production of antibodies probably two substances, one a nucleo-protein, the other a polysaccharide.

The former by itself is antigenic, and varies from species to species, *e.g.*, of bacteria, but is the same in various types of the same organism. The polysaccharide is not in all cases by itself antigenic, but, on the other hand, varies with the type of bacterium.

It will be seen, then, how there is the possibility for the occurrence of specificity not only with species of animals, but also with closely related types of the same species.

THE CHEMISTRY OF RESPIRATION

If the relation of hæm to hæmoglobin be remembered, it will be seen that the possibility of the former being able to combine and part with oxygen depends on its being united to a protein (globin) and on the oxygenation and deoxygenation occurring at certain hydrogen-ion concentrations. This last is of considerable importance regarding the respiratory function of the blood.

The air in the alveoli of the lungs and in the blood of the pulmonary capillaries is separated only by the thin capillary and alveolar walls. The blood parts with its excess of carbon dioxide to the alveolar air, and at the same time receives from it the oxygen which renders it arterial.

The intake of oxygen is the beginning, and the output of carbon dioxide the end, of the series of changes known as respiration. The intermediate steps take place throughout 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 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 carbon dioxide, and with hydrogen to form water. Ultimately, however, carbon dioxide and water pass into the venous blood, most of the carbon dioxide and a portion of the water finding 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.[1]
Oxygen	20·93 vols. per cent.	16·89 vols. per cent.
Nitrogen	79·04 ,, ,,	79·61 ,, ,,
Carbon dioxide	0·03 ,, ,,	3·5 ,, ,,
Water vapour	variable	saturated
Temperature	,,	that of body (37° C.).

The nitrogen remains unchanged in absolute amount, although, owing to diminution in volume of expired air, its percentage increases. The chief change is in the proportion of oxygen and carbon dioxide. The loss of oxygen is about 4, the gain in carbon dioxide 3·5. 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 carbon dioxide would not cause any change in the volume of the gas, for a molecule of oxygen would give rise to a molecule of carbon dioxide 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. Carbohydrate molecules contain sufficient oxygen 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 $\frac{CO_2 \text{ given off}}{O_2 \text{ retained}}$ is called the *respiratory quotient*, R.Q. Normally it is $\frac{4{\cdot}5}{5} = 0{\cdot}9$, but this figure varies considerably with diet.

[1] These are only approximate figures. The composition of expired air varies considerably in different individuals, and in any given individual under varying conditions of rest, work, diet, health, and disease.

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 carbon dioxide in the blood. 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 carbon dioxide, are very soluble.

If a cubic centimetre of water is introduced into a large air-tight bottle containing pure oxygen at atmospheric pressure, and another cubic centimetre of water is similarly placed in a bottle containing pure carbon dioxide at the same pressure, the former would be found at room temperature to have dissolved approximately 0·04 c.c. of oxygen, the latter 1 c.c. of carbon dioxide. 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 N.T.P.

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 :—

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 $0{\cdot}04 \times \frac{1}{5} = 0{\cdot}008$ c.c. ; if it is shaken with nitrogen at a pressure of four-fifths of an atmosphere, it will dissolve $0{\cdot}02 \times \frac{4}{5} = 0{\cdot}016$ c.c. If now a cubic centimetre 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—Aerotonometer.—Numerous instruments have been invented for measuring such tension. They are

called *tonometers* Of these 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.

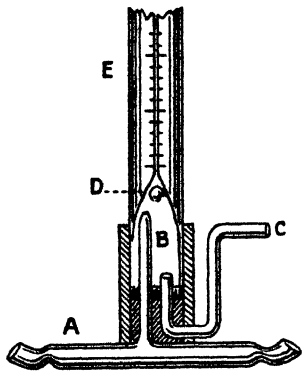


FIG. 31.—Krogh's Tonometer.

As an example, suppose the bubble on analysis proved to consist of 4 per cent. carbon dioxide and 12 per cent. oxygen, together with nitrogen and water 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 carbon dioxide; 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 carbon dioxide 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, and may be collected and measured. In the case of blood, which is the only fluid that need be considered here, this is carried out by means of a mercurial air-pump known as the blood-gas pump.

The total gas obtained is first measured; the carbon dioxide 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.

Another method is the following:—

Chemical Method of Blood-gas Analysis.—When a solution of oxyhæmoglobin is shaken with potassium ferricyanide it yields the same amount of oxygen as it would if placed in a vacuum. In laked

blood the yield in a vacuum is a little greater because then the small amount of oxygen in *solution* in the blood-plasma comes off in addition to that bound to hæmoglobin. Similarly, tartaric acid drives off the carbon dioxide, but a correction has to be made for that which remains in solution. One of the practical exercises in this chapter shows how these gases can be collected in a simple form of apparatus.

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 conveniently 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. 217 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 carbon dioxide *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, about 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 being only 0·24 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 conditions. (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 oxyhæmoglobin 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 gram 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 in the hæmoglobins of 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 gram of iron in hæmoglobin unites with 400 c.c. of oxygen, these figures being 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 the process 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; if it is diminished, oxyhæmoglobin will break up into reduced hæmoglobin and oxygen.

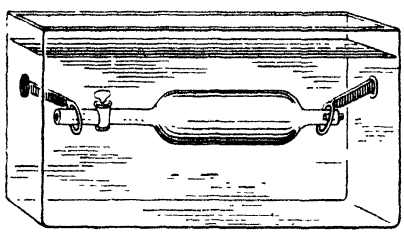


FIG. 32.—Barcroft's Saturator suspended horizontally in warm bath in which it is rotated.

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. The problem before us, therefore, 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 saturator (Fig. 32). Suppose we have six of these tubes, and each contains the same amount (a few cubic centimetres) 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 saturator is rotated in a bath at body temperature for about fifteen minutes till the hæmoglobin and the oxygen are in equilibrium; 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 reduced hæmoglobin .	100	63	45	28	13	6
Percentage of oxyhæmoglobin . .	0	37	55	72	87	94
	100	100	100	100	100	100

This 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. 33), which is called the *dissociation curve of hæmoglobin.*

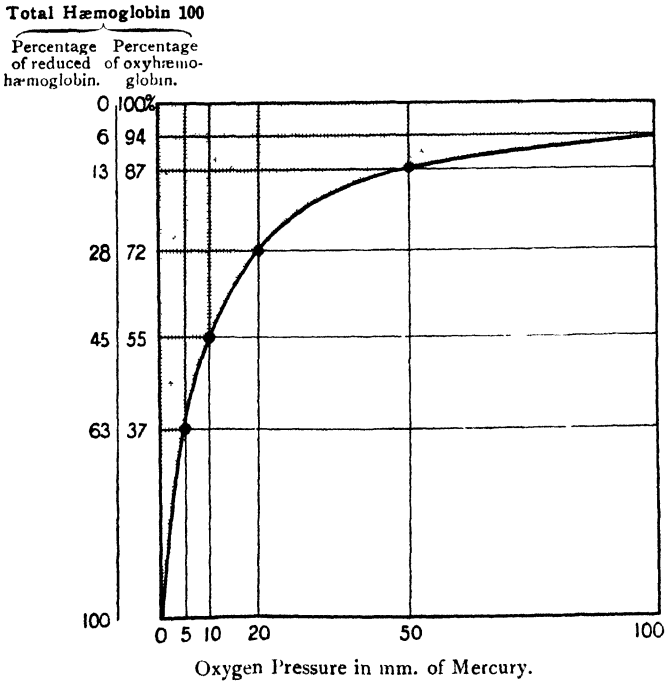


FIG. 33.—Dissociation curve of hæmoglobin at 37° C. The shaded area represents reduced hæmoglobin; the white, oxyhæmoglobin.

A solution of pure hæmoglobin is, however, not the same thing as blood, and the dissociation of oxyhæmoglobin 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, among others, enable the oxyhæmoglobin molecule to break down and to reform more rapidly. It is clearly necessary that the two processes (the union of hæmoglobin with oxygen, and the liberation of oxygen from oxyhæmoglobin) 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 carbon dioxide at 40 mm. pressure, increase the rate of dissociation of oxyhæmoglobin 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 this substance hæmoglobin, which by itself is inefficient for oxygen transport.

The next figure (Fig. 34) shows the dissociation curve of whole blood, and it should be carefully compared with Fig. 33. The two present to the eye graphically the superiority of hæmoglobin as an oxygen carrier when intra-corpuscular 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).

The dissociation curve of hæmoglobin in blood is considerably modified by the amount of carbon dioxide present. Addition of carbon dioxide causes the curve to be shifted to the right, while with dimin-

ished carbon dioxide tension the curve shifts to the left. Hence in the lungs, where the tension of carbon dioxide is minimal, hæmoglobin at a given oxygen tension will combine with more oxygen than in the tissue capillaries, where the carbon dioxide tension is higher. These phenomena are of great importance in facilitating the acquisition of oxygen by the blood in the lungs and its transference to the tissues in the peripheral capillaries.

In order that tissue oxidations may proceed in a normal manner,

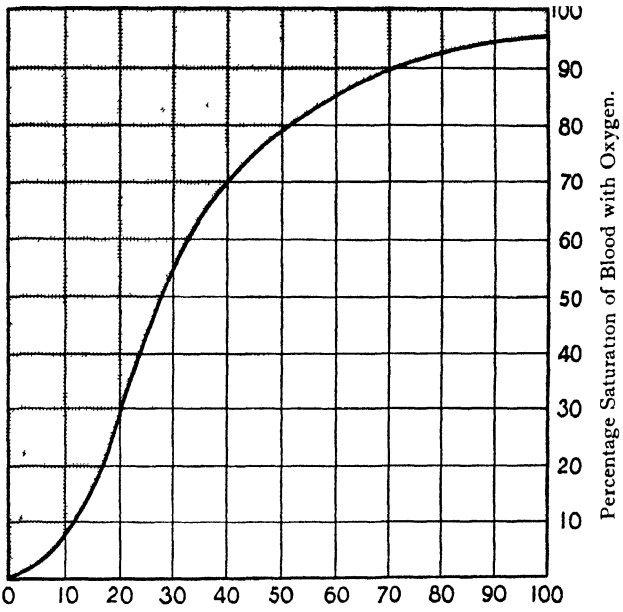


FIG. 34.—Dissociation curve of hæmoglobin in the blood. The shaded area as before represents reduced hæmoglobin, the white area, oxyhæmoglobin.

an adequate oxygen supply at a sufficiently high tension is necessary. The means whereby this supply is provided may be likened to a water system. The hæmoglobin resembles a reservoir, while the plasma and tissue fluids with their dissolved oxygen resemble the distributing pipes. As the oxygen is used in the tissues the tension of dissolved oxygen in the plasma and tissue fluids tends to fall, but this tendency is minimised by the liberation of part of the store of oxygen combined with the hæmoglobin. In a water system the maximum supply of water with a minimum fall in the head of pressure

can be obtained by increasing the area of the reservoir. Fig. 35 shows the shape of the hæmoglobin reservoir. It is obtained by differentiating the equation for the dissociation curve of oxyhæmoglobin, and so determining the amount of oxygen given off for any given fall in oxygen pressure (tension), and allowing for the effect of carbon dioxide. It can be seen at a glance that as the oxygen tension falls towards that of the average venous blood, increasing amounts of oxygen are given off for any given fall in tension.

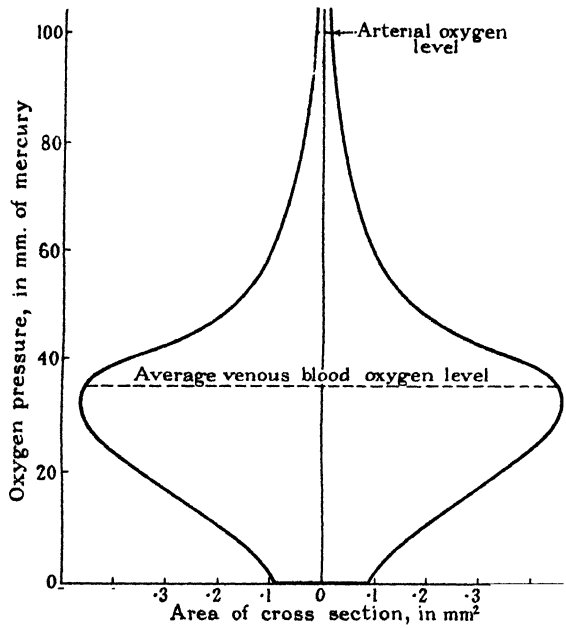


FIG. 35.—The volume of oxygen per 100 c.c. of blood at any oxygen pressure is given by the content of the reservoir when filled to the level of that oxygen pressure. (After Davies.)

The extraordinary efficiency of hæmoglobin as a respiratory pigment can be realised by comparing it with hæmocyanin. This has been done graphically in Fig. 36. This shows the comparison between human blood and crab blood which contains hæmocyanin. When the oxygen tension falls from the arterial to the venous level, human blood gives off approximately 5·5 volumes per cent. of oxygen, whereas the crab's blood gives off only 0·5. This means that at the physiological range the human blood is eleven times the more efficient, and that if the blood of a human being were the same as that of the crab, his heart would need to pump eleven times as

much blood in order to maintain the normal supply of oxygen to the tissues. Thus we can appreciate what Barcroft says regarding hæmoglobin: " But for its existence man might never have attained any activity which the lobster does not possess, or, had he done so, it would have been with a body as minute as the fly's."

Carbon Dioxide in Blood.—Pure distilled water dissociates to a trifling extent into H and OH ions, which, of necessity, are equal in number. Hence we speak of water as neutral, not because it is neither acid nor alkaline, but because it is both in equal degree. Blood, though alkaline to litmus, nevertheless contains H ions, and so has a

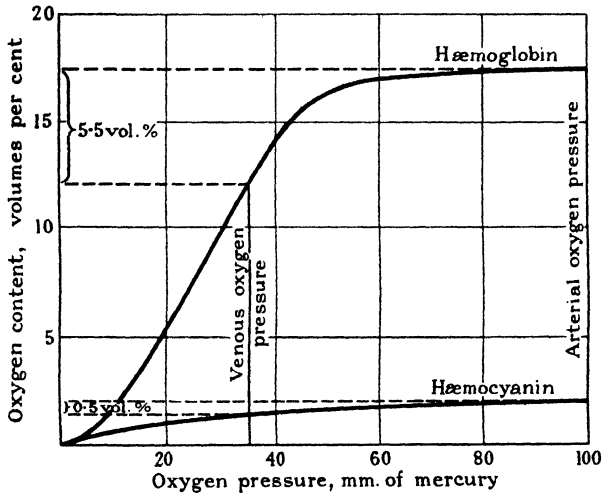


FIG. 36.—Comparison of the dissociation curves of hæmoglobin and hæmocyanin. (After Davies.)

certain degree of acidity. The unit of acidity is the concentration of H ions in a normal solution of HCl (36·5 g. per litre). Compared to this the H-ion concentration in blood is very small indeed, being only 0·000000032. Nevertheless, variations in this figure produce pronounced effects, an increase leading to stimulation of the respiratory centre. The principal acid to which such stimulation is due is carbonic acid (H_2CO_3), and though carbon dioxide is being continually thrust into the blood by the tissues, especially during activity, the H-ion concentration in health varies but little, on account of the buffer substances in the blood. These are principally sodium bicarbonate, sodium phosphate, and proteins. The first-named secures most of the carbon dioxide, while the proteins get

about a third, and among the proteins Buckmaster finds hæmoglobin the most efficient. Whether CO_2-hæmoglobin falls in the same category as other compounds of this pigment with gases is uncertain. Finally, about 5 per cent. is present in simple solution. The *total* carbon dioxide is about equal to what water would absorb at 760 mm. pressure, but most of this is in combination, so small an amount being free that the blood is in equilibrium with a carbon dioxide pressure of only 40 mm. Hg (5 per cent. of an atmosphere). Much in the same way that oxyhæmoglobin dissociates in the tissues, the carbon dioxide compounds dissociate in the lungs, the carbon dioxide being discharged into the air.

Differences between Arterial and Venous Blood. — The average quantities of gases in human blood are as seen in the following table:—

	For 100 volumes of blood.	
	Arterial.	Venous.
Oxygen	19·5	13·5
CO_2	50	56
Nitrogen	2·0	2·0

Nitrogen is simply dissolved from the air and has no physiological significance. The other two gases are important. The numbers for venous blood vary a good deal, tissue activity making venous blood still more venous. But on the average, every 100 c.c. of blood which pass through the lungs gain 6·5 c.c. of oxygen and lose 6 c.c. of carbon dioxide. We have now to study how this interchange is effected.

THE MECHANISM OF GASEOUS EXCHANGE IN THE LUNGS

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 as, 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. Oxygen passes from the alveolar air into the blood-plasma, the oxygen immediately combining with the hæmoglobin, and thus leaving the plasma free to absorb more oxygen. 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 that in the plasma, or that in the lymph which 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.

(i) *The Pressure of Oxygen in the Alveolar Air.*—Haldane and

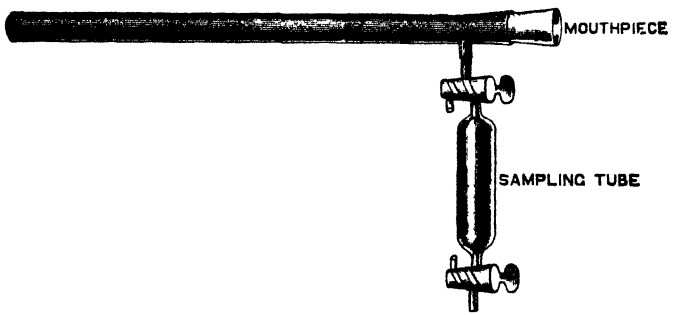


FIG. 37.—Apparatus for obtaining alveolar air.

Priestley introduced a very simple method of collecting alveolar air. A piece of rubber tubing is taken about 1 in. in diameter and about 4 ft. long (Fig. 37). A mouthpiece is fitted into one end. About 2 in. from the mouthpiece a small hole is made, into which is inserted the tube of a gas-receiver, or sampling-tube. 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. 218), or by direct experiment on man, as in the case of Professor Barcroft, who, after living for six days in a respiration chamber, wherein the pressure was reduced to correspond, finally, with an altitude of 18,000 ft., had his radial artery exposed and a cannula inserted to obtain specimens of blood at work and at rest. Simultaneous records of alveolar carbon dioxide and oxygen were made.

This and other 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 correspondingly, but always remains lower than the oxygen pressure in the alveoli by 1 or 2 mm. of mercury.

On the other hand, Haldane and his co-workers have produced strong evidence in favour of oxygen secretion by the pulmonary epithelium after acclimatisation to reduced oxygen pressure and in fit individuals during work.

2. **Carbon Dioxide.** — 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 those in the case of oxygen. This coincides with the ease with which carbon dioxide passes out through the membrane which separates the blood from the air.

It is unnecessary to suppose that the alveolar epithelium actively excretes carbon dioxide, for mere diffusion will explain the passage of that gas from the blood to the alveolar air.

It is pressure differences which regulate the passage of oxygen and carbon dioxide, facilitated by the heat produced in the tissues hastening the reduction of oxyhæmoglobin.

The following table summarises the main facts in relation to the two gases, and the arrows show how they always pass from situations of higher to those of lower pressure :—

Pressure (Tension) of Gases

	Lungs.	Arterial Blood.	Venous Blood.	Tissues
Oxygen	100 mm. →	Just under 100 mm.	→ 35 mm. →	35 mm. to zero.
Carbon Dioxide	40 mm. ←	Just over 40 mm.	← 45 mm. ←	over 45 mm.

Numerous other pieces of apparatus are employed in investigating gaseous exchange. Thus the *spirometer*, a form of gasometer, is used for collecting and measuring the expired air. It has been recently improved by Krogh to make it a recording instrument. The Douglas bag has played an important rôle in investigating respiratory metabolism. This is an empty airtight bag into which the subject breathes; it is portable, and so may be employed during varying conditions of the subject, such as rest and work. The total gases breathed out can thus be collected, and samples withdrawn for analysis.

CAUSE AND CHEMICAL REGULATION OF RESPIRATION

The rhythmic movements of the muscles of respiration are controlled (1) by a specialised small area of grey matter in the floor of the fourth ventricle, termed the respiratory centre; (2) by the vagus nerves; and (3) by the chemical condition of the blood and tissues of the respiratory centre itself.

The respiratory centre is probably multiple, and consists of an *inspiratory* centre and an *expiratory* centre, of which the former is the more important. The centre is normally affected by chemical and nervous stimuli. Only the chemical control of respiration will be mentioned here.

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*. 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 carbon dioxide 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, the alveolar air in man contains a nearly constant percentage of carbon dioxide in the same subject. 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, it was concluded that the percentage varies inversely as the atmospheric pressure, so that the pressure or tension of the carbon dioxide remained constant.

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. Though not strictly accurate, it affords quite a close approximation to facts and compels one to note how small are the changes which affect the respiratory centre. During work the alveolar carbon dioxide pressure increases slightly, and consequently the pulmonary ventilation is 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 a 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 more excitable than normal in the low oxygen pressure.

This is a broad statement and requires certain modification, as at high altitudes the alveolar carbon dioxide can drop to such a level as not to act as a stimulus. It is now agreed that certain fatigue products, such as lactic acid, assist the carbon dioxide in stimulating the respiratory centre. The important stimulus is not any particular acid, but the total hydrogen-ion concentration.

It seems certain that a given rise in hydrogen-ion concentration is more effective if caused by carbon dioxide than by any other acid, possibly owing to the greater facility of penetration of the cells of the respiratory centre by the carbon dioxide. The action of the other acids may well be to liberate carbon dioxide from the bicarbonate of the blood-plasma.

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 vagus nerves) 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 carbon dioxide 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.

Even passage of air over the respiratory mucous membrane may induce inhibition. As these afferent impulses to the respiratory centre pass by the vagus, it is quite uncertain what mechanism exists to maintain respiration when these are rendered inoperative. The mechanism must be assumed to be chemical in the absence of other known afferent paths to the centre.

Breaking Point.—An individual can hold his breath for a varying length of time, depending on the rate of increase in concentration of alveolar carbon dioxide. When this reaches about 8 per cent., he has to take a breath—he has reached the breaking point. On the other hand, if instead of passively restraining respiratory movements he rebreathes his own expired air, the end result, viz., increase of alveolar carbon dioxide, is the same, but the breaking point is reached much later. The explanation of this probably lies in partial obstruction to the circulation in the first case by the voluntary inactivity of the respiratory pump.

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 carbon dioxide 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 sarcolactic 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 gram 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 to weigh the organ 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 carbon dioxide, 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 carbon dioxide put out.

The table on p. 234 shows the coefficients of oxidation for resting organs, and the extent to which they are increased in activity.

Intensity of Respiration.—Most of the figures relating to gaseous metabolism given in this table 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 gram of body-weight. An easier and more practical 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 gram 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

Organ.	Condition of Rest.	Oxygen used per minute per gram of organ.	Condition of Activity.	Oxygen used per minute per gram 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.	0·045 c.c.	...	...

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 gram of body-weight per minute.

NEUTRALITY REGULATION AND ACIDOSIS

As stated above, the reaction of the blood is slightly alkaline, that is the number of hydroxyl (OH) ions present is greater than the number of the hydrogen (H) ions. It will be realised, however, that, though alkaline, hydrogen ions are present, which if in excess would produce an acid reaction. To give a homely simile—a constituency which returns a Liberal to Parliament is termed a Liberal constituency, even though it is not free from Conservatives, and may, in fact, contain almost as many Conservatives as Liberals.

The maintenance of this condition of slight alkalinity is essential for the maintenance of normal health, and the factors which contribute to this are the inherent properties of the blood itself, the action of the renal excretory apparatus, the pulmonary system, and the sundry processes which make up the sum total of those chemical reactions which we term metabolism. We may take these in order.

1. *Inherent Properties of the Blood.*—If to blood one adds either acid or alkali in moderate amount, its reaction changes but slightly. As explained in Chapter V., such a phenomenon is due to the presence of " buffers " in solution, which in blood are proteins, the monosodium and monohydrogen phosphates and sodium bicarbonate.

In short, a buffer can remove hydrogen or hydroxyl ions from solution to form compounds which do not dissociate with the production of these ions. For example, $NaHCO_3$ in the presence of an acid would form H_2CO_3, which is a very weak acid and which thus liberates but few H-ions (*cf.* p. 123).

The sum of all the substances which can act in this manner constitutes the *alkali reserve* of the body. This alkali reserve can be affected in two ways: one when these substances are in excess—this constitutes *alkalosis*; the other when they are reduced—this constitutes *acidosis*. These terms connote the available alkali and have no direct reference to the hydrogen-ion concentration.

In determining whether the condition of acidosis is present in any patient, the important thing is to ascertain the proportion between the amounts of the CO_2 and the $NaHCO_3$ present.

In the fraction $\frac{[CO_2]}{[NaHCO_3]}$ acidosis may be the result of an increase in the numerator or a decrease in the denominator, an alkalosis the reverse; while if both rise or fall to keep the fraction constant, there will be neither acidosis nor alkalosis.

2. *The Renal Excretory Apparatus.*—The kidneys play the major rôle in regulating the ratio of the two sodium phosphates, viz.,

NaH_2PO_4/Na_2HPO_4, and largely by a modification of this ratio are the kidneys enabled to secrete, from a blood of constant reaction, urines of widely differing hydrogen-ion concentration. An example of this is seen in the *alkaline tide* which follows digestion and is due to passage of hydrochloric acid into the stomach.

The monosodium salt is acid in reaction; the dihydrogen salt is alkaline. These salts have a threshold value in the blood beyond which normally they cannot rise owing to immediate removal by the kidneys. In this manner the ratio NaH_2PO_4/Na_2HPO_4 remains practically constant.

3. *The Pulmonary System.*—As stated above, the action of any acid on sodium bicarbonate is to liberate carbon dioxide and thus form the neutral salt. Carbon dioxide acts as a respiratory stimulant, and consequently is eliminated. As the carbon dioxide is formed by an increase in H-ion concentration, it is seen that an acidosis is accompanied by increased elimination of carbon dioxide, while an alkalosis is attended by the reverse.

This can be shown by examination of the alveolar air which was found by Haldane to be constant for any individual, provided that no digestion is proceeding.

In the acidotic state, where carbon dioxide is being rapidly eliminated, the alveolar carbon dioxide is lowered from the normal to a very much lower figure, *e.g.*, 1 to 2 per cent.

4. *Metabolic Products.*—The end of the hydrolysis of proteins is amino-acids, which are deaminised to form hydroxy or ketonic acids, *e.g.*, $CH_3CHNH_2.COOH$ may give rise to $CH_3.CH.OH.COOH$ and the simultaneous formation of ammonia. One view is that this unites with the carbonic acid to undergo conversion into urea. Carbonic acid is a weak acid, and is easily displaced by a stronger acid, such as lactic or hydroxy-butyric acids, with the formation of the ammonium salt, which is excreted. The ratio of ammonia nitrogen to the urea nitrogen or total nitrogen is thus an index of the grade of acidosis. Clinically this is expressed by the ammonia coefficient:—

$$\text{Ammonia N} \times 100/\text{Total N}.$$

The substances found in the blood, and also in the urine, in the condition of acidosis are, as a rule, those derived from fatty acids when the oxidation of these is incomplete. They are:—

β-hydroxy-butyric acid . .	$CH_3.CHOH.CH_2.COOH$
Aceto-acetic acid . . .	$CH_3.CO.CH_3.COOH$
and	
Acetone	$CH_3.CO.CH_3$

The two last, viz., the aceto-acetic acid and the acetone, are ketones, and, as such, may be responsible for the coma which is sometimes found in such a condition as the acidosis of diabetes. On the other hand, the two first can dissociate in the blood with the production of H-ions. The term *ketosis* is sometimes employed, though incorrectly, as synonymous with *acidosis*.

In view of the fact that ingestion of alkali (which may increase the alkali reserve) is frequently beneficial in diabetic coma, it would appear that the coma is due principally to the acidic properties as opposed to their ketonic (enolic) properties.

Furthermore, the estimation of the alkali reserve is the routine method of detecting the onset and determining the grade of diabetic coma, while in the investigation of a suspected case of an acidosis, apart from the qualitative examination of the urine for the " acetone bodies," the alkali reserve and the alveolar carbon dioxide tension are invariably determined.

EXTIRPATION OF THE PANCREAS

Insulin

Complete removal of the pancreas in animals and diseases of the pancreas in man produces a condition of diabetes mellitus together with loss of pancreatic action in the intestine. Grafting the pancreas from another animal into the abdomen of the animal from which the pancreas has been previously removed relieves the diabetic condition during the survival of the graft.

How the pancreas acts otherwise than in producing the pancreatic juice is not as yet 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 *via* the lymph or venous blood, and is then

distributed to minister to parts elsewhere. Removal of such endocrine glands as the thyroid or suprarenal produces disease or death because this internal secretion can no longer be formed. In the pancreas, the external secretion of the pancreas (that is, pancreatic juice) is formed by the cells lining the acini, while the internal secretion, stoppage of which in some way leads to diabetes, is now attributed to the islets of Langerhans.

This internal secretion, prior to its isolation, was termed *insulin* by Sharpey Schafer.

Preparation of Insulin.—The reason for the failure of the earlier workers to obtain insulin rested on the fact that in its preparation from the pancreas by aqueous extraction the trypsin there present destroyed the insulin before it could be isolated. As soon as the discovery was made of the solubility of insulin in 50 per cent. alcohol, this solvent was employed with success.

Since Collip made his original communication on the preparation of insulin, many improved methods have been devised. One of these which is utilised in the commercial production of insulin is that of Dodds and Dickens. These workers ground picric acid with the finely minced pancreas. The resulting insulin picrate is separated from the protein picrate by making use of its solubility in acetone. The solution so obtained is evaporated to dryness and the semisolid material obtained washed with ether to remove the fat and free picric acid. The resulting fairly pure insulin picrate is converted into the hydrochloride, in which form it is further purified.

More recently claims to have obtained it crystalline have been made.

The Action of Insulin.—Experimentally it has been shown that extirpation of the pancreas leads to defective oxidation of carbohydrates and fats. Similarly it is proved that after administration of insulin the oxidation of carbohydrates is increased. So far the action is clear: under the influence of insulin the body removes sugar (glucose) from the circulation and stores it as glycogen, or oxidises it to carbon dioxide and water. In the absence of insulin the glycogen is not stored and the glucose is only incompletely oxidised. Accompanying the defective utilisation of glucose is a defective oxidation of fat, one of the signs of which is the accumulation of ketones and oxyacids in the blood (see above).

The action of insulin appears partly to increase the power of body tissues to oxidise glucose and partly to store it as glycogen for subsequent employment when required.

When injected into a normal animal, insulin causes a lowering of the blood-sugar, a condition known as *hypoglycæmia*, and attended

in rabbits by convulsions when the level has dropped to approximately 0·04 grams per cent. This phenomena forms the basis of the biological method (the only method at present available) for standardising insulin preparations. The international unit adopted is one-third of the amount of insulin which will reduce the blood-sugar of a rabbit 2 kilos. in weight, which has been starved for twenty-four hours, to 0·04 grams per cent. within three hours. It is interesting to note that the preparations of crystalline insulin have a value of 24 international units per milligram.

In diabetics much larger doses are required to bring the blood-sugar below normal. Should, however, convulsions, coma, sweating,

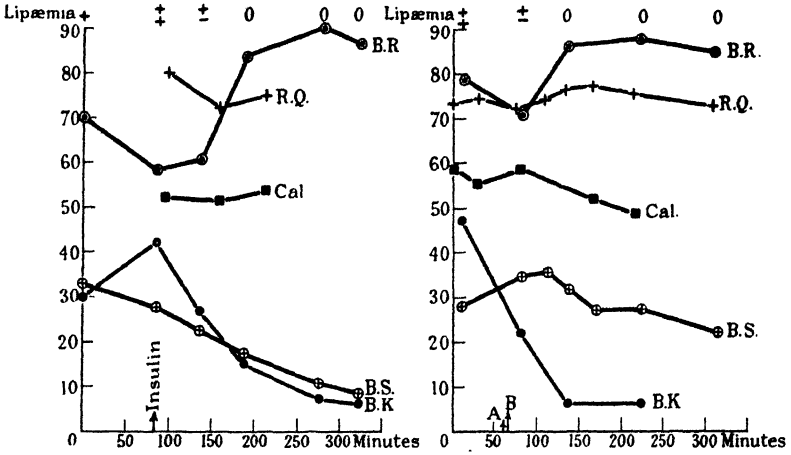


FIG. 38.—Effect of insulin in diabetes mellitus (B.R., basal metabolic rate; R.Q., respiratory quotient; B.S., blood-sugar; B.K., blood ketones; Cal., energy output). (After Davies *et al.*) (From the *British Medical Journal*.)

faintness, or other signs of hypoglycæmia ensue, they may readily be removed by administration of glucose, adrenaline, or pituitary extract.

A graphic record of the action of insulin on blood-sugar, acetone bodies, carbohydrate oxidation, etc., is given in the two subjoined diagrams, which are self-explanatory.

Insulin is probably secreted under the influence of the vagus, and in the normal individual circulates in the blood. This last supposition is suggested by the fact that arterial blood in the non-diabetic contains more glucose than venous, whereas, according to Lawrence, this is not so in a diabetic patient.

Glycosuria (sugar in the urine) occurs in many conditions. It may be a temporary condition, as in alimentary glycosuria due to an

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 thus influences the nervous mechanism which regulates the glycogenic function of the liver. In diabetes mellitus, the body is unable to utilise all the sugar by oxidising it, and so liberating heat and energy; the excess sugar therefore accumulates in the blood and overflows into the urine. In some cases rigid abstention from carbohydrate food makes little or no difference, since sugar can arise 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, carbon dioxide and water. The destruction of sugar by the tissues is termed glycolysis. The formation and storage of carbohydrates is termed glycogenesis.

Many drugs and poisons produce glycosuria, the most potent of these being 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-sugar is usually sub-normal, 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.

CHAPTER IX

TISSUES

MUSCLE

1. THE PREPARATION OF MUSCLE PLASMA.—A rabbit is killed and its blood-vessels are immediately washed out (by opening the abdomen and inserting a cannula in the aorta) with salt solution. The skin is then rapidly removed and the muscles are cut away and minced, the mince being finally extracted with a 5 per cent. solution of magnesium sulphate in a refrigerator overnight. Filter the mixture. The filtrate consists of muscle plasma which may be used for the following experiments.

2. PROPERTIES OF MUSCLE PLASMA.—(a) *Reaction.*—Test the reaction of the plasma to litmus, phenolphthalein, and congo-red.

(b) *Coagulation.*—This may be shown as follows :—Dilute some of the plasma 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 (myosin)—occurs in both, but earliest in that at 40° C.

(c) *Reaction of Clotted Plasma.*—Test the reaction to litmus, phenolphthalein, and congo-red, and determine any difference to the reaction of the uncoagulated fluid.

3. FRACTIONAL COAGULATION OF MUSCLE PLASMA.—Pour 5 c.c. of the muscle plasma into a test-tube and place the latter in a beaker of cold water. Now carefully heat the latter so that its temperature does not rise too rapidly. At 47° C. coagulation takes place. The coagulum consists of *paramyosinogen*. Filter the mixture and again carefully heat the filtrate. At 56° C. flocculi of coagulated *myosinogen* will separate.

4. MYOSIN.—Remove the clot of myosin formed above (2, b) and (a) test its solubility in water, 10 per cent. sodium chloride, 0·2 per cent. hydrochloric acid, 0·2 per cent. sodium hydroxide ; (b) carry out on it the various protein reactions which you know (p. 44). Is myosin an albumin or a globulin ?

5. TO DEMONSTRATE THE PRESENCE OF LACTIC ACID IN SERUM.—Take 5 c.c. of serum in a test-tube and add 0·5 gram of animal charcoal. Boil for one minute and filter into an evaporating basin. Heat on a water-bath to dryness, cool and dissolve in 5 c.c. of concentrated sulphuric acid. Transfer to a *dry* test-tube. Add 3 drops of a saturated aqueous solution of copper sulphate, heat to 100° C. for a minute, cool, and add 3 drops of a 0·2 per cent. solution of thiophene in absolute alcohol. Replace in water-bath at 100° C. Note the formation of a cherry-red colour (Hopkins).

6. GLYCOGEN.—Grind two or three oysters in a mortar with powdered glass or sand. Add 100 c.c. of water, transfer to a flask, boil for fifteen minutes and then strain the mixture through a piece of linen. To the filtrate add sufficient acetic acid to make it faintly acid. Again boil the mixture. Why is a coagulum produced? Now filter the mixture and note the opalescence of the filtrate which should be examined as follows :—

(a) Take 5 c.c. of the glycogen in each of two test-tubes. To one add 0·5 c.c. hydrochloric acid and boil for a few minutes. To the other add the same amount of acid, but do not heat. Cool the first and to both add *the same* volume of iodine solution. Note the differences in colour in the two tubes.

(b) Take 5 c.c. of the glycogen and add 0·5 c.c. concentrated hydrochloric acid. Boil for a few minutes. Cool and neutralise with caustic soda, and test for sugar with Benedict's solution.

(c) Take 5 c.c. of the glycogen and add 0·5 c.c. saliva. Place on water-bath at 37° C. for fifteen minutes. Examine with Benedict's solution.

(d) Take 5 c.c. of the glycogen and add 20 c.c. 95 per cent. alcohol. Let the precipitate settle. Decant the clear fluid and prove the absence from it of glycogen.

7. INORGANIC CONSTITUENTS OF MUSCLE.—Incinerate a small piece of muscle tissue on a piece of porcelain. Dissolve the ash which remains in dilute nitric acid. When most of the ash is dissolved add an equal volume of water and filter. Make the filtrate alkaline with ammonium hydroxide. A heavy white precipitate of phosphates results. Filter, and test the filtrate for chlorides, phosphates, and calcium. To the precipitate on the filter paper add dilute acetic acid. Collect the filtrate, heat it to boiling, add ammonium carbonate and ammonium chloride so long as a precipitate forms. Filter off the calcium carbonate. To the filtrate add disodium phosphate and then

ammonium hydroxide in excess. Note the formation of a precipitate of ammonium magnesium phosphate.

NERVE

1. 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. To the powdered material supplied add 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 reactions by which this substance is identified have already been described.

2. PREPARATION OF LECITHIN.—A finely minced brain is treated for two to four days with cold ether, and filtered. To the filtered extract is added acetone to precipitate lecithin. Filter this off and test it for—

(a) Unsaturated fatty acids by osmic acid (p. 30).

(b) Glycerol by formation of acrolein (p. 30).

(c) Phosphorus by adding to a small quantity five times its bulk of fusion mixture. Heat till all the dark colour has gone, cool, and dissolve in warm water. To this add concentrated nitric acid and excess of ammonium molybdate solution. On warming almost to boiling, a yellow precipitate of ammonium phospho-molybdate is produced.

3. PREPARATION OF PHOSPHATIDES.—Treat finely minced brain with twice its volume of strong alcohol (95 per cent.), and boil under a reflux condenser for half to one hour. Filter hot through a heated funnel, concentrate the filtrate till a cloudiness appears. Allow to stand till next day. Filter the mixture of lipoids.

Examine the solid thus :—

(a) Microscopic appearance.

(b) Hydrolytic products. Place some of the solid in a flask with dilute hydrochloric acid. Boil for at least one hour. Cool, and neutralise with sodium carbonate or hydroxide. Filter and test for reducing sugar with Benedict's reagent.

BONE

1. Drop 2 to 3 grams of crushed bone into some 20 c.c. of dilute nitric acid. Is any gas given off? If so, identify it. Label your experiment and allow it to stand until the next class.

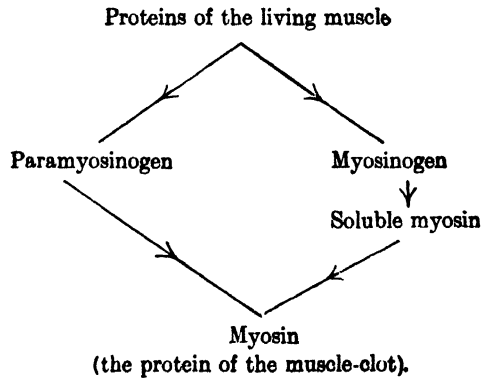
(a) **INORGANIC CONSTITUENTS.**—Decant the liquid fraction obtained in § 1. Use 2 c.c. of it to test for phosphates by the molybdate reaction. To the remainder add ammonium hydroxide until alkaline (litmus), and then make slightly acid with acetic acid. Now add ammonium oxalate until no further precipitation occurs. Boil the mixture and filter. Make the filtrate alkaline with ammonium hydroxide and cool. What is the precipitate? Can you draw any conclusion from your experiment regarding the relative amounts of calcium and magnesium in bone?

(b) Note the physical nature of the material which remains undissolved by the nitric acid (§ 1). Wash it several times with small quantities of water, dilute sodium carbonate, and finally water. To the solid remaining add some 5 c.c. of water, and boil. Cool and apply the various protein tests (p. 44) to portions of the solution. Do your results give you any indication of the type of protein present in solution?

Muscle is composed of solid matter (25 per cent.) and water (75 per cent.). Of the non-aqueous constituents 80 per cent. is protein and 20 per cent. extractives and salts.

The proteins are two in number, viz., *myosinogen* and *paramyosinogen*. The former is present to three times the extent of the latter. They can be separated by fractional heat coagulation, paramyosinogen coagulating at 47° C., the other at 56° C. (In frog muscle a third protein occurs which coagulates at 40° C.) Both proteins give the general reactions of globulins, though myosinogen might be termed an atypical globulin of the nature of the pseudo-globulin of blood-serum. It is not precipitable by dialysis, whereas the other is.

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:—



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° to 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.

Extractives of Muscle.—Under the term *extractives* are included a number of muscle constituents which occur in the tissue and, since they are soluble in such solvents as water, alcohol, and ether, may be extracted from the tissue by such means. They are divided into:—

(*a*) *Non-nitrogenous.*—Glycogen, dextrins, hexose phosphate, lactic acid, inositol, fat, chloride, and bicarbonate.

(*b*) *Nitrogenous.*—Creatine, phosphagen, creatinine, carnosine, adenosine triphosphoric acid, hypoxanthine and its oxidation products, xanthine and uric acid, adenine, guanine, and urea.

Other extractives besides these named have been described, and there are undoubtedly still others whose presence have not been detected.

Non-Nitrogenous Extractives.—The importance of *glycogen* as a constituent of muscle needs no emphasis. The amount of it present varies considerably and is markedly decreased by intense muscular activity. In the liver it forms a reserve which can readily be broken down into glucose and passed into the blood-stream. By this channel it reaches the tissues where it is either oxidised, whereby heat is generated for keeping the animal warm, or it is re-synthesised into glycogen, which plays such an important part in muscular activity.

Glycogen may be prepared by extracting muscle with boiling water and then precipitating the glycogen from the aqueous solution by the addition of alcohol. It resembles starch in forming an opalescent solution and dextrin in being very soluble in water, in giving a reddish colour with iodine, and in being dextro-rotatory.

The most satisfactory method of estimating it in tissues is by Pflüger's method, which is as follows:—20 to 100 grams of the finely chopped liver (or tissue) are boiled for two or 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 allow the mixture 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 the resulting mixture on the boiling water-bath

for three hours, whereby the glycogen is converted into glucose. After cooling, neutralise with 20 per cent. potash, and filter; the filtrate is brought up to 250 c.c. The glucose present in this can be estimated either polarimetrically or volumetrically. The percentage of glucose multiplied by 0·927 gives the amount of glycogen.

Microchemically, glycogen may be detected in the following way:—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 *e.g.*, xylol. 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.

The *lactic acid* occurring in the muscular tissue of vertebrates is often called *sarcolactic acid*. To its presence is due the normal acidity of active muscle. The production of lactic acid in contracting muscle can readily be demonstrated thus:—Remove one hind limb of a pithed frog. Place it on ice till required. 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 with cold scissors. Pound each in an ice-cold 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 ½ gram of animal charcoal. 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.

In resting muscle the normal percentage of lactic acid is about 0·015; during prolonged activity this may be increased tenfold. Hence during exercise the muscle glycogen tends to diminish while the lactic acid, on the other hand, increases. The lactic acid increase in muscle is accompanied by a similar increase in blood where the amount may rise from the normal of about 10 mg. per cent. to even 200 mg. This increase in blood lactic acid is naturally reflected in the urine, where

for a short time after violent exercise there may be a rise from the normal figure of 2 to 600 mg. per cent.

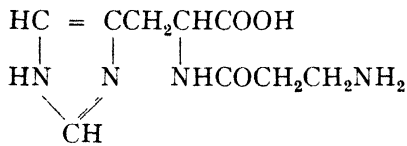
Inositol has previously been described (p. 16). It is found in most tissues. It is also found in plants as inositol phosphoric acid, the calcium magnesium salt of which is called *phytin*.

Nitrogenous Extractives.—Creatine. For the chemistry of creatine see p. 297.

In resting muscle the greater part of the creatine occurs as *phosphagen* (phosphocreatine), and it is open to question whether free creatine is present in any significant amount. Phosphagen is now known to play an important rôle in muscular contraction.

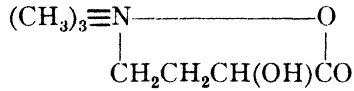
The *purine bases* present in muscle are adenine, guanine, xanthine, and hypoxanthine, substances which are described later (p. 302). Like creatine they probably exist in muscle, at least in part, as components of more complex compounds, *e.g.*, *adenylic* acid (adenine-ribose-phosphoric acid) and *inosinic acid* (hypoxanthine-ribose-phosphoric acid).

Carnosine is a dipeptide which gives on hydrolysis β-alanine and histidine. Its formula is



Its function in muscle is not known.

Carnitine.—This compound is a betaine and possesses the structure



On hydrolysis it yields trimethylamine. Nothing is known of its function in muscle.

The Chemistry of Muscular Contraction.—For long it was considered that muscular activity was essentially a two-stage process, *i.e.*, (*a*) an initial process of contraction whereby glycogen (or a derivative of it) was transformed " explosively " into lactic acid, this reaction providing the energy of contraction; and (*b*) a recovery process during which the lactic acid disappeared, one-fifth being oxidised, the oxidation yielding energy partly used to restore the muscle to its original condition and partly given off as heat, whilst the other four-fifths were re-synthesised into glycogen.

During the past ten years, however, our conception regarding these chemical changes have been drastically revised and considerably amplified.

Starting in 1927, a series of re-investigations of the constituents of muscle disclosed the presence therein of (1) adenylic acid (discovered by Embden and Zimmermann), and (2) a labile organic phosphate (Eggleton and Eggleton), afterwards shown to be composed of creatine and phosphoric acid (Fiske and Subarow), and thereupon called phosphagen. What functions did these substances possess ? Adenylic acid was shown to be a necessary component in the system whereby glycogen is broken down to lactic acid, its rôle being that of a co-enzyme, and, moreover, to be the precursor of the ammonia which is known to be formed in all muscle tissue. It was not, however, until the publication of Lundsgaard's work that we gained a clear conception of the fundamental part played by phosphagen. Lundsgaard showed that, contrary to accepted opinion, a muscle could contract without the production of lactic acid, and that it actually did so if it had previously been poisoned with sodium iodoacetate. A revision of our conceptions regarding the sequence of events in muscular activity became necessary. Very briefly it is as follows :— The break-up of phosphagen into its constituent creatine and phosphoric acid provides the energy for contraction, " fatigue " occurring when all the available phosphagen has been so used up. The reaction is, however, reversible, the energy necessary for the re-synthesis being derived from the decomposition of the adenyl phosphoric acid into inosinic acid, phosphoric acid, and ammonia. Here again we have a reversible process, the required energy of re-synthesis being obtained from the breakdown of glycogen into lactic acid. It is at this stage and onwards that there still remains considerable doubt. It is still generally accepted that four-fifths of the lactic acid is finally reconverted into glycogen *in situ*, the required energy arising from the oxidation of the one-fifth lactic acid. But, in such estimations of the amount of glycogen in muscle during periods of activity, it is doubtful whether sufficient attention has been paid to the amount of lactic acid lost by the muscle to the blood, a loss which may be fairly considerable, as shown on p. 247. Moreover, is it essential to postulate a mechanism of the re-synthesis *in situ* of muscle glycogen from the lactic acid ? There is certainly no evidence for believing that normally there exists a glucose " want " in the supply of this compound by the blood to the muscle for glycogen synthesis. This highly interesting problem is not yet completely solved.

NERVOUS TISSUES

Nervous tissues do not lend themselves so well as others to routine chemical investigation. One or two experiments are described. The most important points which analyses show are: (1) the large percentage of water, especially in the grey matter; in the adult there is 83 per cent., in the fœtus 92; (2) the large percentage of protein. In grey matter, where the cells are prominent structures, this is most marked. Of the solids, protein material comprises about 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*, which contains 0·5 per cent. of phosphorus. 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 neuro-keratin (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 was given in Chapter II. (pp. 35 to 40). They comprise *phosphatides*, *galactosides*, and *cholesterol*.

The following are some analyses of nerve by Falk, the numbers given being 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 sarcolactic 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. Moreover, 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 cell bodies, but in nerve-fibres it is found in patches external to the axis cylinder. 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 being 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.

1. *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 (glucose). The amount of glucose present is slightly less than that in blood. It contains no choline or cholesterol normally, and is practically free from cells, except in certain diseases of the nervous system.

2. *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."

The 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 due to a breakdown in an anatomical sense only, 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 hardening 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 it 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 hydrated oxide. In the later stages of degeneration the Marchi reaction is not obtained, because the fat globules have been absorbed.

In certain diseases of the central nervous system, such as General Paralysis of the Insane, degeneration occurs on a large scale. 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 these conditions shows an excess of protein. Cholesterol can usually be detected, 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. According to the latest view the questionable material is trimethylamine, which is a cleavage product of choline.

Tests for Choline.—These 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; this should be repeated. To the final alcoholic solution, alcoholic platinic chloride is added, and the precipitate so formed allowed to settle and washed with absolute alcohol by decantation; the precipitate is then dissolved in 15 per cent. alcohol, filtered, and the filtrate is allowed to evaporate slowly 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 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 platino-chloride are doubly refracting, whereas the platino-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 grams of iodine and 6 grams 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. Another test is the 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 used in an attempt to distinguish organic from functional diseases of the nervous system; in the latter there is no chemical disintegration leading to the production of bases, *e.g.*, choline.

BONE AND TEETH

Bone and teeth, examples of connective tissue, are unique in having a very high content of inorganic material. Whether the inorganic and organic components are only mixed or actually combined is not definitely known. The bony structure proper, free from other formations, *e.g.*, marrow, consists of cells and a matrix. Concerning the chemistry of the cells little is known.

The *matrix* contains both inorganic (60 per cent.) and organic (40 per cent.) constituents. The inorganic matter is very constant in composition, and consists mainly of calcium, with a small proportion of magnesium, combined with phosphate and carbonate. On X-ray examination it appears to possess a crystalline structure fundamentally that of the mineral apatite. The organic constituents comprise a sclero-protein, a muco-protein, and a keratin. The sclero-protein is **ossein,** which is very similar to the collagen of tendons and skin; the keratin is a very tough elastic fibrous substance similar to elastin found in connective tissue, whilst the muco-protein, *osseo-mucoid*, belongs to the class of conjugated proteins (gluco-proteins) and resembles the tendo-mucoid of tendons.

Ossein.—The precursor of ossein is cartilage, the transformation taking place during ossification. Cartilage is a complex of a protein and chondroitin sulphuric acid, the latter substance being composed of sulphuric acid, acetic acid, glycuronic acid, and chondrosamine in equimolecular proportions.

The Chemistry of Ossification.—Of the several theories which have been advanced to explain the mechanism of calcification in bone, space only permits us to describe broadly that put forward by Robison. His conception is that the enzyme phosphatase, which occurs in bone, teeth, kidney, and intestine, hydrolyses the hexose-phosphates and glycero-phosphates present in the blood. The phosphoric acid so liberated reacts with the calcium salts also present in the blood to form insoluble calcium phosphate, which is deposited in the ossifying cartilage. The reaction takes place in a definitely alkaline medium ($P_H = 8{\cdot}4$). It is noteworthy in this connection

that the bone enzyme is found only in very small amount in unossified cartilage.

As is well known, the calcified tissues of the animal body become abnormal through pathological conditions, unsuitable diet, or abnormal activity on the part of the endocrine organs. Probably the best known of these conditions is *rickets*, characterised by (*a*) enlargements at the epiphyseal junctions, (*b*) softening and bending of bone, (*c*) stunted growth, (*d*) formation of bone-like tissue deficient in calcium salts. This disease may be brought about in the young animal by a dietary deficiency of calcium, phosphate, or vitamin D. Provided that the intake of calcium and phosphorus is normal, rickets may be cured by the addition of vitamin D to the diet or by ultraviolet-light treatment.

It has been said that the bone from a rachitic animal does not give a typical gelatin on boiling it with hot water. Should this be true, it would indicate a degradation of the organic matrix of bone in rickets.

Teeth are composed of enamel, dentine, and cement. Of these, dentine and cement closely resemble bone in chemical composition. The enamel is of epithelial origin. It is the hardest substance found in the animal body and contains only about 5 per cent. of water. It differs from bone in having a higher phosphorus content and a somewhat different organic matrix which, unlike that of bone, does not yield gelatin on boiling it with water.

SKIN

The importance of the skin and its accessory structures to the organism requires no emphasis. Its rôles are numerous. Some of these are as follows:—(*a*) unbroken, it forms an adequate defence against micro-organisms and poisons; (*b*) it is an exceedingly active agent in regulating body-temperature; (*c*) its pigments and other constituents mitigate the action of rays harmful to the delicate structures of the body.

It consists of two principal layers, the lower *dermis* and the upper *epidermis*. The latter consists of four layers the deepest of which is nourished from the blood-vessels. This is the layer in which cells are continually being formed and from which they are displaced towards the surface of the skin. By the time they reach the outer epidermis the cells are dead, and are finally lost by desquamation.

The chief constituent of the epidermis and of its derivatives, viz.,

hair, feather, nail, hoof, etc., is the albuminoid keratin, whose chemical characteristics have already been described (p. 69).

Another important constituent of the skin is the pigment *melanin*. Dispersed, this appears yellowish-brown in colour, but in the mass, black. Raper has definitely shown that the pigment has its origin in the amino-acid tyrosine which, by a series of reactions, is converted by way of dihydroxyphenyl-alanine to a hydrogenated indole-carboxylic acid. This is then oxidised to a quinone which on losing its carboxyl group polymerises to yield the pigment. In nature an enzyme is responsible for this formation of pigment, and it is interesting that the failure of melanin formation in albinos has been associated with the absence of the enzyme.

In addition to these constituents the skin contains a wide variety of substances both inorganic and organic in nature. This includes sodium, calcium, iron, chloride, glucose, glycogen, mucin, urea, etc.

CHAPTER X

URINE

QUALITATIVE SECTION

1. Test the reaction, with litmus paper, of the urine which has been collected under oil to prevent loss of carbon dioxide.

2. Determine the specific gravity of the urine with the urinometer. By means of Long's coefficient (p. 283), calculate the "solid" content of the urine.

3. Test for the presence of the following **INORGANIC SALTS**:—

(a) *Chlorides.*—Acidify a few c.c. with nitric acid and add silver nitrate solution; a white precipitate of silver chloride, soluble in ammonia, is produced. The object of acidifying with nitric acid is to prevent phosphates and urates being precipitated as the silver salts.

(b) *Sulphates.*—Acidify 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. Collect the precipitate, wash it with a little distilled water; then dissolve it in a few c.c. of dilute acetic acid and add a few drops of a solution of potassium oxalate. A precipitate of calcium oxalate immediately separates. The *alkali* (sodium and potassium) phosphates remain in solution.

(ii) Mix another portion of urine with half its volume of nitric acid; add ammonium molybdate, and heat strongly. A yellow crystalline precipitate of ammonium phospho-molybdate is produced.

4. **PREPARATION OF UREA FROM URINE.**—Evaporate about 50 c.c. of urine to small bulk, and finally to complete dryness on the boiling water-bath. Turn out the flame and extract the residue with about 10 c.c. of acetone. The dish may be replaced on the water-bath to concentrate the acetone solution. Pour off the hot acetone extract into a dry watch-glass or evaporating basin. On cooling, urea crystallises

out in silky needles. Examine the crystals so obtained under the microscope.

5. **UREA NITRATE.**—Evaporate some urine in a basin 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.

6. **UREA OXALATE.**—Concentrate the urine as in the last exercise, and add saturated oxalic acid. Crystals of urea oxalate separate out. Examine these microscopically.

7. **TESTS FOR UREA.**—Take some of the urea crystals as prepared by the above method (4), and carry out the following tests on them :—

(a) 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 decomposition of the urea, carbon dioxide and nitrogen being evolved. A similar bubbling, due to evolution of nitrogen, occurs when sodium hypobromite is added to another portion of the solution. The decomposition takes place according to the following equation :—

$$\underset{\text{[urea]}}{CON_2H_4} + \underset{\text{[sodium hypobromite]}}{3NaBrO} \longrightarrow \underset{\text{[carbon dioxide]}}{CO_2} + \underset{\text{[sodium bromide]}}{3NaBr} + \underset{\text{[water]}}{2H_2O} + \underset{\text{[nitrogen]}}{N_2}$$

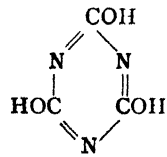
This reaction forms the basis of a method for the quantitative estimation of urea.

(b) Heat some urea crystals gently in a dry test-tube. The urea melts and simultaneously decomposes into biuret and ammonia.

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

After cooling, add a drop of copper sulphate solution and a few drops of 20 per cent. caustic soda or caustic potash. A rose-red colour is produced.

Longer heating eliminates three molecules of ammonia, and three molecules of urea combine to form cyanuric acid,



which sublimes and condenses on the cooler portion of the test-tube. Cyanuric acid gives a violet-coloured insoluble copper salt.

(c) Dissolve some urea in water and add to it a few c.c. of a solution

of mercuric nitrate. Note the formation of a white precipitate. This is a complex salt having the composition $HN.C(OH)NH_2.HgNO_3.HgO$.

(d) To 10 c.c. of a dilute solution of urea in water add an equal volume of glacial acetic acid, and then 2 c.c. of a 10 per cent. solution of xanthydrol in methyl alcohol. Set the mixture aside to stand, when a voluminous crystalline precipitate of di-xanthyl-urea gradually separates. Under certain conditions the precipitation of the urea is complete. This precipitation has been made the basis of methods for the estimation of urea in urine, in blood, and in tissues.

8. SPECIFIC TEST FOR UREA.—To 5 c.c. of a urea solution in a test-tube add 2 or 3 drops of phenolphthalein. Then add about 0·1 gram of soya bean meal. Invert the test-tube in order to mix the contents, and then place it in a water-bath at 50° C. Note that the contents of the test-tube gradually turn pink. The increasing alkalinity is due to decomposition of the urea into ammonium carbonate by the enzyme *urease* which is present in the soya bean. The reaction is generally represented by the equation

$$HOC\begin{matrix}\diagup NH \\ \diagdown NH_2\end{matrix} + 2H_2O \longrightarrow (NH_4)_2CO_3,$$

This reaction forms the basis of important methods for estimating the urea of blood, of urine, and of cerebro-spinal fluid.

9. URIC ACID.—Examine microscopically the crystals of uric acid precipitated from urine to which hydrochloric acid has been added twenty-four hours previously in the proportion of 5 c.c. HCl to 100 c.c. urine. Note that they are deeply coloured with pigment, and to the naked eye look like granules of cayenne pepper.

Microscopically, the crystals are seen to be large bundles, principally whetstones and barrel-shaped, with spicules projecting from the ends. 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. 47).

Murexide Test.—Place a little uric acid, or a urate, on a piece of porcelain; add a little dilute nitric acid and evaporate carefully, without charring, to dryness. A yellowish-red residue is left. Carefully add a little ammonia. The residue turns violet. This is due to the formation of *murexide* or purpurate of ammonia. On the addition of potash the colour becomes a deeper blue.

Schiff's Test.—Dissolve some uric acid in sodium carbonate solution. Put a drop of the solution on filter paper, add a drop of silver nitrate,

and warm gently; a black precipitate of metallic 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 2 or 3 drops of a saturated solution of sodium carbonate to dissolve it. To the clear solution add 1 to 2 c.c. of Folin's phosphotungstic acid reagent, and enough saturated sodium carbonate solution (or a few crystals of sodium carbonate) to render the mixture alkaline. A blue colour develops. This colour reaction has been made the basis of a method for estimating uric acid in urine and other fluids colorimetrically (p. 271).

Reduction of Fehling's Solution.—Dissolve some uric acid by warming it with sodium carbonate solution. Add Fehling's solution and boil again. A white precipitate of copper urate is formed, and on boiling for some time, reduction occurs with the formation of cuprous oxide. Repeat the experiment with Benedict's qualitative reagent (p. 6). No reduction occurs. This demonstrates the advantage of such a solution over Fehling's solutions in testing diabetic urine for small quantities of glucose.

10. DEPOSIT OF URATES OR LITHATES (LATERITIOUS DEPOSIT).—The specimen of urine (obtained from the hospital) contains excess of urates, which have become deposited as the urine cooled. The crystals are tinged with pigment (uroerythrin) and have a pinkish colour, like brickdust; hence the term "lateritious." Examine microscopically. The deposit is usually amorphous. Sometimes crystals of calcium oxalate (envelope crystals—octahedra) are seen also; these are colourless.

The deposit of urates dissolves on heating the urine to body temperature or a little above it.

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 and may be increased. It is, however, soluble in acetic acid. Examine microscopically for coffin-lid crystals of triple phosphate (ammonium-magnesium phosphate), or crystals of stellar (calcium) phosphate, and for mucus. Mucus appears to be flocculent to the naked eye; microscopically it is amorphous.

N.B.—On boiling neutral, alkaline, or even faintly acid urine it may become turbid from the 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 (*cf.* p. 262).

11. CREATININE.—(a) *Weyl's Test.*—Add a little sodium nitroprusside and caustic soda to the urine. A red colour develops, which changes in a short time to yellow. If glacial acetic acid is added to the yellow solution, it becomes green on boiling, and a sediment of Prussian blue forms on standing. (Acetone gives a similar colour reaction, but the colour changes to purple on acidifying.)

(b) *Jaffé's Test.*—Add picric acid and a few drops of strong potash; a deep red colour results owing to the formation of creatinine picrate. This method may be employed quantitatively (see p. 272). If the urine contains sugar, the fluid becomes so dark as to be opaque.

12. INDICAN.—This substance is an ethereal sulphate, the composition and origin of which is described on p. 285.

Jaffé's Test.—Mix 5 c.c. of urine with an equal volume of concentrated hydrochloric acid, and add 2 or 3 c.c. of chloroform. Then add, drop by drop, a dilute solution of an oxidising agent, *e.g.*, potassium chlorate or calcium hypochlorite, shaking vigorously after the addition of each drop. The presence of indican is indicated by the chloroform acquiring a blue colour, due to the solution in it of indigo-blue, formed by oxidation of indoxyl.

13. HIPPURIC ACID.—Preparation by Roaf's method. To 500 c.c. of horse's or cow's urine, add an equal volume of a saturated solution of ammonium sulphate, and then 7·5 c.c. of concentrated sulphuric acid. Allow the mixture to stand for twenty-four hours and then filter off the crystals of hippuric acid which have separated. Recrystallise the product from boiling water. Examine the crystals under the microscope.

14. THE ENZYMES OF NORMAL URINE.—(i) The detection of diastase. Take two small flasks and label them A and B, the latter to serve as the control flask. Into A pour 20 c.c. of urine, and into B 20 c.c. of the same urine which has been previously boiled and cooled. Then to each flask add 1 c.c. of a freshly prepared starch solution and a drop of toluene. Place both flasks in the incubator at 37° for some hours. At noted intervals take small samples from each flask and test for the presence of (a) starch and (b) reducing sugar. Note the time when the starch reaction ceases to appear in the case of the contents of flask A.

(ii) The detection of pepsin in urine may be carried out, using much the same procedure as that in (i), substituting fibrin for starch.

(iii) Trypsin is not usually present in normal urine.

These experiments are the bases of quantitative methods, some of which are described in Chapters VI. and VII.

TESTS ON ABNORMAL (INCLUDING PATHOLOGICAL) URINE [1]

15. URINE A is pathological urine containing albumin. The following two tests for protein are most frequently used in clinical work.

(If the urine is cloudy, it should be filtered before applying these tests.)

(a) Fill a test-tube almost full of the filtered urine A, to it add a *few drops of 0·2 per cent. acetic acid* **and invert the test-tube several times to mix its contents. Then, holding the test-tube by the bottom, heat the upper part of it and notice the formation of a cloud due to the separation of the coagulated albumin. The lower half of the test-tube remains cold and therefore clear. Thus the treated and untreated urine can easily be compared.**

(b) *Heller's Nitric Acid Test.*—**Pour some of the urine gently on to the surface of some concentrated nitric acid in a test-tube. A white precipitate occurs as a ring at the junction of the two liquids. This test is used to detect small quantities of albumin.[2]**

16. URINE B is a 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 it with Benedict's, Fehling's (but see p. 260, § 9), and similar solutions.

17. ACETONE, ACETO-ACETIC ACID AND β-HYDROXY-BUTYRIC ACID are often found in urine in certain forms of "acidosis," especially in diabetes mellitus. The urine provided should be treated 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.[3] This test may also be carried out by pouring a few c.c. of the urine on to the top of**

[1] See p. 314 for a discussion of the term "pathological urine."

[2] In very concentrated normal urine a white ring of urea nitrate may form under these conditions; this is obviously crystalline. Uric acid may also separate out if large excess of urates is present. This can be obviated by previous dilution of the urine with water.

[3] 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.

some 10 per cent. ferric chloride solution ; the claret-like colour appears at the zone of contact.

(b) Acidify the urine with sulphuric acid and shake up with ether ; on standing, the ether 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) Rothera's Test for Acetone and Aceto-acetic Acid.—This is recommended, and is both simple and very reliable. The test is carried out as follows :—Saturate 10 c.c. of urine with ammonium sulphate, add a few drops of dilute sodium nitro-prusside and 2 to 3 c.c. of strong ammonia. A purple colour develops above the layer of undissolved crystals, usually only after standing some minutes. This test is also given by aceto-acetic acid, but is not interfered with by the presence of creatinine.

Note.—Whilst aceto-acetic acid gives a vivid reaction with Rothera's test, aceto-acetic ester, on the other hand, has been found to prevent the development of colour when the free acid or acetone are present.

18. URINE C is from a case of jaundice, and contains bile.

Note.—Urine containing bile often has a characteristic appearance.

(a) *Bile pigment* may be detected by Cole's test, which is performed as follows :—Boil 15 c.c. of the suspected urine, add 2 drops of a saturated solution of $MgSO_4$, then a 10 per cent. solution of $BaCl_2$ drop by drop, boiling between each addition ; continue until no further precipitate is obtained. Allow the mixture to stand for a minute, and then pour off the supernatant fluid. To the precipitate add 3 to 5 c.c. of 97 per cent. alcohol, 2 drops of strong sulphuric acid, and 2 drops of 5 per cent. aqueous solution of potassium chlorate. Boil for half a minute and allow the barium sulphate to settle ; the presence of bile pigments is indicated by the alcoholic solution being coloured a greenish blue. If the alcoholic fluid is poured into a dry tube, mixed with a third of its volume of chloroform and an equal volume of water, the chloroform containing the bluish pigment in solution will separate out on standing.

(b) *Bile salts* may be detected either by (1) Hay's sulphur test (p. 158), or by (2) Pettenkofer's test (p. 157). The latter test is carried out thus :—To 5 c.c. of urine in a test-tube add 5 drops of a 5 per cent. solution of sucrose. After mixing the contents, incline the tube and run 2 to 3 c.c. of concentrated sulphuric acid carefully down the side so that two layers

of liquid are obtained. Note the red ring at the junction of the two layers. Keeping the tube cool under running water, gradually mix the contents of the tube, when the whole solution assumes a red colour.

19. **BLOOD.**—The presence of blood in urine may best be detected by Shore's modification of the Benzidine Reaction. It is as follows :— To a mixture of 3 c.c. of a saturated solution of benzidine in glacial acetic acid (or in alcohol acidified with acetic acid) and 1 c.c. of 3 per cent. hydrogen peroxide, add the urine drop by drop, shaking well after each addition. A blue or green colour indicates a positive reaction.

20. **GLYCURONATES.**—Glycuronic acid is not found free in the urine, but occurs conjugated with certain substances, *e.g.*, phenols, morphine, etc. (p. 315).

Tollen's Test for Glycuronates.—Add 5 c.c. of concentrated hydrochloric acid and 0·5 c.c. of a 1 per cent. alcoholic solution of naphtho-resorcinol to 5 c.c. of urine. Boil the mixture for a minute, by which time it will become darker in colour. Cool and extract with a few c.c. of ether. The supernatant ethereal layer acquires a reddish-violet or violet-blue colour. *Note.*—In pentosuria the ether layer turns yellow or red.

21. **LACTOSE.**—The presence of lactose may be detected thus :— (a) *Osazone Test.*—Decolorise some 25 c.c. of urine by boiling it with charcoal for 30 seconds and filtering. In the filtrate dissolve solid phenyl-hydrazine hydrochloride (2 grams) and sodium acetate (4 grams), and then heat the mixture on the boiling water-bath for thirty to forty-five minutes. Filter quickly. Place the filtrate in ice-cold water for an hour or longer, and then examine the separated solid under the microscope for crystals of lactosazone (p. 18).

(b) *Mucic Acid Test.*—Heat a mixture of urine (50 c.c.) and concentrated nitric acid (12 c.c.) on the boiling water-bath in a fume chamber until the volume is reduced to 10 c.c. Add water (10 c.c.) and allow to stand overnight. A fine white precipitate of mucic acid will separate if lactose or galactose is present. Examine the crystals microscopically.

QUANTITATIVE SECTION

1. **DETERMINATION OF THE P_H.**—For this experiment the urine must be collected under oil to prevent an immediate loss of

carbon dioxide. The technique described on p. 99 for the determination of P_H of fluids must be modified in this case to compensate for the colour of the urine. This is accomplished by the use of the Cole-Onslow

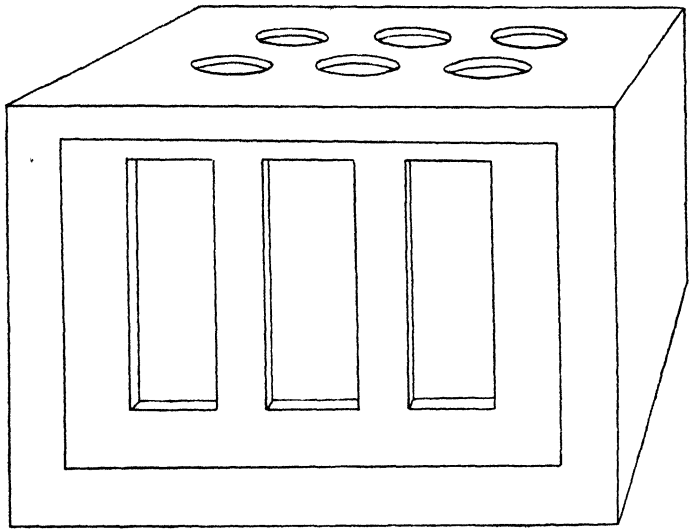


FIG. 39.—Cole-Onslow Comparator.

comparator (Fig. 39). The test-tubes are arranged in the apparatus thus :—

urine (under oil)	water	urine (under oil)
(1)	(2)	(3)
standard indicator	urine + indicator (under oil)	standard indicator
(4)	(5)	(6)

and their contents made up as follows :—

Tubes 1, 3 : 2 c.c. urine + 8 c.c. water (stir with glass rod).

Tubes 4, 6 : Standard indicator tubes (brom-cresol purple and phenol-red) selected from the series provided.

Tube 5 : 7 c.c. distilled water under oil, 10 drops of indicator and 2 c.c. urine (added in this order and finally mixed by stirring with glass rod).

2. TITRATABLE ACIDITY.—Into a 250 c.c. Erlenmeyer flask pipette 25 c.c. of urine. To it add 15 to 20 g. of powdered neutral potassium oxalate. This precipitates the calcium which would form insoluble calcium phosphate as the neutral point is approached during titration. (Calcium phosphate adsorbs the indicator from solution.) Now add 2 drops of phenolphthalein, and shaking the mixture continuously, titrate it with 0·1N sodium hydroxide. Express the result in terms of 0·1N acid per 100 c.c. of urine.

3. TOTAL NITROGEN.—This is determined by the Kjeldahl method already described (p. 79). For the result to be of real value the estimation should be carried out on a sample of urine collected over a period of twenty-four hours, as described on p. 281.

5 c.c. of urine are pipetted into a Kjeldahl flask containing 20 c.c. of concentrated (N-free) sulphuric acid, 5 g. of potassium sulphate, 0·5 g. of crystalline copper sulphate, and the mixture boiled for forty-five minutes. By this time the mixture will be clear and pale greenish-blue in colour. The cooled digest is transferred to the distilling flask, made alkaline as before, 150 to 200 c.c. of the mixture distilled into 50 c.c. of 0·1N sulphuric acid and the free sulphuric acid, remaining after the distillation is complete, determined by back-titration with 0·1N sodium hydroxide. The amount of nitrogen which has distilled over in the form of ammonia is determined by subtraction. From the figure obtained, the amount of nitrogen in 100 c.c. or in the twenty-four samples of urine is calculated.

As an exercise, calculate the amount of protein metabolised by the individual during the twenty-four hours previous to the urine being collected.

4. AMMONIA.—Of the various methods available for estimating ammonia in urine the "formalin" and "aeration" methods are the most generally adopted.[1]

(a) *By the Formalin Method.*—This method is a modification of Sörensen's method for the estimation of amino-acids (p. 155). Neutral

[1] Yet another method is that involving base-exchange. Powdered permutit—a complex sodium aluminium silicate of natural or synthetic origin—is employed to remove the ammonia from the urine. This removal is essentially a replacement of the sodium of the permutit by the ammonium radical. The permutit, having been shaken with a known volume of dilute urine, is removed, washed with distilled water, and then suspended in a measured volume of dilute sodium hydroxide. Such a procedure causes a reversal of the above process, and consequently ammonia is now liberated from the permutit. Once it has passed into solution, it is estimated colorimetrically by means of Nessler's reagent, an alkaline solution of mercuric iodide, giving a characteristic yellow colour with traces of ammonia.

solutions of ammonium salts react with an excess of formaldehyde, an acid salt of hexamethylenetetramine (urotropine) is formed, thus :

$$4NH_4Cl + 6CH_2O \rightarrow \{N_4(CH_2)_6\}.4HCl + 6H_2O.$$

The urotropine is a weak organic base which does not react alkaline to phenolphthalein. Hence the acid can be titrated with 0·1N sodium hydroxide in the presence of this indicator, the end-point being reached when the solution turns pink.

The *method* is as follows :—60 c.c. of urine are stirred with 3 grams basic lead acetate (to remove amino-acids) and filtered ; 2 grams of potassium oxalate (to remove calcium which would interfere with end-point) are added to the filtrate, which is again well stirred and filtered. Ten c.c. of this clear filtrate are diluted with 50 c.c. of distilled water, and a few drops of 1 per cent. phenolphthalein solution are added ; 5 grams of potassium oxalate are added and dissolved with stirring. The mixture, if acid, as it usually is, is neutralised with 0·1N NaOH ; 20 c.c. of 20 per cent. neutralised formalin [1] are added ; this liberates acid as in the equation given above, and the solution is again titrated with 0·1N soda till neutral. Each c.c. of 0·1N NaOH used to restore the pink colour corresponds to 0·0017 gram NH_3.

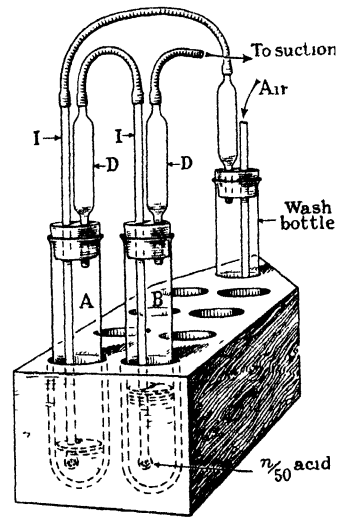


FIG. 40.

(b) *Aeration Method.*—This method is based on the fact that the ammonia of urine normally exists as ammonium salts of weak acids. Addition of potassium carbonate liberates the ammonia, which is driven over by a current of air into a known volume of standard acid, and can be estimated by the method of back titrating the non-neutralised acid with standard sodium hydroxide.

The apparatus used is that depicted in Fig. 40. The wash bottle (a boiling tube) contains 10 per cent. sulphuric acid to remove any ammonia from the air before it enters the apparatus. It is connected

[1] Formalin is a commercial name for an aqueous solution containing 40 per cent. of formaldehyde. There is usually some formic acid present.

in series with the boiling tubes A and B, each closed by a cork which carries (1) a long inlet tube I, ending in a bulb with a large number of perforations and dipping into the fluid placed in the tube; (2) an outlet tube D, which can conveniently be made out of a broken pipette. In order to obtain accurate results, a good suction pump capable of maintaining a rapid stream of air through the apparatus is essential.

The following procedure is recommended. Into A, measure 5 c.c. of urine and to it add a little liquid paraffin or caprylic alcohol to prevent frothing during aeration. Into B, run 20 c.c. of 0·05N sulphuric acid and again add a few drops of caprylic alcohol. Care should be taken that the rubber corks closing the washing bottle and tube B fit tightly, and that the various tubes are correctly joined up. Five grams of dry potassium carbonate are dropped quickly into the urine in A, and the cork immediately placed tightly in position. By means of the pump draw a current of air through the apparatus slowly for three minutes and then as rapidly as possible for thirty to forty-five minutes. Finally gradually stop the pump and, avoiding back pressure, disconnect the tube B. Remove the cork and carefully wash down the inlet tube with distilled water. Add a few drops of de Wesselow's indicator (p. 80), and back titrate with 0·05N sodium hydroxide. The difference between the acid equivalent of the hydroxide used and that originally taken is equivalent to the amount of ammonia liberated from 5 c.c. of urine. Results should be expressed in terms of grams of ammonia per 100 c.c. of urine.

5. UREA.—(a) *The Hypobromite Method.*[1]—This method, which allows a rapid, though not absolutely accurate, determination to be carried out, has for its basis the equation given on p. 258.

The apparatus used is that depicted in Fig. 41. It consists of a bottle connected to a measuring tube by indiarubber tubing. The measuring tube (*e.g.*, an inverted burette) is placed within a cylinder of water, and can be raised and lowered at will. Measure 25 c.c. of a strong 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[2]

[1] Sodium hypobromite reacts with other urinary constituents, *e.g.*, ammonia, uric acid, creatinine, yielding nitrogen.

[2] 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.

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 carrying a spring-clip, seen at the top of the figure. Open the clip and lower the measuring tube until it touches the bottom of the cylinder. Note the level of the water in the burette. Close the clip 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, the gas being thus brought to atmospheric pressure. Read the level of the meniscus as before; the difference between the two readings give the volume of gas (nitrogen) evolved. The carbon dioxide 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 gram 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 mixed. A sample is then taken from the total for analysis, and then, by calculation, the total amount of urea is ascertained.

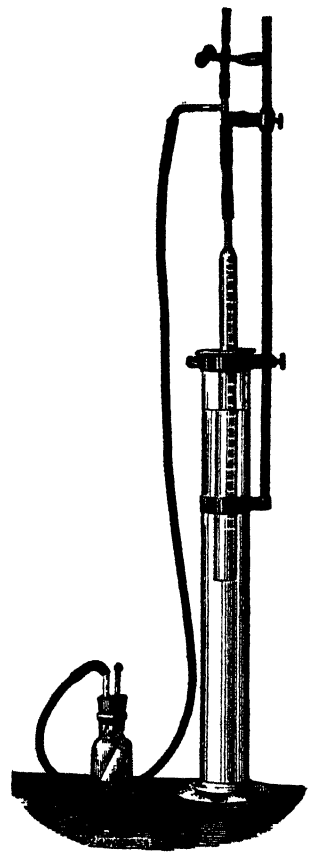


FIG. 41.—Dupré's urea apparatus.

(b) *The Urease Method.*—As was mentioned on p. 259, the enzyme, urease, decomposes urea into ammonium carbonate. Under certain specific conditions the decomposition is both quantitative and rapid. The ammonia, formed by decomposing the ammonium carbonate with sodium carbonate, can be aerated by means of the apparatus on p. 267 into a known volume of standard acid and determined by the usual method of back titration.

The procedure is as follows. The apparatus required is that used for estimating ammonia in urine by the aeration method (Fig. 40, p. 267). Into the tube A pipette 0·5 c.c. of urine add 0·4 gram of finely ground soya bean, 2 c.c. of a 0·6 per cent. solution of acid potassium phosphate, about 2 c.c. of liquid paraffin to prevent frothing, and wash down the sides of the tube with 2 c.c. of water. Connect the tube with the absorption apparatus B, containing 20 c.c. of 0·1N H_2SO_4, and with the wash bottle (containing acid to remove the ammonia from the air current). The tube A is kept at a temperature of 50° C. in a water-bath and an air current is drawn through the series of tubes. After about an hour the rubber connections are disjointed and 3 grams of anhydrous sodium carbonate are dropped into the tube A in order to liberate the ammonia which is present as the ammonium salt. The connections are re-made and the air current is then drawn through for another hour. After this time the excess of the acid in the absorption vessel is titrated with 0·1N sodium hydroxide, using de Wesselow's indicator for determining the end-point. The result includes the ammonia preformed in the urine. This should be estimated separately and subtracted.

Calculation.—From the formula $CO(NH_2)_2 + H_2O \longrightarrow CO_2 + 2NH_3$ it follows that one molecule of urea furnishes two molecules of ammonia, and that 1 c.c. of 0·1N sulphuric acid corresponds to 0·003 gram of urea.

(c) *The Xanthydrol Method of Fosse.*—Ten c.c. of urine are diluted to 100 c.c. with distilled water. Ten c.c. of the diluted urine are placed in a 150 c.c. conical flask, and to it are added 35 c.c. of glacial acetic acid. At intervals of ten minutes, five separate portions of 1 c.c. of a 10 per cent. solution of xanthydrol in methyl alcohol are added, and the mixture allowed to stand for one hour. The crystalline precipitate is collected, washed with alcohol, dried at 100° C., detached from the filter paper, and weighed. The precipitate forms a rigid cake on the filter paper and can easily be detached. The weight of the di-xanthyl-urea divided by seven equals the weight of urea present. Under the above conditions, urea is the only constituent of the urine which gives an insoluble xanthydrol compound.

6. URIC ACID.—(a) *Hopkins-Cole Method.*

Principle of the Method.—After removal of substances of unknown composition by colloidal iron, the uric acid is precipitated as ammonium urate, washed to remove adherent chlorides, and dissolved in hot sulphuric acid. The uric acid is then estimated volumetrically by titration with standard potassium permanganate. The reagents required

are: (*a*) colloidal iron, 0·6 per cent.; (*b*) crystalline ammonium chloride; (*c*) washing fluid composed of 100 grams ammonium sulphate, 10 c.c. concentrated ammonia in 1000 c.c. distilled water; (*d*) sulphuric acid, 45 per cent. by volume; (*e*) 0·05N potassium permanganate, obtained by dissolving 1·58 grams of pure potassium permanganate in 750 c.c. of distilled water and making up to 1000 c.c.

Analysis.—150 c.c. of urine are placed in a small beaker and 30 c.c. of the colloidal iron added with stirring. The solution is filtered into one or two *dry* flasks. With a pipette 100 c.c. of the filtrate (150 if the urine is dilute) are transferred to a dry beaker and ammonium chloride is added to make a concentration of 20 per cent. After complete solution 3 c.c. concentrated ammonia are also added. The mixture is stirred at intervals for twenty minutes. The precipitate of ammonium urate so formed is then filtered either by gravity or by *moderate* suction. Chlorides are removed from the precipitate by treating it on the filter with the washing fluid at least twice. The precipitate is allowed to drain completely and is transferred, by means of hot water, to a flask. The volume is made up to 100 c.c. with water; 20 c.c. of 45 per cent. sulphuric acid are added. Heat the mixture to 65° C. and titrate at this temperature with the standard permanganate. The end-point is a faint pink colour spreading through the liquid. The colour persists only for a second or two. It is not permanent.

Calculation.—Since 1 c.c. of 0·05N $KMnO_4$ is equivalent to 3·75 mg. of uric acid, and since the original urine was treated with $\frac{1}{5}$ of its volume of colloidal iron, if x is the number of c.c. of $KMnO_4$ required to oxidise the uric acid from 100 c.c. of filtrate, then 100 c.c. original urine contain $x \times 3{\cdot}75 \times \frac{6}{5}$ mg. uric acid.

(b) *The Colorimetric Method of Benedict and Franke.*—A much more rapid method of estimating uric acid involves the use of the colorimeter described on p. 318. In this method the deep blue colour which is produced by the action of sodium arseno-phosphotungstate and potassium cyanide on uric acid is made use of for the quantitative estimation of the latter by comparing it with the colour derived from a standard solution of uric acid. Since the colour reaction is very delicate, only a very small volume of urine is required for analysis. With slight modification the method may be applied to the estimation of uric acid in blood and in cerebro-spinal fluid.

5 c.c. of the urine are measured into a 100 c.c. volumetric flask and diluted twenty times by making up to the mark with distilled water. The contents of the flask are well mixed.

Two 50 c.c. volumetric flasks are labelled S (standard solution) and U (unknown solution). Into S measure 10 c.c. of the standard uric acid solution[1] *(i.e.,* **0·2 mg. of uric acid); into U, 10 c.c. of the diluted urine.** *Using burettes and not pipettes, since the solutions are very poisonous,* **add to each flask (1) 5 c.c. of the cyanide solution**[2] **and (2) 1 c.c. of Benedict's reagent.**[3] **By gentle shaking, the contents of the flasks are mixed and then allowed to stand for five minutes. Finally, both flasks are filled to the 50 c.c. mark with distilled water, mixed thoroughly, and the colours compared in the colorimeter.**

Calculation.—$\frac{\text{Reading of standard tube}}{\text{Reading of unknown tube}} \times 0{\cdot}2 = \text{milligrams of uric acid}$ **in 10 c.c. of diluted urine. Finally, a correction must be made for dilution.**

7. CREATININE.—The following colorimetric method (Folin's), employed for the estimation of this compound, is based on the red colour which is produced when an alkaline solution of picric acid is added to a solution of creatinine (p. 261).

Into a 500 c.c. volumetric flask pipette 10 c.c. of urine. To it add 15 c.c. of a saturated solution of picric acid and 5 c.c. of 10 per cent. sodium hydroxide. Mix the contents well and allow to stand for five minutes. Then dilute to the 500 c.c. mark and compare the colour with that of a 0·5N solution of potassium dichromate, the colorimeter plunger being set at 8·0 mm. Careful calibration of the method shows

[1] *Stock uric acid* solution is made up by dissolving 9 grams of pure crystalline disodium hydrogen phosphate and 1 gram of pure sodium dihydrogen phosphate in about 250 c.c. of hot water. Filter if necessary, and make up the volume to 500 c.c. with hot water. Then place 0·2 gram uric acid (accurately weighed) into a litre measuring flask and add a few c.c. of hot water. Pour the hot phosphate solution into the litre flask and shake until all the uric acid is dissolved. Cool the solution and carefully add 1·4 c.c. of glacial acetic acid. Make the volume up to 1 litre with distilled water and mix well. Five c.c. of chloroform should be added as a preservative. Store in a cool place.

Standard Solution.—Pipette 50 c.c. of the stock solution into a 500 c.c. volumetric flask, and dilute to about 400 c.c. with distilled water. Twenty-five c.c of dilute hydrochloric acid (1 in 10) are added. Make up the volume to 500 c.c. with distilled water and thoroughly mix the fluid. This solution may be kept for not more than fourteen days.

[2] The *sodium cyanide* solution is 5 per cent. It should be prepared anew about every two months.

[3] *Uric Acid Reagent.*—Into a litre flask place 100 grams of pure sodium tungstate and to it add 600 c.c. of distilled water. Add 50 grams of pure arsenic acid, 25 c.c. of syrupy phosphoric acid (85 per cent.), and 20 c.c. of concentrated hydrochloric acid. Boil the solution for about twenty minutes. Cool, and make up to 1 litre with distilled water.

that, with this standard set at 8·0 mm., a solution containing 10 mg. of creatinine gives a reading of 8·1 mm.

Calculation.[1]—$\frac{8{\cdot}1}{\text{unknown reading}} \times 10 =$ milligrams of creatinine in quantity of urine used.

8. CHLORIDES.—The Volhard-Arnold procedure 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 gram of sodium chloride (29·075 grams of fused silver nitrate in a litre of distilled water).

ii. Solution of potassium thiocyanate (8 grams 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 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. Let the volume necessary for this be x c.c.

Analysis.—Pipette 10 c.c. of the urine in a 100 c.c. measuring flask ; 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 25 c.c. 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. Let the volume so used be a c.c. This must by multiplied by 4 to represent what the total fluid (100 c.c.) would have required.

[1] The simple relation of the reading to the concentration holds, in this case, only over a limited range of readings, *i.e.*, 6 to 12 mm. If the reading is less than 6, make another determination on the urine, accurately diluted ; if greater than 12, take a larger volume of the urine.

In the previous standardisation of the thiocyanate solution x c.c. of it were found to be equivalent to 10 c.c. of the silver solution; therefore $4a$ c.c. are equivalent to $\frac{10 \times 4a}{x}$ of the silver solution, and this represents the amount of silver solution not used in precipitating the chlorides. Therefore $20 - \frac{10 \times 4a}{x}$ 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 $20 - \frac{40a}{x}$ c.c. of the standard silver nitrate for their precipitation, and as each c.c. of the standard solution is equivalent to 0·01 gram of sodium chloride, the total chlorides in the 10 c.c. of urine (expressed as sodium chloride) is $\left(20 - \frac{40a}{x}\right) \times 0{\cdot}01 = 0{\cdot}2 - \frac{0{\cdot}4a}{x}$ grams. If one multiplies this by 10, we obtain $2 - \frac{4a}{x}$ grams, 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.

9. **PHOSPHATES.**—The method depends upon the fact that, in a very slightly acid solution (ensured by the addition of sodium acetate and acetic acid), uranium salts react with phosphates to yield insoluble uranium phosphate. When all the phosphate has so reacted the uranium salt will form a brown uranium ferrocyanide with the potassium ferrocyanide reagent added as an indicator.

The reagents required are :—

(i) A standard solution of uranium nitrate: 35·5 grams of the nitrate are dissolved in water, the volume of the solution made up to 1 litre. One c.c. corresponds to 0·005 gram of phosphoric acid (P_2O_5).

(ii) Acid solution of sodium acetate. Dissolve 100 grams of sodium acetate in 900 c.c. of water; to this add 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

[1] In using uranium nitrate it is imperative that addition of 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.

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 of phosphoric acid (expressed as P_2O_5) in the urine.

10. 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. One of the methods which can be recommended for the estimation of total phosphorus is that of Neumann 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 milligrams. If multiplied by 1·268 the result is expressed in terms of milligrams 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,[2] transferred into a flask, and a measured quantity of half-normal

[1] The solution is prepared as follows:—A solution of 75 grams 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 grams 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.

[2] 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

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, $x \times 0{\cdot}553$ = milligrams of phosphorus present; and $x \times 1{\cdot}268 = P_2O_5$.

11. INORGANIC AND ETHEREAL SULPHATES.

A. GRAVIMETRIC METHOD. Method of Folin.

(i) *Estimation of total (inorganic and ethereal) sulphates.*—25 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 decomposition 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 are 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 tared Gooch crucible (a porcelain crucible with a perforated bottom on which is spread a layer of asbestos). The precipitate is washed with water, ignited, and weighed. The increase in weight is the amount of barium sulphate obtained.

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 gram of total barium

five minutes: Filter pulp is prepared by shaking up 30 grams 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.

sulphate; this multiplied by 0·343 = 0·171 of total SO_3. Another 100 c.c. of the same urine gave 0·45 gram of barium sulphate from inorganic sulphates; this multiplied by 0·343 = 0·154 of SO_3 in inorganic combination. If this is subtracted from the total SO_3 (0·171 − 0·154), we see that 0·017 gram of SO_3 was in combination as ethereal sulphate, or about one-tenth of the total, which is the average proportion in normal urine.

(ii) *Estimation of inorganic sulphates.*—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.

(iii) 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. 125 c.c. of the filtrate are then gently boiled for about 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.

B. VOLUMETRIC METHOD (modification of the method of Fiske, Rosenheim and Drummond).—*Principle.*—The sulphates of the urine are precipitated from the faintly acidified urine as benzidine sulphate by the addition of a solution of benzidine ($NH_2.C_6H_4.C_6H_4.NH_2$) in hydrochloric acid. As benzidine is a weak base its salts with acids are readily dissociated, and the sulphuric acid contained in benzidine sulphate may be quantitatively titrated with standard alkali solutions, using phenolphthalein as an indicator.

The total sulphates (inorganic and ethereal) are similarly estimated after previous hydrolysis of the ethereal sulphates by boiling with hydrochloric acid.

The ethereal sulphates are represented by the difference between the total and inorganic sulphates.

Analysis.—(a) *Estimation of inorganic sulphates.*—Twenty c.c. of urine are measured into a flask and acidified with dilute hydrochloric acid (1 : 4) until the reaction is just acid to congo-red paper; 100 c.c. of benzidine solution are then run in and the precipitate, which forms in a few seconds, is allowed to settle for ten minutes. (The benzidine solution is prepared by rubbing 4 grams of benzidine into a paste with water, and transferring it with 500 c.c. of water into a 2-litre flask. Five c.c. of concentrated HCl are added, and the solution made up to 2 litres with distilled water.) The precipitate is filtered under pressure on paper pulp with the precaution of not allowing at any time the precipitate to be sucked dry on the filter. This is washed with 10 to 20 c.c. of water saturated with benzidine sulphate. The precipitate and filter paper are transferred into the original precipitation flask with about 50 c.c. water and titrated with 0·1N sodium hydroxide solution, after the addition of a few drops of a saturated alcoholic solution of phenolphthalein. One c.c. of decinormal NaOH = 4·9 mg. H_2SO_4.

(b) *Estimation of total sulphates.*—Add 2 c.c. of 25 per cent. HCl to 20 c.c. of urine and boil gently for twenty to thirty minutes. After cooling the flask add sodium hydroxide until neutral to litmus. Then add the dilute hydrochloric acid (1 : 4) until the reaction of the mixture is just acid to congo paper. Then follow the procedure described under (a).

12. 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 the sulphur to sulphuric acid is completed by heating with copper nitrate and potassium chlorate. The solution finally obtained is used to estimate the sulphuric acid by either the gravimetric or volumetric methods.

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 grams copper nitrate, and 50 grams 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, due to the formation of cupric 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 either of the above procedures. The filtrate from the barium sulphate

precipitate may also be used for the estimation of phosphorus by Neumann's method (p. 275).

13. GLUCOSE.—Four main methods are available for the quantitative determination of glucose in urine :—(a) the polarimetric method (p. 12) ; (b) the fermentation method ; (c) the gravimetric method ; and (d) volumetric methods employing Benedict'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 carbon dioxide 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.

Of the *volumetric methods* that of Benedict is described below.

BENEDICT'S METHOD.—For preparation of Benedict's quantitative reagent see p. 10.

Analysis.—Three or 4 grams of anhydrous sodium carbonate are placed in a 300 c.c. flask, then 25 c.c. of the above solution. This is kept boiling over a small flame, and the urine 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 reading on the burette is 10 c.c., then this amount of urine contains 0·05 gram of glucose, therefore 100 c.c. contains 0·5 gram. Should it be found that the first titration gives a result indicating that the percentage is greater than 1 per cent., the urine should be diluted quantitatively so as to bring the concentration between 0·5 and 1·0 per cent. A further example will make the calculation clear. 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 gram of glucose.
1 c.c. „ „ contains $\frac{0{\cdot}05}{2}$ „
and 100 c.c. „ „ contain $\frac{0{\cdot}05 \times 100}{2}$ „
= 2·5 glucose per cent.

ESTIMATION OF ALBUMIN BY ESBACH'S ALBUMINOMETER.—Esbach's reagent for precipitating the albumin is made by dissolving 10 grams of picric acid and 20 grams 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 (Fig. 42) up to the mark U; then the reagent up to the mark R. Close the tube with a cork, and to ensure

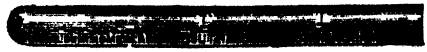


FIG. 42.—Esbach's albuminometer.

complete mixture tilt it to and fro a dozen times without shaking. Allow the corked tube to stand upright for twenty-four hours and read off on the scale the height of the precipitate. The figures roughly indicate the number of grams 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 grams per litre, or 0·3 gram 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 present is less than 0·05 per cent., it cannot be estimated by this method.

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 may be regarded as 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. The process which occurs here is generally spoken of as a filtration. During the passage of this dilute urine through the rest of the renal tubule it becomes quantitatively changed into the urine as found in the bladder.

GENERAL CHARACTERS OF URINE

Quantity.—An individual of average weight and height passes from 1200 to 1800 c.c. daily. This contains about 60 grams (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 closely covered by a ground-glass plate to exclude dust and to avoid evaporation. A few drops of chloroform or toluene should be added to prevent putrefaction. 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, being, like bile pigment, an iron-free derivative of hæmoglobin containing the pyrrole ring. The bile pigment (and possibly also the hæmatin of the food) is in the intestine converted into stercobilin, most of which leaves the body in the fæces; the remainder is reabsorbed and is excreted with the urine as urobilin. Urobilin is very like the artificial reduction product of bilirubin called hydrobilirubin (p. 171). 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 light and 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 p. 307).

Reaction.—The total twenty-four-hour sample of urine from a normal individual usually reacts acid to litmus, its P_H being about 6. Nevertheless, the hydrogen-ion concentration may vary within wide limits (P_H 5·5 to 8·0). The reaction of urine is the sum of the reactions of its acidic and basic constituents, which are both inorganic and organic in nature. Of these, however, the mono-hydrogen (alkaline) and di-hydrogen (acid) phosphates of sodium and potassium play by far the greatest rôle. In this connection it will be remembered that the renal excretion of acid or alkali phosphates is one of the factors regulating the neutrality of the blood and of the organism in general.

Variations in the reaction of urine from the normal are found:—

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. Leathes states that respiration is a more important factor than gastric secretion in producing the change of reaction. During sleep, respiration is comparatively inactive, hence carbon dioxide accumulates, and the increase in H-ion concentration is reflected in the urine. With the activity associated with daytime this effect passes off.

2. During strenuous physical exercise.

3. 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 render it alkaline.

4. In certain pathological conditions, *e.g.*, cardio-renal disease.

Specific Gravity.—This should be determined on a sample of the twenty-four hours' urine. The specific gravity varies inversely as the quantity of urine passed. Normally it lies between 1015 and 1025. A specific gravity below 1010 should excite suspicion of hydruria; one over 1030 of a febrile condition, or of diabetes mellitus when 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. A rough estimate of the total solids of

the urine (grams per litre) may be obtained by multiplying the last two figures of the four-figured number expressing the specific gravity by 2·66 (Long's coefficient).

Composition.—The following table gives the average amounts of the urinary constituents passed by a man (taking an ordinary diet containing about 100 grams of protein) in the twenty-four hours :—

Total quantity of urine	1500·00	grams
Water	1440·00	,,
Total solids	60·00	,,
Urea	35·00	,,
Uric acid	0·75	,,
Hippuric acid	0·7	,,
Creatinine	1·0	,,
Sodium chloride	16·3	,,
Sulphuric acid	2·01	,,
Phosphoric acid	3·16	,,
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 thus water, urea, and sodium chloride. In the foregoing table the student must not be misled by seeing the acids and metals separated. These are combined to form salts, *e.g.*, sodium urate, calcium phosphate, etc.

THE INORGANIC CONSTITUENTS OF URINE

The inorganic or mineral constituents of urine are chiefly chlorides, phosphates, and sulphates. Carbonates may also be conveniently included here, the bases with which these are in combination being sodium, potassium, ammonium, calcium, and magnesium. The total amount of these salts excreted daily varies from 19 to 25 grams. The most abundant is sodium chloride, which averages in amount 10 to 16 grams *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 the result of metabolism. The sulphates are derived from 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 beginning of this chapter.

Chlorides.—The chief chloride is sodium chloride. Its ingestion is followed by its appearance in the urine, part on the same day, part on the next day. Some is decomposed to form the hydrochloric acid of the gastric juice. In passing through the body, sodium chloride fulfils the useful office of stimulating metabolism and excretion. During starvation and in febrile conditions, *e.g.*, pneumonia, there is generally a marked retention of chloride by the organism, with the result that chlorides may almost be entirely absent from the urine.

Sulphates.—Sulphur is present in the urine in three forms :—

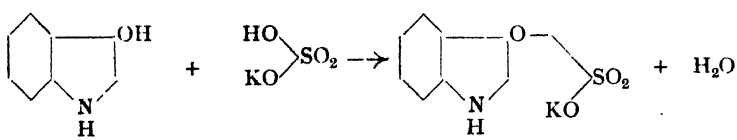
1. *Inorganic Sulphate.*—By far the greatest part of the inorganic sulphates are derived from the metabolism in the body of the sulphur-containing proteins, only the slightest trace entering the body with the food. The amount excreted daily varies in amount from 1·5 to 3 grams.

2. *Ethereal Sulphate.*—These, which vary considerably in daily amount, are esters formed by the union of sulphuric acid with the products (or oxidation products) of bacterial action on such amino-acids as tyrosine and tryptophan in the intestine. These products are toxic, and the organism, not being able to oxidise them completely, to protect itself *conjugates* them with the sulphuric acid, or potassium hydrogen sulphate, already at hand, to render them less harmful and more soluble. Such a conjugation or protective synthesis is represented by the following equation :—

$$C_6H_5OH + SO_2\langle{}^{OH}_{OK} \rightarrow SO_2\langle{}^{OC_6H_5}_{OK} + H_2O$$

[phenol] [potassium hydrogen sulphate] [potassium phenyl-sulphate] [water]

Indole, on the other hand, is first oxidised to indoxyl, which substance is conjugated with potassium hydrogen sulphate thus :—



[indoxyl] [potassium hydrogen sulphate] [potassium indoxyl-sulphate] [water]

Since it gives rise to indigo on oxidation, potassium-indoxyl-sulphate is called *indican* (p. 261). In using this term, however, care must be taken not to confuse it with the indican of plants, which is a glucoside, and which also yields indigo on oxidation. It should be remembered, moreover, that these ethereal sulphates will not yield, as do the inorganic sulphates, a precipitate of barium sulphate immediately on treatment with barium chloride in acid solution. Such a precipitate will only be obtained if the esters *are first hydrolysed by boiling* them with, for example, dilute hydrochloric acid.

3. *Neutral Sulphur*.—In addition to the above-mentioned inorganic and ethereal sulphates, there is always present in the urine a certain amount of sulphur in a less oxidised form than that of sulphate. Into this fraction falls the incompletely metabolised sulphur compounds such as cystine, taurine, methyl sulphide, thiocyanates, etc. This fraction will only yield a precipitate with barium chloride if it is *first oxidised to* sulphate, and only by such a procedure can it be estimated.

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 consequently rendered alkaline. Urine containing carbonates becomes, like saliva, cloudy on standing, the precipitate consisting of calcium carbonate together with 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.

The earthy phosphates may be 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 ammonium - magnesium phosphate ($NH_4MgPO_4+6H_2O$). This crystallises in " coffin-lid " crystals (see Fig. 43) 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 give a precipitate of calcium phosphate when heated; this precipitate is amorphous, and is liable to be mistaken for albumin. It may be distinguished readily from albumin as it dissolves on adding a few drops of acetic acid, whereas coagulated protein does not dissolve.

FIG. 43.—Ammonium-magnesium or triple phosphate.

The phosphoric acid in the urine chiefly originates from the phosphates of the food, but is partly a decomposition or katabolic product of the phosphorised organic materials in the body, such as lecithin and nuclein. As has already been shown earlier in this chapter, the excretion of phosphates, both acid and alkaline, plays a very large part in the maintenance of the neutrality of the blood. The amount of P_2O_5 in the twenty-four hours' urine varies from 2·5 to 3·5 grams, to which the earthy phosphates contribute about half (1 to 1·5 gram).

UREA

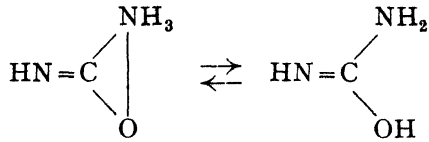
Urea is a crystalline substance, melting at 132° C., readily soluble in water, alcohol, and acetone. It has a saltish taste, and its aqueous solution reacts neutral to litmus.

It was first prepared synthetically by Wöhler in 1828 from ammonium cyanate, $(NH_4)CNO$, a substance which has the same empirical, but not the same structural, formula as urea.

Addition of excess of nitric or oxalic acid to a concentrated urine leads to the separation in crystalline form of urea nitrate or urea oxalate respectively, the former being lozenge-shaped tablets or hexagons (Fig. 44 (*a*)); the latter, prisms (Fig. 44 (*b*)). Analyses of these salts show that their formulæ are $CON_2H_4.HNO_3$ and $(CON_2H_4)_2.H_2C_2O_4$ respectively; in other words, there appears to be only one basic group in the urea molecule to which the acid can attach itself.

On the basis of such evidence Werner argued that the old

carbamide formula $CO(NH_2)_2$ does not account for the chemical behaviour of urea. Instead, he concluded that the structure of urea might be represented in two ways :—



the former representing its structure in alkaline, neutral, or weakly acid (acetic) solution, the latter in strongly acid (hydrochloric) solution.

Under the action of certain micro-organisms, such as the *Micrococcus ureæ*, which grows readily in urine, urea takes up water,

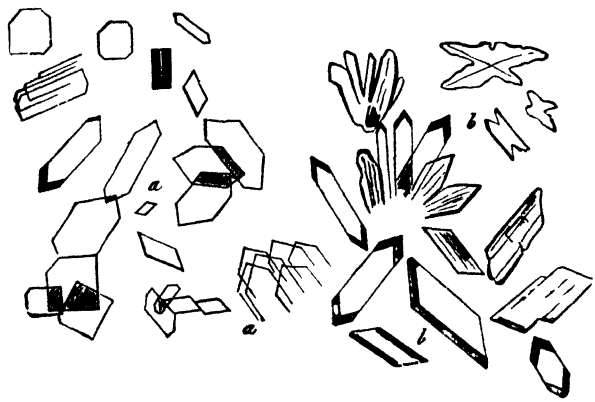


FIG. 44.—*a*, nitrate ; *b*, oxalate of urea.

and is converted into ammonium carbonate $[CON_2H_4 + 2H_2O = (NH_4)_2CO_3]$. For this reason putrid urine has an ammoniacal odour.

Many leguminous seeds, especially the soya bean, contain an enzyme (urease) which similarly converts urea into ammonia and carbon dioxide ; this process may be used for estimating urea (p. 269).

By means of nitrous acid, urea is broken up into carbon dioxide, water, and nitrogen. This may be used as a test for urea. Addition of fuming nitric acid (*i.e.*, nitric acid containing nitrous acid in solution) to a solution of urea, or to urine, causes an abundant evolution of nitrogen and carbon dioxide to take place. The equation representing the main reaction which takes place between sodium

hypobromite and urea has already been given (p. 258). Side reactions of a complex nature also occur, and under the usual experimental conditions a certain amount (0·7 per cent.) of carbon monoxide is mixed with the nitrogen. The main reaction is, however, important, for on it is based one of the readiest methods for estimating urea. If the experiment is performed as directed on p. 268, the carbon dioxide being absorbed by excess of soda, then the amount of gas collected may be used as a measure of the amount of urea present.

The quantity of urea excreted, as will be seen later, is somewhat variable, the chief cause of variation being the amount of protein food ingested. This can well be illustrated by the following table of Folin's :—

Constituent of Urine.	Normal Protein Diet.			Starch Cream Diet.		
	Weight. Grams.	Nitrogen. Grams.	Per Cent. of Total N or S.	Weight. Grams.	Nitrogen. Grams.	Per Cent. of Total N or S.
Urea	31·6	14·7	87·5	4·72	2·2	61·7
Ammonia . . .	0·6	0·49	3·0	0·51	0·42	11·3
Creatinine . . .	1·55	0·58	3·6	1·61	0·60	17·2
Uric acid . . .	0·54	0·18	1·1	0·27	0·09	2·5
Undetermined N . .	...	0·85	4·9	...	0·27	7·5
Total N . . .	...	16·8	100·0	...	3·6	100·0
Inorganic SO_3 . .	3·27	...	90·0	0·46	...	60·5
Ethereal SO_3 . .	0·19	...	5·2	0·10	...	13·2
Neutral SO_3 . . .	0·18	...	4·8	0·20	...	26·2
Total SO_3 . . .	3·64	...	100·0	0·76	...	100·0

In a man taking the usual Voit diet containing about 100 grams of protein (which will contain about 16 grams of nitrogen), the quantity of urea excreted daily averages 33 grams. The amount in human urine, normally, is approximately 2 per cent.; nevertheless this varies, because the volume and concentration of the urine is inconstant even in health. The excretion of urea is usually at a maximum three hours after a meal, especially after one rich in protein.

Muscular exercise has but little effect on the amount of nitrogen eliminated. This is strikingly different from what occurs in the case of carbon dioxide; the more work the muscles perform, the more carbon dioxide passes into venous blood. This is rapidly discharged in the expired air. Muscular energy is derived normally from the combustion of non-nitrogenous material, 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 protein material.

The Site of Formation of Urea in the Animal Body

The importance of urea in nitrogenous metabolism was first established by Roulle in 1773 by his discovery of urea in the urine; in 1823 it was shown by Prévost and Dumas to be a constituent of the blood.

The problem of the site, as well as that of the nature, of the transformation of nitrogenous metabolic products into urea has engaged the attention of investigators for a long time. Originally it was considered that urea was formed in the kidneys, just as carbon dioxide was thought to be formed in the lungs. Prévost and Dumas were the first to insist that, despite complete extirpation of the kidney, the formation of urea and other waste products still continues, and that they accumulate in the blood and tissues, to give rise ultimately to the pathological condition known as *uræmia*. At the same time it was a well-known fact that when certain degenerative changes, *e.g.*, cirrhosis or acute yellow atrophy, occur in the liver, the amount of urea formed is much reduced, and may be almost entirely absent from the urine. Such facts were interpreted in different ways, and so we find, until quite recently, three very well-defined schools of thought regarding this question. In the first place, there were those who urged that urea was formed only by the liver; the second school thought that urea was formed by the tissues, but chiefly by the liver; and finally, those who denied any special rôle to the liver in the formation of urea. These were the ideas prevalent when the American workers Bollman, Mann, and Magath undertook, in 1922, a re-investigation of this and kindred problems.

It was already a well-known fact that after the removal of the liver in such animals as frogs, urea formation almost ceases, and in its stead ammonia is found in the urine. Similar experiments on other animals, *e.g.*, the dog, invariably failed, owing to death occurring soon after the removal of the liver. Mann and his

colleagues evolved a technique whereby they were able to keep their animals alive for thirty or more hours after the operation, an ample period for carrying out the large number of blood and urine analyses which the workers considered necessary for the purpose of their experiments.

The following graphs (Figs. 45 and 46), modified from one of their papers, clearly indicate the main results obtained. Fig. 45 shows the simultaneous decreases that took place in the blood and urine urea nitrogen following removal of the liver. The line O represents the blood urea-N in milligrams per litre; the rectangles the milli-

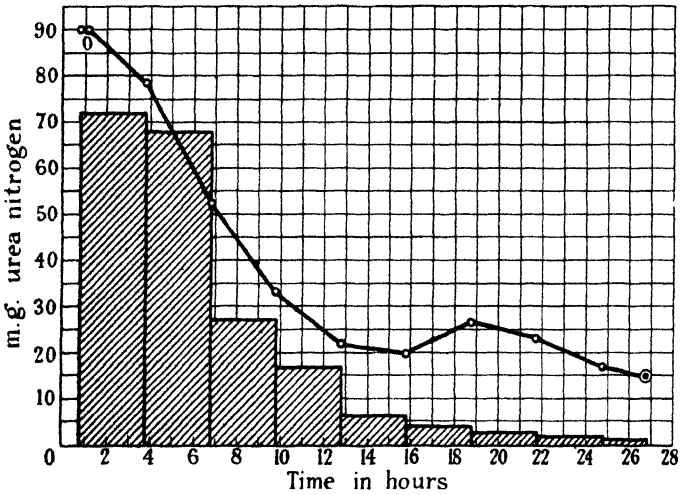


FIG. 45.—Urea nitrogen of blood and urine after hepatectomy. (After Bollman, Mann, and Magath.)

grams of urea-N excreted during each hour of the intervals when urine was collected. Fig. 46 gives curves showing the effect of the removal of the liver at O on the blood urea-N at different levels; in curve 1, the liver was removed at O twenty-four hours after removal of both kidneys; curve 2 is similar to curve 1, the liver being removed eight hours after removal of both kidneys; curve 3, the effect of simultaneous removal of liver and both kidneys; curve 4, the effect of removal of liver only, urinary secretion being maintained. Such evidence must be accepted as proving conclusively that the liver is the one and only seat of the formation of urea in the animal organism.

Uræmia.—This term was originally applied on the erroneous supposition that it is urea or some antecedent of urea which acts as the poison in the pathological condition known by this name. There

is fair support for the belief that the poison is not found in 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 after the simultaneous blocking of both renal arteries by clot, or of both ureters by stones, again uræmia does not follow. On the

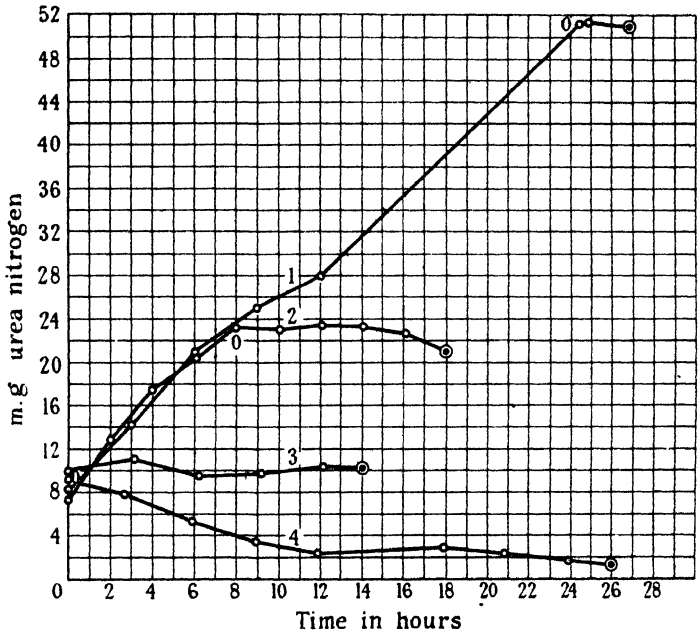


FIG. 46.—Blood urea nitrogen. For explanation see text. (After Bollman, Mann, and Magath.)

other hand, uræmia may occur even while a patient with diseased kidneys 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.

Exogenous and Endogenous Metabolism

Reference to Folin's table (p. 288) will show that the amounts of urea, uric acid, and inorganic sulphur present in the urine vary considerably with the amount of protein in the diet. We can indeed accept it as a fact that the composition of the urine is intimately bound

up with the composition of the diet. Further examination of Folin's figures show, however, that in the case of creatinine, the figure remains fairly constant whatever kind of diet the individual was taking. This variability of the urea, etc., and the constancy of the creatinine was considered by Folin to have a special significance, the meaning of which may well be considered at this point. Before proceeding further, the student would be well advised to read again the paragraph on p. 174 regarding protein absorption.

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, but that nitrogenous katabolism is of two kinds. One varies with the food, and is therefore variable in amount, occurring 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 deaminised, 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* katabolism. The final product is in part urea, but the waste nitrogen finds its way out of the body in other forms also, one 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, 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. The decomposition of the protein is generally regarded as being one involving hydrolysis, and during which there is 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 grams of nitrogen daily (that is, the amount corresponding to an intake of 100 grams 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 directly into the blood-stream. An increased formation of urea results. In the case of arginine the exact chemical decomposition which takes place is known. We have already seen that arginine can be hydrolysed *in vitro* into urea and a second amino-acid ornithine. By means of its specific enzyme *arginase* the liver is also able to effect this decomposition and thus liberate urea, a process to which reference will again be made. In addition to this, the ornithine itself is further decomposed, thus liberating an extra quantity of urea. Not all the amino-acids absorbed from the intestine undergo this conversion into urea in the liver, for, apart from those required for the resynthesis of new tissue, a certain number of them are necessary for the elaboration of special compounds, *e.g.*, thyroxine (from tyrosine) required for essential body processes.

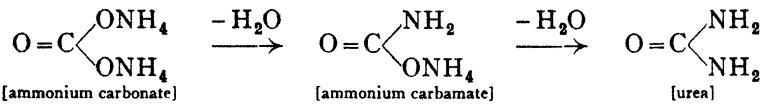
Deamination.—The enzyme action by which the liver splits off the amino-group from an amino-acid, leaving a non-nitrogenous residue, is called *deamination*. At one time the process was considered to be hydrolytic in character but now it is known to involve oxidation, and the degradation of the amino-acid may well be represented :

$$\underset{\substack{|\\ NH_2}}{CH_3CHCOOH} \xrightarrow{+O} \underset{\substack{\|\\ NH}}{CH_3CCOOH} \xrightarrow{+H_2O} CH_3COCOOH + NH_3$$

[alanine] [pyruvic acid] [ammonia

Should the deamination of amino-acids actually follow such a path, it will be highly interesting, especially in view of the fact that *certain* (not all) amino-acids may be formed by perfusing a surviving liver with the ammonium salts of their corresponding *keto*-acids. Thus ammonium pyruvate gives rise in this way to alanine.

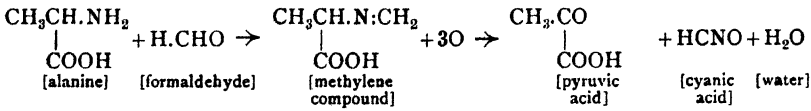
The ammonia which is liberated during the process of deamination is converted into urea, but how this conversion takes place has even yet not been satisfactorily answered. The oldest view is that the ammonia thus liberated combined with carbon dioxide to form ammonium carbonate, which compound gave rise first to ammonium carbamate and then to urea by succeeding stages involving, in each case, the elimination of a molecule of water thus :—



[ammonium carbonate] [ammonium carbamate] [urea]

Such a scheme of formation of urea from the amino-acids is, however, open to so many objections that alternative schemes have been advanced.

One such scheme is based on the fact that Wöhler, in his synthesis of urea, used ammonium cyanate as his starting-point, the process essentially being one involving molecular rearrangement. Fearon and Montgomery, investigating the possible relationship of the formation of cyanic acid to oxidative deamination, found that amino-acids, *e.g.*, glycine, alanine, were oxidised by hydrogen peroxide in alkaline solution to yield cyanic acid and urea. Better yields of these substances were obtained if potassium permanganate was used instead of hydrogen peroxide, while the formation of cyanic acid was specially marked when glucose or formaldehyde (or some such aldehydic substance) was present. Such observations have led these workers to put forward the following scheme as indicating the possible line of breakdown of an amino-acid :—



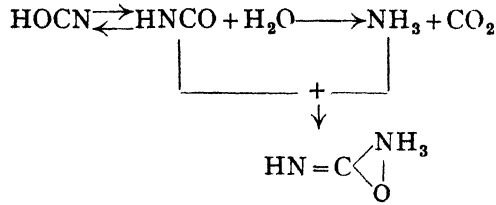
[alanine] [formaldehyde] [methylene compound] [pyruvic acid] [cyanic acid] [water]

It will be seen that cyanic acid is one of the products of such decomposition. It will be remembered, moreover, that this substance is an extremely reactive compound undergoing the following changes :—

I. In acid solution, it is hydrolysed to ammonia,

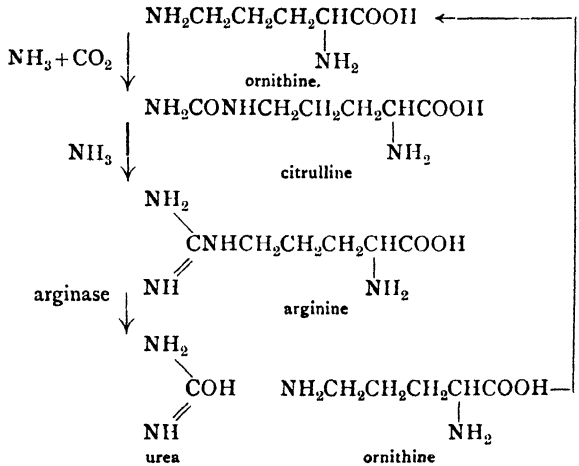
$$HOCN + H_2O + HCl \longrightarrow NH_4Cl + CO_2.$$

II. In neutral aqueous solution, it is partly hydrolysed to ammonia and carbon dioxide, the ammonia so liberated combining with unchanged cyanic acid to yield urea :—



The importance of cyanic acid in the animal organism may not cease here, since, as we shall see when we come to the question of the presence of ammonia in urine, cyanic acid may also be the precursor of urinary ammonia.

It will be seen that this scheme has many points in its favour, the main drawback to its general acceptance being the absence of definite proof that cyanic acid itself occurs in the animal organism.



Much more recently Krebs and Henseleit have put forward experimental evidence in support of yet another method whereby urea may be formed in the animal body. Accepting the view that ammonia is the primary product of deamination, they maintain that urea is a product of a cycle of chemical operations in which arginine, ornithine, citrulline (first isolated from the water melon), ammonia, and carbon dioxide are the factors concerned. Ornithine, they say, unites with carbon dioxide and ammonia to form citrulline, which, in turn,

unites with another molecule of ammonia to give arginine. This amino-acid is then broken down by the arginase of the liver yielding urea and ornithine, whereby the series of operations is completed, the ornithine being available for another cycle of reactions.

AMMONIA

The urine of man and carnivora contains small quantities of ammonium salts. In man the daily amount of ammonia excreted varies between 0·3 and 1·2 grams: the average is 0·7 gram. 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.

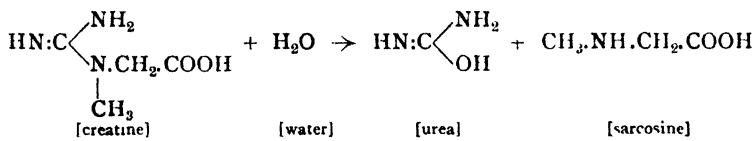
In normal circumstances the amount of ammonia depends on the balance between the production of acid substances in metabolism and the supply of bases in the food. The necessity for the supply of the so-called "fixed" bases (*i.e.*, Na, K, Ca, etc.) is for certain specific purposes, *e.g.*, the maintenance of the buffering-power of the blood and other body fluids, for bone formation, etc. Only when these various requirements have been met will excretion of fixed bases normally take place. On the other hand, the amount of acids produced by the body depends on a number of factors, *e.g.*, the amount of protein metabolised, the type of work carried out by the body, etc., and naturally varies considerably from time to time. It can be visualised then that sometimes there is a large excess of acids over bases to be excreted, other times the amounts may be almost equal, and at other times (especially on a vegetarian diet) the bases excreted are in excess of the acids. When the production of acids is excessive and there is therefore not normally a sufficiency of the fixed bases for their neutralisation, the body utilises ammonia for the purpose. Hence we may regard ammonia as the physiological remedy for a deficiency of fixed bases utilisable for neutralisation purposes. Thus we find that when the production of acids is excessive (as in diabetes mellitus, or when mineral acids are given by the mouth or injected into the blood-stream), the amount of ammonia in the urine is high compared with the average figure; on the other hand, under the opposite conditions—namely, excess of alkali, either in food or given as such—the urinary ammonia is low or actually non-existent.

We have now to consider where this ammonia arises and from what it is formed. In more recent times the increasing accuracy of quanti-

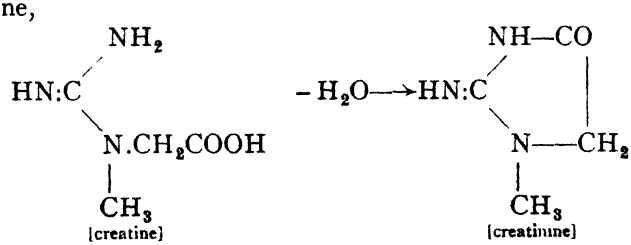
tative methods has shown the amount of blood ammonia to be exceedingly low (0·07 mg. per cent.). It has been considered that this ammonia plays very little part in the neutralisation of the acids excreted by the kidneys. Once that fact was realised it was soon found that the kidney itself possessed the ability of producing the ammonia necessary for the neutralisation of the acids which it has to excrete. As regards the second part of our question there is as yet no entire agreement. Some believe, on the one hand, that the kidney possesses a " urease " system whereby it can obtain its ammonia by the decomposition of the urea which reaches it from the blood, whilst others are equally emphatic that the organ can, like the liver, deaminise amino-acids and thus produce ammonia. Hence we must regard the problem of the precursor of the urinary ammonia as being far from settled.

CREATINE AND CREATININE

Creatine is a constant constituent of muscle, being present therein to the extent of 0·4 per cent. It closely resembles in structure and behaviour the amino-acid arginine. The latter compound, it will be remembered, may be hydrolysed by barium hydroxide to urea and a second substance, ornithine. Creatine on hydrolysis also yields urea, but here the other product is sarcosine (methyl-glycine). Like arginine, therefore, it can be regarded as a derivative of guanidine; in fact, it is methyl-guanidine-acetic acid.



On boiling with acids it undergoes dehydration and yields creatinine,



In muscle it has been found linked with phosphoric acid. The compound is phosphagen (creatine-phosphoric acid) which plays an important part in muscular activity (see p. 249).

Creatine is absent from normal urine, but it is present in the urine

of infants; also 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.

For long, attempts to substantiate the view that urinary creatinine had its origin in creatine yielded no decisive answer. Finally Benedict and Osterberg showed that if small quantities of creatine were fed to a dog over a long period (their experiment lasted seventy days) the excretion of creatinine, which first remained constant, ultimately increased. Apparently there is a period of storage within the organism before the conversion occurs.

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 have 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 view of E. Mellanby may be followed as a 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 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 this organ is responsible for creatinine formation.

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.

FIG. 47.—Uric acid crystals.

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 : 15,000 and at 18°, 1 : 39,500. The forms which uric acid assumes when precipitated from human urine, either by the addition of hydrochloric acid or in certain pathological conditions vary, the most frequent being the whetstone shape; there are also bundles of crystals resembling sheaves, barrels, and dumb-bells (see Fig. 47).

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*.

Although uric acid does not contain the carboxyl (COOH) group typical of organic acids, yet, as its name indicates, it is capable of forming salts with bases. It is a weak acid and forms two series of salts, *i.e.*, the acid (*primary*) salts and neutral (*secondary*) salts.

The secondary salts, however, only exist in the solid condition or

in the presence of strong alkali. Water decomposes them at once into primary salt and alkali; by carbon dioxide 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.

The quantity of uric acid excreted by an adult daily varies from 7 to 10 g. (0·5 to 0·75 gram). The method used for estimating uric acid is based on the discovery made by Hopkins, that when urine is saturated with ammonium chloride, and then made strongly alkaline with ammonia, 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 by titrating it with standard potassium permanganate.

Origin of Uric Acid.—It has long been known that when the kidneys of an animal are removed, uric acid continues to be formed in the body, and accumulates in such organs as the liver and spleen. The credit for proving conclusively that the liver is the organ concerned in the destruction of uric acid in the dog lies with Mann and his colleagues. These workers used much the same technique as that already described on p. 290 in connection with their investigation into the formation of urea.

In mammals, uric acid is the chief end-product of the katabolism of cell nuclei or of nucleic acid, of the principal constituents of the nuclei (p. 70).

There is also a certain amount of evidence recently accumulated which indicates that there may be some metabolic relationship between arginine and histidine on the one hand, and uric acid on the other.

The mention of uric acid leads us to a study of the **Purine Bases,** of which class of substances it is a member.

Emil Fischer showed that among the decomposition products of nuclein are derivatives of a substance he named purine. The empirical formulæ for purine, the purine bases, and uric acid are as follows:—

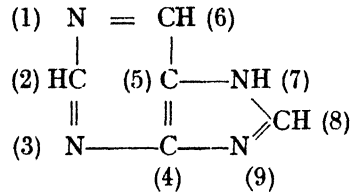
Purine . .	$C_5H_4N_4$		
Hypoxanthine .	$C_5H_4N_4O$	Monoxypurine	Purine bases.
Xanthine . .	$C_5H_4N_4O_2$	Dioxypurine	
Adenine . .	$C_5H_3N_4.NH_2$	Amino-purine	
Guanine . .	$C_5H_3N_4O.NH_2$	Amino-oxypurine	
Uric acid . .	$C_5H_4N_4O_3$	Trioxypurine	

The above list includes the chief purine derivatives which we shall

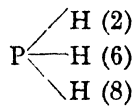
encounter in our more immediate studies. Nevertheless it does not exhaust by any means the number of all such substances of physiological interest. Of these, some are of importance because they occur as various dietary constituents, and in this connection mention may be made of theophylline (dimethyl-xanthine), theobromine (also a dimethyl-xanthine), caffeine (trimethyl-xanthine); these latter occur in tea, cocoa, and coffee.

We may now trace the various relationships which exist between the various members of the above table.

Purine.—As the table shows, purine may be regarded as the parent compound of all these substances. It has never been discovered in the animal organism. Its structural formula is

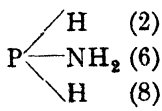


or, in so far as the purines of chief physiological importance have the hydrogen atoms in positions 2, 6, 8 replaced by other atoms or groups, we might represent the purine molecule shortly thus:—



the hydrogen atoms being attached to the atoms of the nucleus occupying the positions indicated by the figures in brackets.

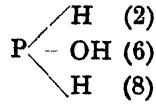
Adenine.—This compound is found in tissues, blood, and urine. Chemically it is 6-amino-purine. Hence its shortened formula is



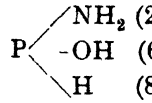
The reactive group in this compound is the amino-group. Under the deaminising action of the enzyme *adenase*, also present in the animal body, this group is replaced by an (OH), and there is formed

Hypoxanthine.—This compound, like adenine, is found in the

tissues, in blood, and in urine. From its mode of formation its formula is

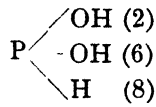


Guanine.—Like adenine, this is an enzymic decomposition product of the nucleins. Combined with calcium it gives the brilliancy to the scales of fishes. Is also found in the bright tapetum of the eyes in these animals. It is a constituent of guano, and is probably derived from the fish eaten by marine birds. It is 2-amino-6-oxypurine, and hence its formula (according to the scheme adopted) is



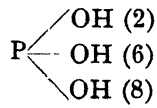
Guanine suffers a like fate to adenine. It is acted on by the deaminase, *guanase*, and yields

Xanthine, or 2, 6-dioxypurine whose shortened formula is

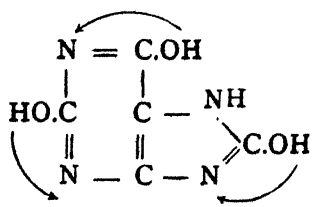


In the body it is found together with hypoxanthine. The relationship of these two compounds is even closer, for both are oxidised by an enzyme, *xanthase*, present in man, to

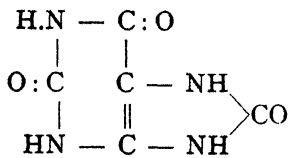
Uric Acid.—The relationship of this compound to purine is indicated by its chemical name, 2, 6, 8-trioxypurine, and may be represented thus:



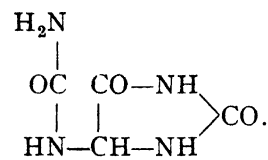
Hence the complete structural formula is



Uric acid is a typical example of a compound exhibiting tautomerism. The above formula represents one modification. Under certain conditions the hydrogen atoms of the hydroxyl groups wander in the direction of the arrows, when a second modification is obtained. The structural formula of this substance is



Uric acid is the main end-product of purine metabolism in man. This is, however, not true of all animals. In the dog, for example (with the exception of one particular type), all the uric acid produced is oxidised (by the enzyme, uricase, found in the liver) to a simpler substance, **allantoin,** the formula of which is



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, moreover, may be exogenously or endogenously formed. Certain kinds of food (for instance, sweetbreads) increase uric acid intake because they contain nuclein in abundance, or free purine bases. 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 pathologically, as in the various leucæmias. The uric acid that arises from such 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 should be remembered that nuclein metabolism of all cells must contribute to uric acid formation.

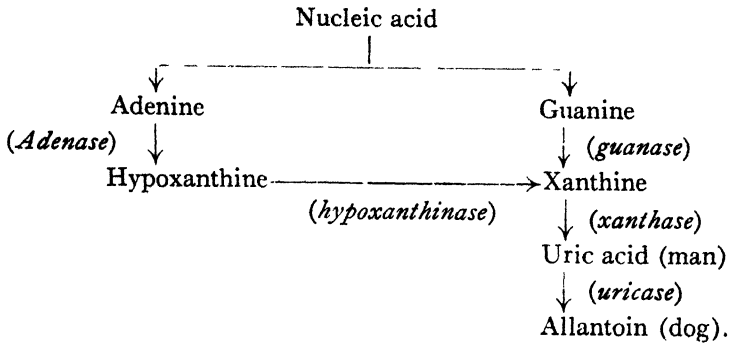
A study of uric acid formation forms a useful occasion on which to allude to the rôle of enzyme action in metabolism generally. Enzymes are not confined to the interior of the alimentary canal; but most of the

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 *glycogenase* which enables the liver cells to turn glycogen into sugar is the one which has been known longest. The enzyme *arginase* (see p. 52) which hydrolyses arginine into urea and ornithine, is one of the more recently discovered. Other examples which may be mentioned are proteoclastic and peptoclastic enzymes (tissue erepsin, etc.) found in many organs.

The formation of uric acid from nuclein is perhaps the best instance of all such enzyme action, 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, but 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 apart the base and carbohydrate; *deaminases* which remove the amino-group from the purine bases so set free. One of these called *adenase* converts adenine into hypoxanthine, while another, called *guanase*, converts guanine 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 the organs (especially the liver) of some animals there is a capacity to destroy uric acid after it is formed, and so such 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 or *uricase*. 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.

The changes intermediate between nucleic acid and certain

end-products, uric acid and allantoin, may be summarised thus :—



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 these latter containing substances belonging to the aromatic series. If, for example, either benzoic acid or a benzoate is administered to man, combination with glycine occurs with the elimination of one molecule of water and the resulting hippuric acid is excreted in the urine :—

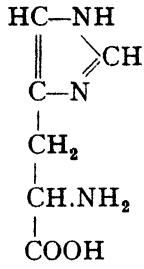
$$\underset{\text{[benzoic acid]}}{C_6H_5COOH} + \underset{\text{[glycine]}}{\begin{matrix}CH_2NH_2\\|\\COOH\end{matrix}} \longrightarrow \underset{\text{[hippuric acid]}}{\begin{matrix}CH_2NH.CO.C_6H_5\\|\\COOH\end{matrix}} + \underset{\text{[water]}}{H_2O}$$

This is another well-marked instance of a protective synthesis (or conjugation) carried out in the animal body, and experimental investigation shows that it is accomplished by the cells of the kidney itself. For if a mixture of glycine, benzoic acid, and defibrinated blood is perfused through a surviving kidney (or mixed with a minced kidney just removed from the body), the two former are found to have been condensed to hippuric acid.

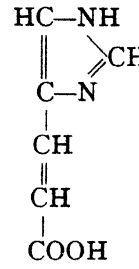
Hippuric acid may be crystallised from horses urine by evaporating the urine to a syrup and saturating with hydrochloric acid. The crystals are dissolved in boiling water, filtered, and on cooling the acid again crystallises out. It melts at 186° C., and on further heating gives rise to the odour of bitter almonds.

UROCANIC AND KYNURENIC ACIDS

These two acids are found in the urine of the dog, but are absent from human urine. The former is obviously derived from the amino-acid, histidine, as a comparison of their formulæ will show—

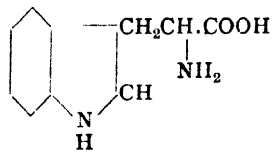


[histidine]

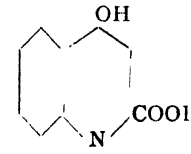


[urocanic acid]

Kynurenic acid has its origin in the amino-acid tryptophan. The formulæ for these two substances are given below :—



[tryptophan]



[kynurenic acid]

AMINO-ACIDS IN URINE

Normal urine contains traces of amino-acids, although the amount is so small as to be detectable only by colorimetric tests. Under certain conditions definite increases of the amount of individual amino-acids are found. For instance, during extensive disintegration of tissue protein, such as occurs in *acute yellow atrophy* of the liver, leucine and tyrosine may be present in excessive quantities. Cystine is another amino-acid which is also sometimes found in increased amounts. When the increased excretion takes place over a period, the condition is termed *cystinuria*. One of the peculiarities of this abnormality is that it runs in families, and this can very well be seen in the following genealogical tree of a cystinuric patient studied by Robson.

From the point of view of protein metabolism the condition is interesting, because, as is now generally accepted, free cystine given

to a cystinuric by the mouth is almost completely oxidised to inorganic sulphate, while protein (containing combined cystine) administered in the same way leads to an increased excretion of cystine in the urine.

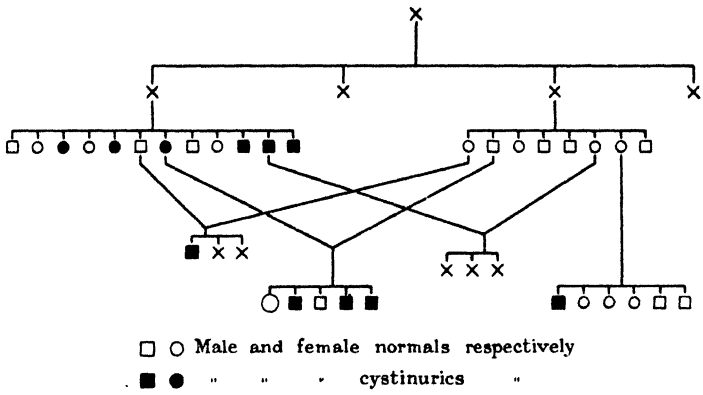
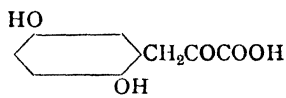


FIG. 48.—Genealogical tree in a cystinuric family. (Robson.)
(From the *Biochemical Journal*.)

Another anomaly of metabolism is *alkaptonuria*. Here the urine, when first passed, has a brownish tint, but subsequently rapidly darkens on exposure to the air. This is due to the presence of the compound *homogentisic acid*.



This compound has its origin in the tyrosine of the diet; apparently alkaptonurics have not the power of fully oxidising tyrosine in the normal way.

Cystinuria, pentosuria, and alkaptonuria have been grouped together by Garrod as " inborn errors of metabolism."

THE URINARY PIGMENTS

The chief pigments normally present in urine are *urochrome*, *urobilin*, and *uroerythrin*.

1. **Urochrome.** — Freshly voided urine contains a colourless substance, *urochromogen*, which gives the Erhlich diazo reaction and which, on oxidation, yields the yellow urinary pigment termed *urochrome* by Thudichum. Urochrome is an acid and reacts acid to

litmus. It contains 11·15 per cent. N and 5·09 S, the latter element not being present in the form of cystine, although most of it is easily split off as sulphide. It shows no absorption bands, but cuts off that violet part of the spectrum which urobilin allows to pass. It does not fluoresce with zinc salts as does urobilin. It yields a pyrrol derivative which is not identical with hæmopyrrol, and so urochrome is probably not related to urobilin. The urochrome pigments are supposed to have their origin in globin and possibly in the tryptophan of that protein. Weiss maintains that on further oxidation it yields uromelanin.

2. **Urobilin.**—Urobilin is a derivative of the blood pigments, and is identical with stercobilin (pp. 171, 177). 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.

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. Urobilinogen is regarded as a complex pyrrol compound. In numerous pathological conditions urobilin is abundant.

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. 49, spectrum 4).

Urobilin, like most animal pigments, shows acidic properties, forming compounds with bases from which it is liberated by 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. 49, 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 if filtered off. When it is redissolved the ordinary band alone is visible.

3. **Uroerythrin (Purpurin).**—This is the colouring matter of pink deposits of urates (p. 299). It may be isolated 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 amyl 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. 49, spectrum 7). It gives a green colour with caustic potash, and a

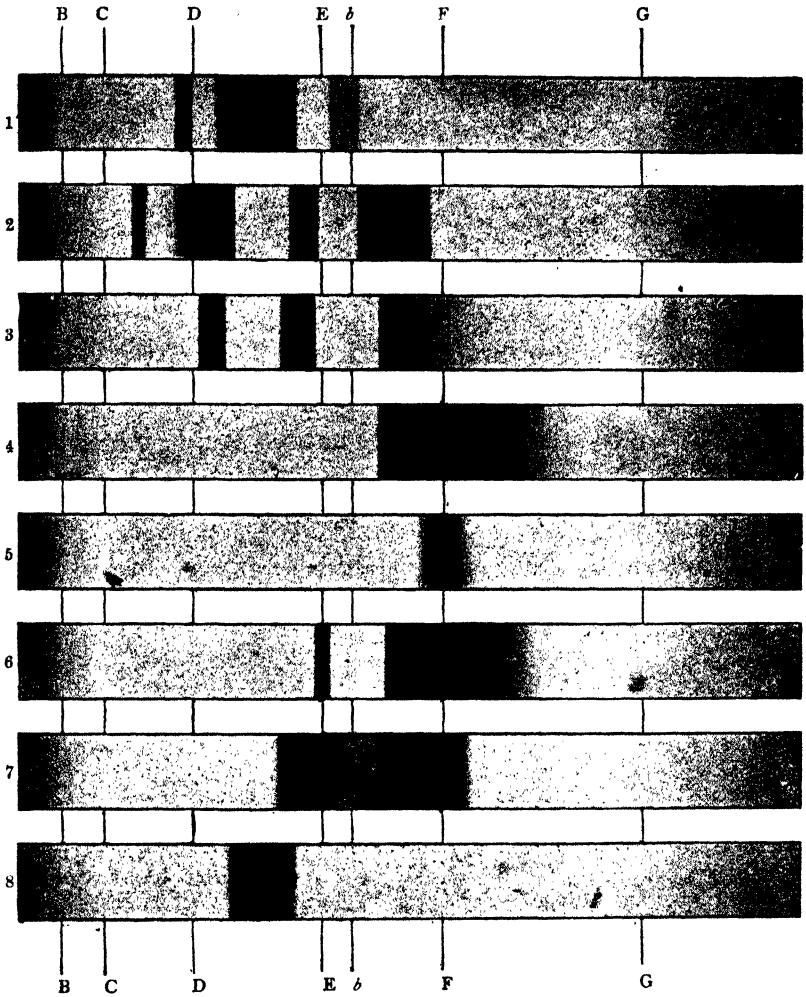


FIG. 49.—Chart of absorption spectra; 1, acid hæmatoporphyrin; 2, alkaline hæmatoporphyrin; 3, hæmatoporphyrin as found sometimes in deposits of urates; 4, acid urobilin, concentrated; 5, acid urobilin, dilute; 6, the E band spectrum of urobilin; 7, uroerythrin; 8, urorosein concentrated—on dilution the band shrinks rapidly from the red end of the spectrum. (After F. G. Hopkins.)

red or pink with mineral acids. Uroerythrin appears to be a small but constant constituent of urine, its amount being increased in fevers

and certain diseases of the liver. 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, which points to the existence of a colourless chromogen. It may be separated from the urine as follows:—Caustic alkali is added to the urine. The phosphates so precipitated carry down the pigment, which may be dissolved out with chloroform. The chloroform is evaporated, the residue washed with alcohol, and finally dissolved in acid alcohol. Urines rich in the pigment yield it easily on shaking with amyl alcohol.

When the urine is sufficiently rich in the pigment, the bands shown are those of alkaline hæmatoporphyrin (Fig. 29, spectrum 11). On adding sulphuric acid, the spectrum of acid hæmatoporphyrin is seen (Fig. 29, spectrum 10). Occasionally urate deposits are pigmented with a form of the pigment which shows two bands in the spectrum, very like that of oxyhæmoglobin (Fig. 29, spectrum 2); 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, others are:—(*a*) *Indoxyl.*—The origin of this substance from indole is mentioned on p. 284. It is easily oxidised to indigo-blue or indigo-red.

$$2C_6H_4\underset{\text{[indoxyl]}}{\langle^{C.OH}_{HN}\rangle CH} + O = C_6H_4\langle^{CO}_{NH}\rangle C : C\underset{\text{[indigo-blue]}}{\langle^{CO}_{NH}\rangle C_6H_4} + 2H_2O.$$

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 mostly excreted as a conjugated sulphate, indican, which resists oxidation. The presence of indican in the urine may be demonstrated by Jaffé's method, which has been described in the practical section (p. 261). (*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 amyl alcohol, but not in ether. The colour is destroyed by alkalis. It shows an absorption band between the D and E lines (Fig. 49, spectrum 8).

6. **Pathological Pigments.** — The most frequently appearing 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 catechol and hydroquinone 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. 307.

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. The majority of these, however, are pathological and are detected chiefly by means of the microscope.

The **chemical substances** are uric acid, urates, oxalates, carbonates, and phosphates. Rarer substances also found 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. 47, p. 299) 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.

Deposit of Urates.—This is much commoner, and may, in a concentrated urine, occur normally 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 urine after it has been passed, which leads to the same result. The chief constituent of the deposit is the primary or monosodium urate.

This deposit may be recognised as follows :—

1. It has a pinkish colour due to the pigment *uroerythrin*, one of the pigments of the urine (p. 308).

2. It dissolves upon warming the urine.

Microscopically it is usually amorphous, but crystalline forms similar to those depicted in Figs. 50 and 51 may occur.

Crystals of calcium oxalate may be mixed with this deposit (see Fig. 52).

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.

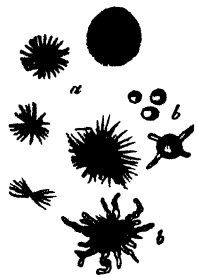


FIG. 50.—Monosodium urate.

FIG. 51.—Mono-ammonium urate.

FIG. 52.—Envelope crystals of calcium oxalate.

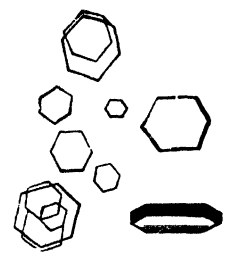


FIG. 53 —Cystine crystals.

Deposit of Cystine. — The amino-acid, cystine, is recognised by colourless six-sided crystals, which appear only in acid urine (Fig. 53). Cystine, in quantity, cannot be regarded as a constituent of normal urine.

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 this be absolutely sterile), will in time become alkaline owing to an enzyme formed during the growth of the *Micrococcus ureæ* which converts ammonium carbonate urea into

$$\underset{\text{[urea]}}{CO(N_2H)_2} + \underset{\text{[water]}}{2H_2O} = \underset{\text{[ammonium carbonate]}}{(NH_4)_2CO_3}$$

The ammonia so formed 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 ammonium-magnesium phosphate, $MgNH_4PO_4 + 6H_2O$: coffin-lids and feathery stars (Fig. 54).

3. Crystalline calcium phosphate, $CaHPO_4$, in rosettes of prisms, in spherules, or in dumb-bells (Fig. 55).

4. Magnesium phosphate, $Mg_3(PO_4)_2 + 2H_2O$, occurs occasionally and crystallises in long plates.

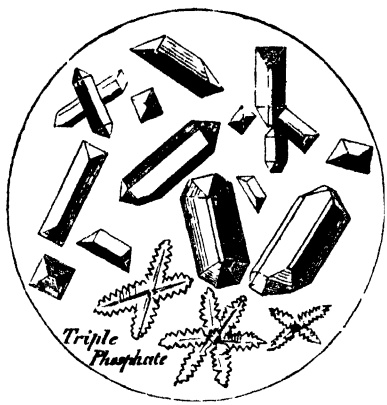


FIG. 54.—Triple phosphate crystals

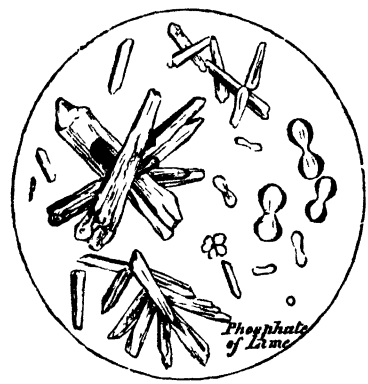


FIG. 55.—Crystals of phosphate of lime (stellar phosphate)

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 (20 per cent.) dissolves magnesium phosphate away from the edges ; it has no effect on the triple phosphate. A calcium phosphate ($CaHPO_4 + 2H_2O$) may occasionally be deposited in acid urine. Pus in urine is apt to be mistaken for phosphates, but can be distinguished microscopically.

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. 282). 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. 47).

Urates. — Generally amorphous. The primary urate of sodium (Fig. 50) and of ammonium (Fig. 51) may sometimes occur in star-shaped clusters of needles or spheroidal clumps with projecting spines. Tinged brick-red. Soluble on warming.

Calcium Oxalate.—Octahedra, so-called envelope crystals (Fig. 52). Insoluble in acetic acid.

Cystine.—Hexagonal plates (Fig. 53). Rare.

Leucine and Tyrosine.—Rare (pp. 49, 50).

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. 43 and 54).

Calcium hydrogen phosphate, $CaHPO_4$. Rosettes, spherules, or dumb-bells (Fig. 55).

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 (pp. 49, 50).

ABNORMAL CONSTITUENTS OF URINE

Note on the Use of the Term " Pathological."—Although the term " pathological " has been used in the practical section, it must be remembered that in many cases no strict line of demarcation in the qualitative sense can be drawn between the constituents of physiological and pathological urines. To quote an instance—it has been shown that the urine of patients suffering from diabetes mellitus may contain, among other compounds, acetone, aceto-acetic acid, etc. Nevertheless the absurdity of calling all urine which may contain acetone, etc., pathological, is evident when it is remembered that such compounds are present in the urine of a person on a carbohydrate-free diet, or of a person even after a relatively short period of fasting, the compounds being the result of a normal physiological process, and are excreted simply because the next step of their oxidation to carbon dioxide and water does not take place under such conditions. The

difference between " normal " and " pathological " is quantitative rather then qualitative.

PROTEINS IN THE URINE

There is little or no protein 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 section of this chapter. 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 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 so little sugar that it is not recognisable by ordinary tests, and therefore for clinical purposes it may be considered absent. It occurs in the urine in diabetes mellitus, and can be artificially produced as briefly referred to on p. 239.

The methods usually adopted for detecting and estimating the sugar have been given in the practical section. Almost invariably the sugar present is glucose. Lactose may occur in the urine of pregnant women and nursing mothers. Sucrose and fructose may be found after excessive amounts of these sugars have been consumed.

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 (p. 262). 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. Glycuronic

acid ($C_6H_{10}O_7$), if present, is also likely to be confused with sugar by Fehling's test. This acid, possibly an oxidation product of glucose, does not occur free in the urine, but always in combination with such substances as phenol, cresol, indoxyl, etc. Traces of these are found in normal urine, and are the result of yet another method of protective synthesis (conjugation), of which we have already seen examples in the case of ethereal sulphates and hippuric acid. The excretion of glycuronic acid may be increased in varying degree under certain conditions, for example, (1) in cases of intestinal stasis; (2) in diabetes mellitus; and (3) especially after the administration of drugs such as chloral, camphor, salicylates, sulphonal, morphine. Glycuronates are distinguished from glucose by the fact that they are not fermentable by yeast.

The best confirmatory tests for reducing sugars are the formation of the phenyl-osazone (p. 7) 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. 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 oxy-hæmoglobin are seen.

If only a small quantity of blood is present, the urine—especially if acid—has a characteristic reddish-brown colour, termed " 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 form of methæmoglobin mixed with more or less oxyhæmoglobin. These substances are usually identified spectroscopically.

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 certain white blood corpuscles, in which, in fact, they originate. 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 test 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.

COLORIMETRY

From his studies of inorganic and organic chemistry the student will have learned that the presence of a substance in a solution can be **detected** by its reacting with a reagent to produce (*a*) a precipitate, (*b*) a colour, or (*c*) an evolution of gas of characteristic properties. Moreover he will have learned various methods of estimating elements or compounds by, for example, (*a*) the collection, drying, and weighing of a precipitate, or (*b*) the collection and measurement of a volume of gas, or (*c*) some method involving titration with standard alkali or acid, oxidising or reducing agent, etc.

In the practical section of this chapter, however, he is introduced to another method of estimating the amount of a particular substance in solution, *i.e.*, that of measuring the intensity of colour which that substance produces when it reacts with a specific reagent. The intensity of colour produced will depend on the concentration of the substance in solution. It will at once be evident that a requisite of such a method is a standard intensity of colour with which the colour produced by such means may be compared. Theoretically the most satisfactory standard is one prepared from a solution of the substance to be estimated, treated in exactly the same way as the unknown solution. Other standards, however, are sometimes available, *e.g.*, a particular dye in solution or " fixed " in a glass plate.

Beer's law, upon which colorimetry is based, states that light in passing through a coloured medium is absorbed in direct proportion to the concentration of the coloured substance. It follows that the

intensity of the observed colour is **directly proportional to the concentration** of the pigment in solution and **inversely proportional to the depth** of solution through which the light passes. In other words a column 20 mm. long of a 1 per cent. solution of dye should transmit the same intensity of colour as one 10 mm. long of a 2 per cent. solution of dye.

Hence if L_1 and L_2 are the lengths (read on the colorimeter

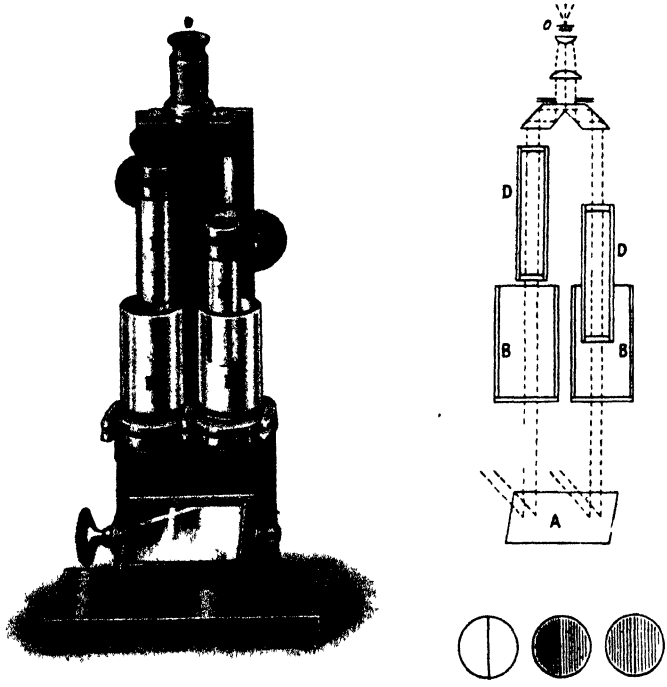


FIG. 56.—Colorimeter.

scale) of the unknown and standard solutions and C_1 and C_2 their respective concentrations, then

$$\frac{C_1}{C_2} = \frac{L_2}{L_1}$$

or C_1 (concentration of unknown) $= C_2$ (concentration of known) $\times \frac{L_2}{L_1}$.

The actual comparison of colour intensities can be done, as Benedict and others originally did it, in graduated test-tubes, diluting one or other of the solutions with water until a match is obtained.

Modern practice, however, makes use of the colorimeter, a form of which is shown in Fig. 56. The solutions to be compared are placed in cups B and B, the standard in one cup, the unknown in the other. The plungers are lowered, by means of the rack and pinion, into the solutions, that dipping into the standard being fixed at a given height which can be read by means of the scale on the back of the instrument.

Equality of colour tone in both hemispheres of the field seen through the eye-piece O is attained by raising or lowering the plunger dipping into the unknown solution. Then reference to the scale will give the length of the column of the unknown liquid lying below its plunger. The calculation of the concentration of the unknown solution is made according to the formula given above.

CHAPTER XI

DETECTION OF SUBSTANCES OF PHYSIOLOGICAL IMPORTANCE

Subsequent information may be very usefully employed in testing for the various substances the properties of which have been previously studied.

The student should realise that to attempt to memorise, however perfectly, the following or any other detailed scheme is misdirected energy and waste of time. Only by acquiring a thorough knowledge regarding the physical properties and chemical reactions of the substances sought for can their identification be effected.

1. *Preliminary Examination.*

A. If the substance is solid, heat a small portion of it on a piece of porcelain. Carbohydrates (p. 5), fats (p. 29), proteins (p. 41), urea (p. 286), and uric acid all give characteristic odours on being heated, and this often gives a useful lead as to the course the subsequent examination should follow. Prolong the heating to burn off most of the carbonaceous matter and notice if a residue remains. Extract the carbonaceous matter with water. Utilise the solutions so obtained for detecting the presence of Na, K, Ca, Cl, SO_4, etc.

B. If the solution is a liquid.

(*a*) Note its reaction, colour, clearness or opalescence, taste, smell. Coloured liquids suggest blood, bile, urine, etc. Opalescent liquids suggest starch, glycogen, or certain proteins.

(*b*) Evaporate a small quantity of it to dryness on a boiling-water bath or carefully over a flame. Note any odour given off. If a residue remains, submit it to the preliminary tests mentioned above (A).

2. To a neutral or faintly acid solution 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. 8. **Remember: the iodine test for starch, etc., fails in alkaline solutions, or if the solution be warm.**

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 pp. 5, 8).

(*b*) Blue solution: no reduction on boiling; boil some of the original solution with 10 per cent. hydrochloric acid, make the solution neutral by the addition of sodium hydroxide and then test with Fehling's or Benedict's solutions. Again boil the mixture; abundant yellow or red precipitate: Sucrose. Confirm by HCl test (see p. 7).

4. To the solution under examination, add two drops of 1 per cent. copper sulphate solution and then sodium hydroxide.

(*a*) Violet solution: **Proteins** (albumins, globulins, metaproteins). In presence of magnesium sulphate the potash causes also a white precipitate of magnesia.

(*b*) Pink solution; biuret reaction. **Peptones** or **proteoses.** In presence of ammonium sulphate very large excess of potash is necessary for this test.

5. 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.**

6. 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.**

7. 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.**

8. 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.

9. To a fresh portion add nitric acid:

(*a*) No precipitate, even though excess of sodium chloride is also added: **Peptone.**

(*b*) Precipitate which disappears on heating and reappears on

cooling: **Proteoses.** This is a distinctive test for proteoses, and is given by all of them.

(*c*) Precipitate disappearing on longer heating and not reappearing on cooling: **Native Protein.**

In (*a*), (*b*), and (*c*) when a benzene ring is present nitric acid *plus* heat causes a yellow colour turned orange by the addition of concentrated ammonia (xanthoproteic reaction).

10. Confirmatory tests for proteins:—

(*a*) Millon's test (p. 44).

(*b*) Adamkiewicz's reaction or, better, Rosenheim's modification (p. 44).

(*c*) To test for fibrinogen:—
- (i) It coagulates by heat at 56° C.
- (ii) It is changed into fibrin by thrombin 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.
- (iii) Boil with Neumann's mixture (p. 42) and test resulting solution for phosphate.

11. **If blood** (or derivatives of hæmoglobin) is suspected:—

If solid, make a solution in water:—(*a*) Note colour (red or brown) and reaction to litmus paper. Then examine spectroscopically (diluting if necessary) according to following scheme:—

- Red
  - Acid—hæmatoporphyrin (two bands).
  - Neutral
    - two bands between D and E lines; add a reducing agent.
      - Bands remain—CO-hæmoglobin.
      - One band replaces the two—oxyhæmoglobin.
  - Alkaline—reduced hæmatin (two bands) unaltered by reducing agent.
- Brown
  - Acid—acid oxyhæmatin (band in red).
  - Neutral—methæmoglobin (gives HbO_2, then reduced Hb on reduction with $(NH_4)_2S$).
  - Alkaline—alkaline oxyhæmatin (gives reduced hæmatin on reduction).

The best reducing agent to employ in the foregoing tests is sodium hydrosulphite (p. 183), except in the case of methæmoglobin; here warming with ammonium sulphide is the best.

(*b*) Heat a few drops with glacial acetic acid and a crystal of

sodium chloride on a glass slide under a cover glass, repeat if necessary. When cold, hæmin crystals are seen.

(*c*) Try guaiacum test and Adler's benzidine test (p. 180).

(*d*) If the blood is old and dry, and its hæmoglobin converted into hæmatin:

(i) Try hæmin test.
(ii) Extract with water and try guaiacum test and Adler's test.
(iii) Dissolve it in potash, add reducing agent, and examine for spectrum of reduced hæmatin.

12. **If bile** is suspected:

(*a*) Try Gmelin's test for bile pigments (p. 157); also Cole's test (p. 263).

(*b*) Try Pettenkofer's and Hay's tests for bile salts (pp. 157, 263).

13. 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. 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 usually does not give the xanthoproteic test. On heating it does not coagulate, but dissolves. 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 small portion, add strong nitric acid, and examine for crystals of urea nitrate. Solid urea heated in a dry test-tube gives off ammonia, and the residue is biuret, which gives a rose-red colour with copper sulphate and caustic potash. For the specific test for urea (p. 259).

(*e*) Uric acid. Very insoluble in water hot or cold; soluble in alkali, and precipitated in crystals from this solution by hydrochloric acid. Uric acid crystals precipitated from urine are deeply pigmented red. Try the murexide, Folin's and Schiff's tests (p. 259).

(*f*) **Cholesterol.** Characteristic flat crystalline plates. For various colour tests (p. 31).

14. **Urine.** Normal constituents.

(*a*) Chlorides. Acidify with nitric acid; add silver nitrate; white precipitate soluble in strong ammonia.

(*b*) Sulphates. Acidify with nitric acid or hydrochloric acid; add barium chloride; white precipitate.

(*c*) Phosphates. Acidify with nitric acid; add ammonium molybdate; boil; 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; pigmented crystals of uric acid are deposited. For tests see above. A more rapid test is to saturate with ammonium sulphate, collect the precipitate on a filter, and test it for uric acid.

(*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 (p. 305).

(*g*) Creatinine. For colour tests (p. 261).

15. **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, Cole's, Hay's, and Pettenkofer's tests.

(*d*) *Pus.* White deposit. Microscope (pus cells). Add potash; it becomes stringy. Benzidine test is positive before, negative after, boiling.

(*e*) *Albumin.* (i) If acid, precipitated 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*) *Glucose.* (i) Reduces Benedict's and Fehling's solution. (ii) Add picric acid and potash; boil; the urine becomes a dark opaque red; the similar slight coloration in normal urine is due to creatinine. (iii) The urine has a high specific gravity. (iv) Ferments with yeast. (v) Brown colour on heating with alkali (Moore's test).

(*g*) *Aceto-acetic acid and acetone.* For tests (p. 262).

(*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.

16. **Enzymes.**

If the presence of an enzyme is suspected, prepare various substrates such as :—

(*a*) Fibrin suspended in 0·2 per cent. hydrochloric acid.

(*b*) Fibrin suspended in 0·5 per cent. sodium carbonate.

(*c*) 1 per cent. starch paste in water.

(*d*) Boiled milk coloured with phenolphthalein.

(*e*) 2 per cent. urea containing a small amount of phenolphthalein (p. 259).

To each of the above add some of the suspected material, and to each prepare a control in which the suspected material has been boiled prior to addition. Incubate at 37° for half an hour, and then examine.

If protein hydrolytic products are formed in (*a*), pepsin is present; if in (*b*), trypsin is present. Test (*c*) with iodine; if there is no coloration, then an amyloclastic enzyme is present—ptyalin or amylase. If (*d*) is clotted, a rennet-like enzyme is present. If the milk is decolorised, then lipase is present. If (*e*) becomes coloured, urease is present. In each case the control tube must not show any indication of a change occurring.

APPENDIX

IMPORTANT DATA

H-ion Concentration, etc.

P_H of 0·1 HCl	1·0		
0·1 NaOH	13·0		
M/3 NaH_2PO_4	4·5		
M/3 Na_2HPO_4	9·1		
Saliva	6·2—7·6	Opt. P_H of Ptyalin	6·7
Gastric Juice	1·2—4·5	Pepsin	1·2
Duodenal Contents	7·7	Trypsin	8·1
		Erepsin	7·8
		Steapsin	8·0
Blood	7·25—7·45		
Urine	5·5—8·0		

Iso-electric Point

of Casein	4·7
Egg-albumin	4·8
Hæmoglobin	6·8

Constituents of Blood (per 100 c.c.).

Glucose	70—100 mg.
Total Protein	6·5—8·2 gm.
Hæmoglobin	15·6 gm.
Total Nitrogen	3·0—3·7 gm.
Non-protein Nitrogen	25—35 mg.
Urea Nitrogen	10—15 mg.
Ammonia Nitrogen	0·1—0·2 mg.
Uric Acid	2—3·5 mg.
Creatine	3—7 mg.
Creatinine	1—2 mg.
Undetermined Nitrogen	4—18 mg.
Total Fatty Acids	290—420 mg.
Cholesterol	150—190 mg.
Chlorides (as NaCl)	450—500 mg.
Calcium (serum)	9—11 mg.

Constituents of Blood (per 100 c.c.)—*continued.*

Magnesium (serum)	2—3 mg.
Sodium (serum)	330 mg.
Potassium (serum)	16—22 mg.
CO_2 capacity (plasma)	55—75 vols. per cent.
CO_2 content (arterial blood)	45—55 ,, ,,
CO_2 ,, (venous blood)	50—60 ,, ,,
O_2 capacity	16—24 ,, ,,
O_2 content (arterial blood)	15—23 ,, ,,
O_2 ,, (venous blood)	10—18 ,, ,,

Blood Volume—5·6 litres; approx. 1/12—1/14 of body-weight.

Constituents, etc., of Urine.

Volume	1200—1800 c.c.
Sp. Gr.	1·015—1·025
Total Solids	45—90 gm.
Total Nitrogen	12—18 gm.
Urea	30—35 gm.
Ammonia	0·75 gm.
Uric Acid	0·5—1·0 gm.
Creatinine	1·0—1·5 gm.
Amino-acid Nitrogen	0·1—0·2 gm.
Chloride (as NaCl)	10—15 gm.
Phosphate (as P_2O_5)	2·5 gm.
Sulphur (as SO_3)	1·5—3·0 gm.
Sodium	3·0—4·5 gm.
Potassium	1·5—2·5 gm.
Glucose	0·1 gm.

Factor for conversion of Nitrogen to Protein, 6·25.

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