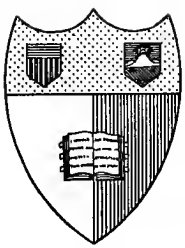

ALLEN'S
COMMERCIAL ORGANIC ANALYSIS

FOURTH EDITION REWRITTEN AND REVISED

EDITED BY HENRY LEFFMANN, M. A., M. D., PROFESSOR OF CHEMISTRY AND TOXICOLOGY IN THE WOMAN'S MEDICAL COLLEGE OF PENNSYLVANIA; W. A. DAVIS, B. Sc., A. C. G. I., FORMERLY LECTURER AND ASSISTANT IN THE CHEMICAL RESEARCH LABORATORY, CITY AND GUILDS COLLEGE, IMPERIAL COLLEGE OF SCIENCE AND TECHNOLOGY, LONDON; AND SAMUEL S. SADTLER, S. B., VICE-PRESIDENT OF THE AMERICAN ELECTRO-CHEMICAL SOCIETY; MEMBER AMERICAN INSTITUTE OF CHEMICAL ENGINEERS.

IN many respects this edition of Allen is a new work. The field of Commercial Organic Analysis has been so enlarged and specialised during the last few years that it has been found necessary to rewrite many parts and add much new matter. Obsolete methods are omitted; what little of the old text remains has been carefully revised and many new illustrations added.

To accomplish the object in view, namely, the furnishing of a modern work of the greatest practical value to the analyst, it was deemed advisable to secure the services of an English and an American editor and to organise a corps of writers particularly versed in the subjects discussed.

The general arrangement of the volumes remains as before, only such changes have been made as will bring the text into line with the latest scientific classification. Great care has been exercised by the editors and contributors in the choice of methods and only those of the highest degree of accuracy and rapidity selected. Effort has been made to secure uniformity in weights and measures, nomenclature and abbreviations. References are to original sources, not to translations or abstracts.

The work is issued in eight volumes, numbered consecutively, as follows:—

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VOLUME VIII

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ALLEN'S COMMERCIAL

ORGANIC ANALYSIS

A TREATISE ON

THE PROPERTIES, MODES OF ASSAYING, AND PROXIMATE ANALYTICAL EXAMINATION OF THE VARIOUS ORGANIC CHEMICALS AND PRODUCTS EMPLOYED IN THE ARTS, MANUFACTURES, MEDICINE, Etc.

WITH CONCISE METHODS FOR

THE DETECTION AND ESTIMATION OF THEIR IMPURITIES, ADULTERATIONS, AND PRODUCTS OF DECOMPOSITION

VOLUME VIII

Enzymes, Proteins and Albuminoid Substances, Milk and Milk Products, Meat and Meat Products, Hæmoglobin and Blood, Proteoids, Fibroids

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FOURTH EDITION. ENTIRELY REWIRTTEN

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PREFACE.

In this volume, the last edition of which was issued fifteen years ago, very extensive alterations have been made, the greater part of the volume being entirely new matter. The order of arrangement has, however, been retained for the reasons stated in the earlier volumes. A brief introductory article on *enzymes* has been added.

The methods of separation of amino-acids dealt with under *Proteins* and certain matter included under *Proteins of Digestion* may perhaps be regarded as, in some measure, duplicating material included in Volume VII; but the Editors have advisedly retained this matter in Volume VIII not only because it presents the information from another point of view, but because its insertion was essential for the completeness of these sections.

It is proposed to issue, at a later date, a supplementary volume which shall contain not only a complete reference index of the whole work, but shall bring the latter completely up to date by the incorporation of all processes of analysis and information regarding analytical processes which have appeared in the interval that has elapsed since the revision of the work was first taken in hand.

CONTENTS.

MILK.

HENRY LEFFMANN.

MILK PRODUCTS.

CECIL REVIS AND E. RICHARDS BOLTON.

MEAT AND MEAT PRODUCTS.

W. D. RICHARDSON.

ENZYMES.

By E. FRANKLAND ARMSTRONG, Ph.D., D.Sc., F.C.G.I.

The detection and relative estimation of enzymes is in most cases a matter requiring some experience, particularly in such complicated materials as plant and animal tissues. As a rule, it suffices to demonstrate the action of the enzyme on the particular substance to which it is related, but a control experiment should be made at the same time with the boiled enzyme material. Any change in this is deducted from that observed in the first case.

Enzymes enclosed in tissues with a living cell wall cannot as a rule be extracted until the protoplasmic structure is destroyed. This may be effected in a number of ways:

(1) By drying the material quickly at a low temperature, 20–30°, and if necessary subsequently warming the partially dried material to 50° or more.

(2) By autolysis of the material, usually with the addition of an antiseptic to prevent bacterial action.

(3) By rapid dehydration brought about by stirring with absolute alcohol or acetone.

(4) By the mechanical disintegration of the living tissue. This is effected in a Buchner press or by rotating the cells very rapidly with sand. Soft organisms like bacteria can be hardened by freezing with liquid air and then ground.

In general, aqueous or glycerol extracts of the materials, prepared in some such manner as described, are used as sources of enzymes. In special cases the solid material is used directly. The extracts may be purified from crystalloids by dialysis. The dissolved enzyme can be precipitated with alcohol or acetone, washed with alcohol and ether and dried in a vacuum, and is thus obtained as a soft colourless powder. The process generally entails a loss of activity.

The better studied enzymes are:

Sucroclasts..... Invertase, lactase, maltase
Amyloclasts..... Diastase
Glucosidoclasts... Emulsin, myrosin[1]

[1] See vol. 7, art. *Glucosides*.

Proteoclasts..... Pepsin, trypsin, erepsin, rennin
Lipoclasts.....Lipase
Oxydases .....Laccase,[1] tyrosinase, peroxydase, catalase

Enzymes being measured by their activities, it is fundamental that the amount of enzyme shall be proportional to the change produced. The numerous investigations on the kinetics of enzyme action have established that this relation only holds when the amount of enzyme is relatively small compared to the amount of the substance on which it acts and, further, the change must not exceed the conversion of about 30 or 40% of the original substance. The latter part of this statement, which virtually includes the first part also, is commonly referred to as Kjeldahl's law of proportionality. Kjeldahl stated originally, that the amount of reducing sugar formed by the action of malt extract upon an excess of starch was a true measure of the diastatic power, so long as the digestion was not carried beyond the point corresponding to 40% conversion of the starch. He showed that up to this point the velocity curve could be expressed by a straight line.

Enzymic activity may be expressed as (1) the amount of change produced by a given amount of enzyme in a given time, (2) the amount of enzyme required to produce a given change in a given time, or (3) the time required for a given amount of enzyme to effect a given change. In addition to these three factors the temperature of the change and all other conditions must be maintained constant, particularly those likely to introduce alkaline impurities, *e.g.*, the use of ordinary glass apparatus. Lastly, the hydrolyte must either be chemically pure or such bulk of it be acquired as will suffice for a large number of comparative experiments.

Neglect of these precautions in the case of diastase or invertase may lead to altogether false values.

In investigating extracts of leaves or plant material for enzymes the influence of tannin in rendering the enzymes inactive must not be overlooked. Brown and Morris (*Trans.*, 1893, **63**, 604) have shown that the comparatively weak diastatic action of some leaves is due to the tannin they contain. The writer has found this to apply to emulsin and other enzymes including the oxydases. The tannin also interferes with the estimation of sugars by Fehling's method. It is precipitated from the leaf extract by means of basic lead acetate; the solution is filtered, the volume of the filtrate noted and the lead precipitated by

[1] Since the specific nature of the oxydases is most uncertain, it is preferable to use the general term oxydase instead of laccase.

hydrogen sulphide. The precipitate is filtered off, washed, the filtrate evaporated to remove hydrogen sulphide and the residue made up to the original volume.

Diastase (Amylase).—This enzyme is technically of greatest importance and is the one most often examined in analytical practice. Pancreatin, malt diastase and taka diastase are official pharmaceutical preparations. Malt extracts are largely used by bakers and in the textile industry for which purpose a high diastatic power is required. The importance of diastatic power determinations in the case of malt has already been dealt with (Vol. 1, page 136).

The methods in use are of two kinds: (1) the saccharification methods in which the maltose produced by the enzyme acting on an excess of soluble starch is measured; (2) the liquefaction methods in which the power of the enzyme to form products which no longer give a colouration with iodine solution, is determined.

Lintner Value.—It is usual to estimate the amount of enzyme necessary to produce a given change, the temperature, time and concentration of the substrate being fixed. The simplest and most rapid process is to ascertain when starch is absent by the iodine test, but it is more accurate to estimate the maltose formed.

This process is carried out as described in Vol. 1, p. 137. A series of test-tubes each receives 10 c.c. of a 2% solution of soluble starch and progressively increasing quantities of enzyme solution—0.1, 0.2 c.c., etc. After an hour 5 c.c. of Fehling's solution (Vol. 1, p. 318) are added to each tube and after mixing they are heated in the water bath for 10 minutes. On examination one of the tubes will prove to be colourless, those on either side of it being faintly blue or yellow. 0.1 c.c. of enzyme is taken as having a diastatic power of 100, when the maltose it produces is equivalent to 5 c.c. of Fehling's solution. If x c.c. of enzyme were required in the experiment the diastatic power is $\frac{0.1 \times 100}{x}$.

The procedure adopted by the malt analysis committee of the Institute of Brewing is given in Vol. 1, p. 136.

Sherman, Kendall and Clark[1] (*J. Amer. Chem. Soc.*, 1910, **32**, 1073) have submitted the older methods to careful criticism; when testing commercial preparations of pancreatic diastase they find that most of them give unsatisfactory results. Apparently these pancreatic preparations are too poor in electrolytes to function normally when caused to act upon pure starch dissolved in pure water. Accordingly sodium chloride and disodium phosphate are always added so as to

[1] The recent work of these authors on amylase, *J. Amer. Chem. Soc.*, 1910, **32**, 1087; 1911, **33**, 1195, is of very great importance in this field.

obtain the optimum activity of the enzyme. The results so obtained were about twenty times as high as when no electrolyte was added.[1]

Their procedure is as follows: pure soluble starch and water are used at 40°; 0.3 grm. of sodium chloride and 7 c.c. of $N/5$ disodium phosphate are added per 100 c.c. of reaction mixture. 100 c.c. of 2% soluble starch are digested with the enzyme solution for exactly 30 minutes.

All the vessels and solutions are preheated at 40°. Several experiments may be started simultaneously with varying proportions of enzyme. At the expiration of the time 50 c.c. of Fehling's solution is added, the flask immersed in a bath of boiling water for 10 minutes and the reduced copper determined by any of the usual methods. The weight of cuprous oxide must not exceed 300 mg.; it is corrected by the value found in a blank without the enzyme.

By plotting the velocity curve of diastatic action, which is not a straight line, with time as abscissæ and yield of reducing sugar as ordinates, a scale is obtained which permits of an expression of true diastatic power based on the weight of cuprous oxide obtained. 300 mg. of cuprous oxide is taken as 100 on this scale and the corresponding scale values of K are given in the following table.

Cuprous oxide		Cuprous oxide		Cuprous oxide		Cuprous oxide	
Mg.	K	Mg.	K	Mg.	K	Mg.	K
30	9.1	100	31.2	170	54.1	240	78.3
40	12.2	110	34.4	180	57.5	250	81.8
50	15.3	120	37.6	190	60.9	260	85.4
60	18.4	130	40.9	200	64.3	270	89.0
70	21.6	140	44.2	210	67.8	280	92.6
80	24.8	150	47.5	220	71.3	290	96.3
90	28.0	160	50.8	230	74.8	300	100.0

The values of K are divided by the respective weights of enzyme. When any other time is used the standard time, 30 minutes, is divided by the actual time in minutes and multiplied by the value of K. Thus in 30 minutes 0.15, 0.30, 0.45, 0.60 mg. of pancreatin yielded respectively 76, 147, 217, 286 mg. of cuprous oxide, whence $K = 23.5$, 46.5, 70.2 and 94.8; then the diastatic power is 156, 155, 156, 158 units.[2]

Iodine Methods.

Robert's Method (*Proc. Roy. Soc.*, 1881, **32**, 145).—In this the time taken at 40° to convert a 1% starch solution into achroodextrin, the first point at which no colour is given by iodine solution, is measured.

[1] The acceleration is more probably due to the effect of the acid phosphate in neutralising alkaline impurities.

[2] See Appendix.

This is known as the achromic point; the time taken to reach it must be between 2 and 10 minutes, failing which, the quantity of enzyme solution must be suitably altered.

Five cubic centimetres of enzyme solution and 5 c.c. of starch solution are warmed separately to 40° and mixed at a given time; the mixture is tested with iodine every minute until the achromic point is reached. The method is very rapid though less accurate than the others described.

Modifications of this method have been described by Francis (*Bulletin Pharmacy*, 1898, **12**, 52), Takamine (*J. Soc. Chem. Ind.*, 1898, **17**, 118, 437) and Johnson (*J. Amer. Chem. Soc.*, 1908, **30**, 798).

Sherman, Kendall and Clark (*J. Amer. Chem. Soc.*, 1910, **32**, 1073) use a 1% solution of potato starch which they have boiled for 2 hours. 250 c.c. of this are digested with the enzyme at 40° until 0.25 c.c. when removed and mixed with 5 c.c. of dilute iodine[1] solution in a test-tube shows no colour differing from that of the untreated iodine solution. The amount of enzyme is found which completes the digestion in 30 minutes (± 1 min.) and the result expressed by dividing the weight of starch (2.5 grm.) by the weight of enzyme required to digest it. This method gives parallel results to the Lintner method.

Wohlgemuth (*Biochem. Zeitsch.*, 1908, **9**, 1) proposes to make use of the iodine colouration by working in the following manner:

Increasing quantities of the enzyme solution are filled into a series of test-tubes and the whole placed in ice while 5 c.c. of 1% starch solution are added to each. The tubes are kept 30 or 60 minutes at 40° and replaced in ice water. They are then nearly filled with water, a drop of $N/10$ iodine solution added to each and the contents shaken. Colours varying from dark blue, bluish-violet, reddish-yellow to yellow are obtained. The tube in which the violet colour just shows is taken as indicating complete hydrolysis to dextrin and the amount of enzyme in it recorded. If this, for example, contained 0.02 c.c. saliva the diastatic power is expressed as D $40°/30 = 250$, *i.e.*, 1 c.c. saliva at 40° in 30 minutes hydrolyses 250 c.c. of 1% starch solution.

It has been suggested to use starch paste in Metts' tubes for diastatic power determinations but this method does not give satisfactory results.

In the case of malt extracts suitable for the textile industries it is the liquefying power which is of importance, comparisons based on the Lintner standard being valueless. The analysis of such malt

[1] 2 grm. iodine and 4 grm. potassium iodide in 250 c.c. water. For use 2 c.c. are diluted to 100 and 5 c.c. taken for each test.

extracts is now being studied by a commission appointed by the Society of Dyers and Colourists. (See Appendix.)

SUCROCLASTS.

Invertase is obtained by extracting fresh yeast with chloroform water, air-dried yeast with water or by filtering yeast which has been allowed to autolyse. By precipitating with alcohol it is obtained free from maltase and most other enzymes.[1]

Maltase is only obtained by extracting air-dried yeast with water. It is destroyed on precipitation with alcohol.

Emulsin is best obtained from sweet almonds. The oil is expressed and the press cake extracted with water at about 10–20°. Protein is removed from the extract by means of a little acetic acid (2 drops per 100 c.c. of liquid); to the filtrate an equal volume of alcohol is gradually added and a granular precipitate of enzyme obtained. This is washed with alcohol and immediately redissolved in water; a colourless clear liquid is so obtained admirably suited for polarimetric work. (See Armstrong, *Proc. Roy. Soc.*, 1908, Series B, **80**, 324.)

Lactase is obtained in a similar manner from some but not all preparations of kephir.

Qualitative.

Invertase.—The enzyme solution is incubated with 10% sucrose for half an hour at 37° and the reducing power tested with Fehling's solution. With active preparations the hydrolysis is very rapid.

Emulsin.—The enzyme solution is incubated for a few hours with 2% salicin. Hydrolysis is shown by the solution reducing Fehling's solution and giving a purple colouration with ferric chloride.

Alternatively amygdalin may be used as test material and the formation of hydrogen cyanide identified with picric paper (filter paper soaked in a solution of 10 grm. sodium carbonate and 1 grm. picric acid per 100 c.c.) which becomes a deep brick red. This test is especially suitable when the enzyme is to be identified in plant or animal extracts containing reducing and colouring substances which obscure the test with salicin.

Lactase, Maltase.—Lactose and maltose are used as test materials; 5% solutions are incubated some hours at 37°. Change is demonstrated by the use of Barfoed's reagent (Vol. 1, page 333) which is reduced by dextrose and not by the disaccharides. A more certain

[1] Compare Vol. 1, page 314, also O'Sullivan and Tompson, *J. Chem. Soc.*, [1890], 57, 834.

method is the preparation of the osazones by heating with phenylhydrazine acetate for an hour; the separation of the insoluble dextrose osazone is unmistakable.

Quantitative.

The methods apply to all the enzymes; the appropriate hydrolyte must be used in each case.

(1) *Measurement of the Increase in the Reducing Power.*—This may be determined by any of the methods described in Vol. 1. Latterly Bertrand's method has been largely used; the copper oxide precipitate is dissolved in acid ferric sulphate solution and the ferrous sulphate formed estimated with permanganate. The method has the advantage over the gravimetric method that it avoids the double weighing of the crucible and is therefore very quick. (Compare Vol. 1.)

The procedure is as follows: Having precipitated the cuprous oxide in the usual way, it is filtered rapidly through a small Gooch crucible or Soxhlet tube, the precipitate being kept back as far as possible; it is thoroughly washed with hot water. The iron solution (ferric sulphate 50 grm., concentrated sulphuric acid 200 grm. to 1 litre) is added to the precipitate in the beaker until the black precipitate first formed is completely dissolved on shaking. This solution is run through the Gooch crucible followed by a little fresh iron solution and finally by water. The iron filtrate is returned to the original beaker and permanganate (5 grm. in 1 litre) run in until the colour changes from green to purple. The colour change is not permanent and the titration should be performed quickly. The volume of permanganate required multiplied by the factor obtained on standardising it gives the weight of copper reduced which is converted into sugar by the usual tables. (For tables given for Bertrand's conditions, see *Bull. Soc. Chim.*, 1906, **35**, 1285.)

To standardise the permanganate, 0.25 grm. ammonium oxalate are dissolved in 100 c.c. of water and 2 c.c. of concentrated sulphuric acid. This is warmed to 60° and the permanganate added till the colour changes. The weight of oxalate multiplied by 0.8951 gives the equivalent in reduced copper. 1 c.c. of permanganate equals about 0.01 grm. of copper.[1]

The iron solution should show a colouration with a single drop of permanganate; if not, permanganate is added to the bulk till this point is reached.

[1] In the writer's experience as also stated in Vol. 1, p. 325, this method of standardisation will give low results. The permanganate is therefore not standardised against cane sugar. (See Appendix.)

Pavy's method gives good results when used to measure the hydrolysis of lactose or maltose, but it is less satisfactory when applied to sucrose. (See Croft Hill, *Trans.*, 1898, **73**, 634; Armstrong, *Proc. Roy. Soc.*, 1904, **73**, 500.)

(2) *Optical Methods.*—The change in the specific rotation is measured. Care must be taken in cases in which change proceeds rapidly to prevent the errors due to *mutarotation* by adding a drop of alkali shortly before polarising.

With invertase and sucrose it is convenient to determine the time taken under standard conditions for the rotation to become $\pm$ 0°.

Emulsin, maltase and lactase are somewhat, invertase is extremely sensitive to small quantities of acid or alkaline impurities. Very small amounts of impurity may easily vary the rate of action of the enzyme to one-half or one-third of its previous value. It is therefore absolutely necessary to make measurements under carefully standardised conditions and on account of this the greater number of the existing determinations of the activity of invertase are valueless. In all operations only hard glass must be used; this is a remark which in the writer's experience applies generally to enzyme work. Small quantities of weak acids neutralise the effect of alkaline impurities and therefore appear to accelerate enzyme action. (Compare Ford, *J. Soc. Chem. Ind.*, 1904, **23**, 414.) Measurements of the activity of invertase are therefore conveniently made in the presence of amphoteric amino-acids, as has been suggested by Ford for diastase (*Trans.*, 1906, **89**, 76) and by Armstrong for invertase (*Proc. Roy. Soc.*, 1907, Series **B. 79**, 362).

Euler, who has reinvestigated the subject especially with regard to the optimum concentration of acid (first determined by O'Sullivan and Tompson in 1890, *Trans.*, **57**, 843) allows his invertase preparations to act on a mixture of 5 c.c. of 5% sodium dihydrogen phosphate with 200 c.c. of 20% sucrose. Action is stopped by alkali and the solution polarised. The time taken for the rotation to become $\pm$ 0° is expressed in minutes as the enzyme strength (*Zeit. physiol. Chem.*, 1911, **73**, 335).

Zymase.—The activity of yeast juice or acetone yeast is estimated by determining the amount of carbon dioxide liberated from sucrose. 20 c.c. yeast juice, 8 grm. sucrose and a little toluene are mixed in an Erlenmeyer flask closed by a Meissl tube and the loss in weight determined from day to day at 22°. This amounts to from 1–2 grm. When more accurate measurement is required the carbon dioxide is replaced by air and the volume or weight measured. (Compare

Vol. 1, page 222, see also Harden, Thompson and Young, *Biochem. J*, 1910, **5**, 230.)

For detecting the formation of very minute quantities of alcohol the so-called Pasteur's drop method adapted by Hansen and recently studied by Klöcker (*Centr. Bakt.*, 1911, **31**, 108) is of value. 5 c.c. of the liquid are placed in a test-tube closed by a cork through which a glass tube 80 cm. long can pass. The whole is supported obliquely over a tiny flame and the liquid slowly warmed—the characteristic oily drops denoting alcohol are clearly seen in the tube and the less alcohol present the higher up in the tube they appear. In this way 0.002 volume % of alcohol can be identified with certainty.

Proteoclastic (Proteolytic) Enzymes.

Pepsin is obtained by extracting the mucous membrane of the stomach with glycerol. It breaks down proteins to albumoses and peptones, but not to amino-acids. It is inactive in an alkaline medium and more stable in acid than in neutral solution. The optimum temperature is 40°.

Trypsin is obtained by extracting minced pancreas with glycerol. It breaks down proteins to polypeptides and amino-acids; it is active in alkaline solution (0.2 to 0.5% sodium carbonate), only slightly active in neutral and inactive in acid solution. It is very unstable in aqueous solution, but is protected by the presence of the substrate and the products of hydrolysis. The optimum temperature is 40°.

Erepsin is present in the expressed juice of intestinal mucous membrane; it decomposes albumoses, peptones and polypeptides to amino-acids, but does not attack proteins with the exception of casein. It is active only in an alkaline medium.

Rennin, the milk-clotting enzyme of the mucous membrane of the stomach, is active only in acid solution. Hydrochloric acid is the most favourable; the others in order of activity are nitric, lactic, acetic, sulphuric and phosphoric acids. The optimum temperature is 37–39°. It is obtained by extracting the fourth stomach of the calf or the stomach of the pig with glycerol. It is used commercially in making junket and cheese. It has been suggested that the action of rennin is identical with that of pepsin in neutral solution. This is still a matter of controversy.

Trypsin.—The quantitative measurement of protein hydrolysis may be made in a variety of ways.

(1) *Determination of the amino nitrogen before and after hydrolysis with the apparatus devised by van Slyke* (see page 486 and Vol. VII, page 263).

(2) *Optical Method.*—The change in rotatory power of the solution after hydrolysis may be studied directly or after precipitation of the unchanged protein.

(3) *Electrical Conductivity Method.*—The conductivity of the solution increases as hydrolysis proceeds. (See Bayliss, *J. Physiol.*, 1907, **36**, 221.)

(4) *Sörensen's Method* (*Biochem. Zeitsch.*, 1908, **7**, 45).—This is based on the fact that carboxyl groups can be titrated with barium hydroxide in presence of amino groups if these latter are fixed by excess of formaldehyde which forms methylene compounds. Thymolphthaleïn 0.05 grm. in 100 c.c. of 93% ethyl alcohol or phenolphthaleïn are used as indicators. (See also page 488.)

The formaldehyde solution is freshly prepared by mixing 50 c.c. 40% formaldehyde, 25 c.c. absolute alcohol, 5 c.c. thymolphthaleïn and $N/5$ barium hydroxide till the colour is faint blue. 200 c.c. of boiled water are used for the control titration. 15 c.c. of the formaldehyde mixture and 5 c.c. of barium hydroxide are added and the mixture titrated with $N/5$ hydrochloric acid until it has a bluish opalescence. Two drops of barium hydroxide now cause a blue colouration and two more drops a strong blue. This is taken as the end point. 200 c.c. of the hydrolysed protein are used, 15 c.c. formaldehyde are added, then a slight excess of barium hydroxide and the mixture is titrated with hydrochloric acid until the colour is weaker than the control. A few drops of barium hydroxide are then added until the colour of the control is attained.

The number of c.c. of barium hydroxide used multiplied by 2.8 gives the amount of hydrolysed nitrogen in milligrams.

When phenolphthaleïn is used the end point is taken with a distinctly red colour. Titration may also be made with $N/10$ or $N/5$ sodium hydroxide.

A more simple method of procedure is as follows: The formaldehyde is neutralised by the alkali until its colour is just red to phenolphthaleïn. 10 c.c. are added to the test solution which is titrated until a distinctly red colour is obtained to match a control, made by adding a known excess of alkali to a little phenolphthaleïn.

Sörensen, Henriques and Gjaldbaek (*Zeitsch. physiol. Chem.*, 1911, **75**, 363) carry out the titration in four stages. The test liquid is made neutral to litmus paper and phenolphthaleïn. It is then titrated

to a faint rose (first stage) and distinct red to match the control (second stage); formaldehyde is then added and titration continued to a faint rose (third stage) and finally to a distinct red (fourth stage). When the ratio of the figures in the fourth and first stages are high, amino-acids are indicated; when low, the products are of polypeptide nature.

Jacoby (*Biochem. Zeits.*, 1908, 10, 229) takes 1 grm. ricin in 100 c.c. of 1.5% sodium chloride solution and adds to a series of tubes, each containing 2 c.c. of this solution, increasing quantities of trypsin solution. Each tube is made up to 3 c.c. with water and 0.5 c.c. of 1% sodium carbonate added. All the tubes are opaque—those containing trypsin gradually become clear according to the quantity.

Pepsin.—The methods in use are described in detail on pages 492 to 494. In addition the following may be mentioned: Jacoby (*Biochem. Zeits.*, 1906, 1, 58) dissolves 0.5 grm. of ricin in 5% sodium chloride solution and filters. The opalescent solution becomes cloudy on the addition of $N/5$ hydrochloric acid. This solution is mixed with varying quantities of gastric juice and placed in a thermostat (37°) for 3 hours. The solution which has become clear is taken as that containing the minimum amount of enzyme able to digest the protein.

Rennin is measured by the amount required to coagulate a given quantity of milk in 30 minutes at 40°. Since ordinary milk varies very considerably in quality, it is preferable to use an artificial milk made by taking 3 grm. of milk powder and 27 grm. water. The milk powder is first made into a paste with a little water and the rest of the water then added; the milk dissolves almost entirely (Blum and Field, *Biochem. Zeit.*, 1907, 4, 62). Varying quantities of gastric juice are added to equal quantities of this milk, and the whole kept at the ordinary temperature (17.5° C.) for two hours. A drop of 20% calcium chloride solution is added and the tubes immersed for 5 minutes at 40°. The coagulated solution to which least enzyme had been added gives a measure of the activity of the enzyme. (See also p. 130.)

Lipase.—Castor-oil seeds are shelled, freed from oil and powdered, the milk is separated and allowed to stand 24 hours when an emulsion rises to the surface. A portion of this is triturated with olive oil and a trace of manganese sulphate; the formation of fatty acid is measured by titration. Other lipases are allowed to act on ethyl butyrate and the acid formed estimated by titration or by the conductivity method.

Pancreatic lipase is tested towards neutral olive oil or on an aqueous emulsion of egg yolk; the free acid formed in either case is titrated. The activity is accelerated by the addition of a little bile.

Castor-oil lipase is only active in strongly acid solution; it is insoluble in water.

A glycerol extract of pancreas is made as follows: a fresh pancreas of a pig is freed from fat, finely minced, ground with sand, and extracted for 24 hours with a mixture of 90 parts of glycerol and 10 parts of 1% sodium carbonate, 10 c.c. of the mixture being used for every gram of pancreas. The fluid is strained through muslin and kept at 0°.

Oxydases.[1]—Enzymes are supposed to take part in a large variety of oxidative changes undergone by such different materials as sugars, fats, amino-acids, aldehydes, alcohols, phenols, etc. A number of reactions are proposed for their detection. None of these are really satisfactory nor are they given by all oxydases; often the test is given slowly when no oxydase is present. Very considerable experience is therefore necessary in working with them and a more satisfactory test is greatly to be desired. The difficulty is perhaps in part due to the peculiar nature of oxydases and it has been suggested that they are active in virtue of containing inorganic catalysts such as manganese or iron salts.

The more important oxydases are:

(1) *Tyrosinase*, which acts only on tyrosine and allied compounds.

(2) *Oxydase* or *laccase*,[2] which acts on a number of phenols.

(3) *Peroxydase*, which is only active in presence of hydrogen peroxide and then behaves in the same way as oxydase. It is probable that *oxydase* consists of peroxydase and an organic peroxide.

(4) *Catalase*, which liberates oxygen from hydrogen peroxide.

Preparation.

Oxydase.—Aqueous solutions are readily obtained from the potato tuber, from maize and from fungi, particularly *Lactarius* species. The aqueous extract of almost any plant contains peroxydase. The extract sometimes contains both tyrosinase and oxydase; by heating at 70° for a short time the tyrosinase is destroyed.

Tyrosinase.—The principal sources of vegetable tyrosinase are fungi, particularly Russula and Agaricus species and wheat bran. The material is macerated with chloroform water and the extract filtered. The enzyme can be precipitated by alcohol and the precipitate collected, washed with alcohol and dried *in vacuo*.

[1] A complete account has been given by J. H. Kastle. *The Oxydases*, *Bull.* 59, Hyg. Lab. U. S. Pub. Health and Mar. Hosp. Ser., Washington, 1910.

[2] It is desirable to use the general term oxydase rather than the more specific laccase as it is improbable that there are specific oxydases for each class of compounds.

Animal tyrosinase is conveniently obtained from the ink sac of the cuttle fish.

The tests for oxydases are all based on colour reactions; the chief reagents are given in the following pages with references to the original papers. Usually a few drops of the reagent are added to 2 or 3 c.c. of the suspected enzyme solution. A direct test indicates oxydase; if it appears only after the addition of a few drops of hydrogen peroxide, peroxydase is indicated.

The technique for the macro- and microscopic identification of oxydases in the flowers and vegetative parts of plants by means of benzidine and α-naphthol has been described by Keeble and Armstrong (*Proc. Roy Soc.*, 1912. Series, B, **85**, 214).

Reagents.

1. **Guaiacum.**—1% in ethyl alcohol. This should be freshly prepared or it may be freed from peroxides by boiling with animal charcoal. It turns blue with oxydase. (See Moore and Whitley, *Biochem. J.*, 1909, **4**, 136.)

2. **α-Naphthol.**—1% in 50% ethyl alcohol. It gives a lavender colour when oxidised. This is suitable for microscopic work (Bourquelot, *Compt. Rend.*, 1896, **123**, 423).

3. **p-Phenylenediamine Hydrochloride.**—1% in water. It yields a greenish colour when oxidised.

4. **Indophenol.**—(Rohmann and Spitzer, *Ber.*, 1895, **28**, 567) 2 or 3 drops of 1% α-naphthol in 50% alcohol and 1% aqueous *p*-phenylenediamine hydrochloride are added to the enzyme extract which is then made slightly alkaline with sodium carbonate. This causes the purple oxidation product to dissolve. Vernon (*J. Physiol.*, 1911, **42**, 402) has adapted this test for quantitative work.

5. **Phenolphthalin.**—A pinch is dissolved in 1 c.c. of $N/10$ sodium hydroxide, 25 c.c. of water are added, and the solution filtered and made up to 100 c.c. 5 c.c. are left for 18 minutes with the oxydase and the mixture acquires the red colour due to phenolphthaleïn (Kastle, *Amer. Chem. J.*, 1908, **40**, 251).

6. **Phenol.**—5% aqueous solution. It becomes reddish-brown in 24 hours (Bourquelot, *Compt. Rend.*, 1896, **123**, 315).

7. **Guaiacol.**—Oxidation in 2% alcoholic solution to red tetraguaiacoquinone which can be measured colourimetrically (Bourquelot, *Compt. Rend. Soc. Biol.*, 1896, **48**, 893).

8. **Quinol.**—Oxidation to quinone (Bertrand, *Compt. Rend.*, 1894, **118**, 1215).

9. **Benzidine** (Schreiner, *U. S. Dept. Agric., Bureau of Soils, Bull.* 36, 1909). 1% in 50% ethyl alcohol gives an intense blue colouration changing to a brown precipitate.

10. **Salicylic Aldehyde.**—Oxidation to salicylic acid (Schmiedeberg, *Arch. f. exp. Path.*, 1881, **14**, 288, 379; Jaquet, *Ibid.*, 1892, **29**, 386; also Jacoby, *Zeitsch. physiol. Chem.*, 1900, **30**, 135).

Oxydase has been determined quantitatively by the following methods:

(*a*) Oxidation of potassium iodide and acetic acid, the iodine liberated being measured with thiosulphate. (Bach, *Ber.*, 1904, **37**, 3785).

(*b*) Oxidation of pyrogallol to insoluble purpurogallin (Bach and Chodat, *Ber.*, 1903, **36**, 1756; 1904, **37**, 1342).

(*c*) Oxidation of the leucobase of Malachite Green to the blue-green pigment (Czyhlarz and von Fürth, *Beitr. chem. physiol. Path.*, 1907, **10**, 358).

(*d*) Oxidation of vanillin to dehydrovanillin (Herzog and Meier, *Zeitsch. physiol. Chem.*, 1908, **57**, 35; 1909, **59**, 57; 1911, **73**, 258) 150 c.c. of 0.96% solution of vanillin, 5 c.c. of H_2O_2 (10 volumes); the enzyme solution and water to 400 c.c. are mixed and allowed to stand for a convenient period of 12 to 24 hours. The precipitate of dehydrodivanillin is collected on a tared baryta filter, washed, dried at 100° and weighed. The writer has found this method to give satisfactory results when comparing the activity of several enzyme solutions. It is of course not in any way an absolute measure of the activity.

(*e*) Oxidation of the leucobase of Brilliant Green (Herzog and Polotzky, *Zeitsch. physiol. Chem.*, 1911, **73**, 247).

Tyrosinase is identified by the production of black insoluble melanin when it acts on tyrosine (0.05%) dissolved in dilute sodium carbonate (0.04%). Bach (*Ber.*, 1908, **41**, 216, 221) has shown that the amount of change can be determined quantitatively by titration with potassium permanganate (0.002/N) and sulphuric acid (10%). Von Fürth and Jerusalem determine the melanin spectrophotometrically (*Beitr. Chem. physiol. Path.*, 1907, **10**, 131–173).

The guaiacum test is used to distinguish between boiled and unboiled milk. Fresh milk gives a blue colouration with guaiacum tincture and peroxide, boiled milk gives no reaction.

With 5 drops phenylenediamine (2%) and a drop or two of dilute peroxide (1 in 10) fresh milk (5 c.c.) gives a yellow colour changing rapidly to emerald green and prussian blue; boiled milk only gives the reaction after several hours. With concentrated hydrogen peroxide

the boiled milk gives a blue colour immediately (Nicholas, *Bull. Soc. Chem.*, 1911, **9**, 266–269).

Catalase.—The decomposition of hydrogen peroxide by purified aqueous solutions of catalase is best measured by titration with potassium permanganate ($N/100$). The hydrogen peroxide is chosen about $N/20$–$N/50$ and rendered acid before titration. When dealing with animal and plant extracts the oxygen liberated may be measured volumetrically.

The catalase of blood (hæmase) has been studied by Senter (*Proc. Roy. Soc.*, 1904, **74**, 201). It is most active in neutral solution; acids hinder it. Loew (*U. S. Dept. Agric.*, 1901, *Bull.* 68) has made an exhaustive study of the enzyme of tobacco leaves.

Van Laer (*J. Inst. Brewing*, 1906, **12**, 313) has applied the measurement of the catalase of malt to malt analysis. He claims that a high catalytic activity is an indication of a high diastatic activity, although it is not possible to measure one by the other exactly. The practical utility of the method is that it only takes a few minutes. 3 grm. of ground malt are brought together with 25 c.c. of hydrogen peroxide (1.7%), which must be free from acids, and the amount of oxygen evolved in five minutes is measured.

In a further paper (*Ibid.*, 1909, **15**, 553) 6 grm. of malt and 25 c.c. of 0.85% hydrogen peroxide are used and the gas measured 1 minute after mixing. For the precautions necessary and the discussions of the results the originals should be consulted; they have an important bearing on malt analysis.

THE PROTEINS AND ALBUMINOID SUBSTANCES.[1]

By S. B. SCHRYVER, Ph.D., D.Sc.

Under the generic name of proteins is classed a large number of highly complex nitrogenous substances which form a considerable part of the solid contituents of blood, muscles, glands, and other organs of animals and which occur in smaller quantities in almost every part of plants.

The typical member of the class is albumin, the chief protein constituent of the white of egg and at one time the term "albumin" was employed to designate all other members of the group. It was decided, however, by a joint committee of the physiological and chemical societies of London, and by a committee of the American Society of Biological Chemists to use the generic name of "protein." The same committee also recommended a general method of classification of the proteins,[2] which is adopted in the following pages.

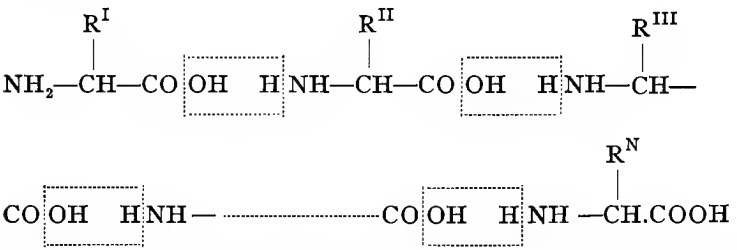
The structure of the proteins has formed the subject of numerous investigations in recent years by Emil Fischer and his pupils, and a great number of their researches have been collected together in an important monograph (E. Fischer, *Untersuchungen über Aminosäuren Polypeptide und Proteine*, Berlin, 1906). Not only have many points in the structure been elucidated, but several substances of protein-like characters have been synthetically prepared, although no naturally occurring protein has as yet been obtained in this way.

From these investigations it is now known that proteins belong to the class of chemical substances designated as "polypeptides," which

[1] So much of our positive knowledge of protein chemistry is of such recent date that comparatively few routine chemical methods exist for the analysis of these substances. The rational basis of the methods depends, however, on much of this knowledge so recently acquired, and the routine processes evolved are to a large extent founded upon the operations involved in scientific investigation, and are being continually added to and modified. For this reason, it is almost impossible at the present time to confine an article of this description to purely routine analytical methods. It has been thought, therefore, necessary to give sketches of certain experimental methods of scientific research, as an appreciation of these methods will aid very materially in understanding the newer suggestions for analytical technique which are constantly appearing in modern literature.

[2] The classifications adopted by the English and American societies differed slightly from one another. That suggested by the Americans contained a larger number of classes and was more complete. With one or two minor alterations, it has been adopted therefore, in preference to the English classification.

are formed by the condensation of several amino-acids according to the scheme:



R in the above formula may represent either a simple alkyl or aryl radicle, or a similar group in which a hydrogen atom is substituted by either an amino group or a carboxyl group.

The proteins differ from one another very markedly in their physical properties and they also differ from one another chemically, in that they yield varying amounts and varying numbers of amino-acids on hydrolysis. The original conception of Mulder to whom the name "protein" is due, that only one such substance exists, which forms the basis of all the albuminoid substances, which are said to differ from one another only in physical properties, is therefore incorrect. This view, which was also shared by Liebig, was no doubt due to the fact that the various members of the class differed but slightly from one another in empirical composition. By the elaboration by Emil Fischer and his pupils of the method for the approximately quantitative separation of the hydrolysis products of the proteins, the way was indicated for distinguishing with some degree of certainty the individual proteins.

The following amino-acids have been isolated as protein hydrolysis products:

Hydrolysis Products of Proteins.

A. *Mono-aminomonocarboxylic Acids.*

1. *Glycine,* $C_2H_5O_2N$, or aminoacetic acid.
   $(NH_2).CH_2.COOH$
2. *Alanine,* $C_3H_7O_2N$, or α-aminopropionic acid.
   $CH_3CH(NH_2).COOH$
3. *Valine,* $C_5H_{11}O_2N$, or α-aminoisovaleric acid.
   $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH.CH(NH_2).COOH$

4. *Leucine*, $C_6H_{13}O_2N$, or α-aminoisocaproic acid.

$$\begin{matrix} CH_3 \\ \\ CH_3 \end{matrix}\!\!\rangle CH.CH_2.CH(NH_2).COOH$$

5. *Isoleucine*, $C_6H_{13}O_2N$, or α-amino-β-methyl-β-ethyl-propionic acid.

$$\begin{matrix} CH_3 \\ \\ C_2H_5 \end{matrix}\!\!\rangle CH.CH(NH_2).COOH$$

6. *Phenylalanine*, $C_9H_{11}O_2N$, or β-phenyl-α-aminopropionic acid.

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

7. *Tyrosine*, $C_9H_{11}O_3N$, or β-*p*-hydroxyphenyl-α-aminopropionic acid.

$$HO.C_6H_4.CH_2.CH(NH_2).COOH$$

8. *Serine*, $C_3H_7O_3N$, or β-hydroxy-α-aminopropionic acid.

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

9. *Cystine*, $C_6H_{12}O_4N_2S_2$, or dicysteine, or di-(β-thio-α-amino-propionic acid).

$$HOOC.CH(NH_2).CH_2.S-S.CH_2.CH(NH_2).COOH$$

B. *Mono-aminodicarboxylic Acids.*

10. *Aspartic acid*, $C_4H_7O_4N$, or aminosuccinic acid.

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

11. *Glutamic acid*, $C_5H_9O_4N$, or α-aminoglutaric acid.

$$HOOC.CH_2CH_2.CH(NH_2).COOH$$

C. *Diaminomonocarboxylic Acids.*

12. *Arginine*, $C_6H_{14}O_2N_4$, or α-amino-δ-guanidinevaleric acid.

$$HN=C\!\!\begin{matrix} NH_2 \\ \diagup \\ \diagdown \\ NH.CH_2.CH_2.CH_2.CH(NH_2).COOH \end{matrix}$$

13. *Lysine*, $C_6H_{14}O_2N_2$, or $\alpha\epsilon$-diaminocaproic acid.

$$H_2N.CH_2.CH_2.CH_2.CH_2.CH(NH_2).COOH$$

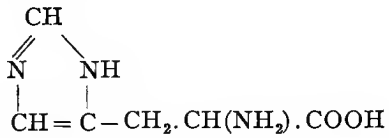
D. *Diamino-hydroxy-monocarboxylic Acid.*

14. *Caseinic acid*, or diaminotrihydroxydodecanic acid.

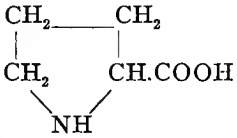
$$C_{12}H_{26}O_5N_2$$

E. *Heterocyclic Compounds.*

15. *Histidine*, $C_6H_9O_2N_3$, or β-iminazole-α-aminopropionic acid.

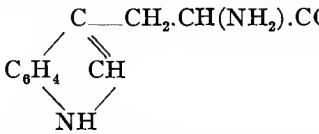


16. *Proline*, $C_5H_9O_2N$, or α-pyrrolidinecarboxylic acid.



17. *Hydroxyproline*, or hydroxypyrrolidinecarboxylic acid. $C_5H_9O_3N$

18. *Tryptophan*, $C_{11}H_{12}O_2N_2$, or β-indole-α-aminopropionic acid.



Several of the above substances exist conjugated in the protein molecule in an optically active form.

PERCENTAGES OF AMINO-ACIDS DERIVED BY HYDROLYSIS FROM CERTAIN TYPICAL ANIMAL PROTEINS

	Salmine[1]	Egg albumin[2]	Serum albumin[3]	Serum globulin[4]	Case-inogen[5]	Gelatin[6]	Keratin from ox-horn[7]
Glycine	.....	0	0	3.5	0	16.5	0.4
Alanine	.....	2.1	2.7	2.2	0.9	0.8	1.2
Valine	4.3	.....	.....	+	1.0	1.0	5.7
Leucine	.....	6.1	20.0	18.7	10.5	2.1	18.3
Isoleucine	.....	.....	.....	.....	.....	.....	.....
Phenylalanine	.....	4.4	3.1	3.8	3.2	0.4	3.0
Tyrosine	.....	1.1	2.1	2.5	4.5	0	4.6
Serine	7.8	.....	0.6	.....	0.23	0.4	0.7
Cystine	.....	0.3	2.5	0.7	0.06	.....	6.8
Proline	.....	2.3	1.0	2.8	3.1	5.2	3.6
Hydroxyproline	.....	.....	.....	.....	0.25	3.0	.....
Aspartic acid	.....	1.5	3.1	2.5	1.2	0.6	2.5
Glutamic acid	.....	8.0	7.7	8.5	11.0	0.9	3.0
Tryptophan	.....	+	+	+	1.5	0	.....
Arginine	87.4	.....	.....	.....	4.84	7.6	2.3
Lysine	.....	.....	.....	.....	5.80	2.8	.....
Histidine	.....	.....	.....	.....	2.59	0.4	.....

[1] Kossel, *Zeitsch. physiol. Chem.*, 1904, 40, 311; Abderhalden, *Ibid.*, 1904, 41, 55; Kossel and Dakin, *Ibid.*, 1904, 41, 1907.

[2] Abderhalden and Pregl, *Zeitsch. physiol. Chem.*, 1905, 46, 24; Mörner, *Ibid.*, 1901, 34, 207.

[3] Abderhalden, *Zeitsch. physiol. Chem.*, 1903, 37, 495; Mörner, *Ibid.*, 1901, 34, 207.

[4] Abderhalden, *Zeitsch. physiol. Chem.*, 1905, 44, 17; Abderhalden and Samuely, *Ibid.*, 1905, 46, 193; Mörner, *Ibid.*, 1901, 34, 207.

[5] Abderhalden and Funk, *Zeitsch. physiol. Chem.*, 1907, 53, 16; E. Fischer, *Ibid.*, 1903, 39, 155; Fischer and Abderhalden, *Ibid.*, 1904, 42, 540; Hart, 1901, 33, 347.

[6] Fischer, Levene and Aders, *Zeitsch. physiol Chem.*, 1902, 35, 70; Kossel and Kutscher, *Ibid.*, 1901, 31, 161.

[7] Fischer and Dörpinghans, *Zeitsch. physiol. Chem.*, 1902, 36, 462.

The Hydrolysis of the Proteins and the Separation of the Amino-acids.

As stated above, the proteins differ from one another in the number and quantities of the amino-acids which they yield on hydrolysis. The quantitative estimation of the hydrolysis products is therefore an important operation, when it is necessary to establish the identity or difference of proteins of various origins. Unfortunately, the operation of separating the various amino-acids, especially the mono-amino-acids, is for the most part a very complex and difficult one, requiring relatively large amounts of materials, and yielding, at any rate in the case of the mono-amino-acids, barely approximately quantitative results. It cannot therefore be included among the ordinary routine methods for the analytical investigations of the proteins. Certain individual amino-acids, such as tyrosine and ammonia can be, however, estimated fairly readily, and certain groups, such as the mono-amino- and diamino-acids can also be easily estimated. The processes employed in these estimations will be described below, when dealing with the routine analytical methods. As, however, the estimation of the amino-acids is a process of such fundamental importance for the general chemistry of the proteins, and is continually forming the subject of new investigations, which may finally result in simplification and greater accuracy if not with all, at any rate with certain of the amino-acids, it is thought advisable to give a somewhat detailed account of the methods, although they can only be applied when large amounts of material are available, and a somewhat lengthy research is possible (compare Vol. 7, pp. 218 and 262).

A. The Separation of the Mono-amino-acids.

The principle of the process as devised by Emil Fischer and his pupils consists in the esterification of the amino-acids after hydrolysis, and the separation of the esters into fractions by distillation under very low pressure.

The hydrolysis can be carried out by either 25% sulphuric or 25% hydrochloric acid, and more recently Hugonnenq and Morel have employed hydrofluoric acid (15–25%). In this case the reaction is carried out in lead vessels at the temperature of the water-bath and the hydrolysis must in certain cases be continued for 150 hours in order to get complete scission of the amino-acids. The hydrofluoric acid is afterward removed as calcium salt. (Reports, *Intern. Congress*

Applied Chem., 1910, Sect. IV, A, 2, 82.) When hydrochloric and sulphuric acids are employed, the protein is first gently warmed on a water-bath with about 3 times its weight of acid until a nearly clear solution is obtained, and the hydrolysis is then completed over a free flame under a reflex condensor. This process lasts generally 30 hours. (See, however, criticisms by Osborne below, page 28.) When sulphuric acid is employed, the mixture at the end of the hydrolysis is diluted with water and the acid is then quantitatively removed by barium hydroxide. On concentrating the solution *in vacuo* and allowing to stand (after quantitatively neutralising when hydrochloric acid is used) *tyrosine* and *cystine* separate out, when these substances are present in sufficient quantities. When cystine is yielded in large quantities on hydrolysis, as happens in the case of keratin, hair, and other scleroproteins, hydrochloric acid can be employed as hydrolysing agent; after hydrolysis, sodium acetate is added in excess, when the greater part of the cystine separates and can be purified by recrystallisation (Folin, *J. Biol. Chem.*, 1910, **8**, 9).

After separation of the cystine and tyrosine (and diaminotrihydroxydodecanic acid, when this is present) the solution is evaporated to a small bulk, saturated with hydrogen chloride gas, and allowed to stand for some days at 0°. The greater part (but not all) of the *glutamic acid* separates then in the form of its hydrochloride and is filtered with a pump, after the addition of an equal bulk of ice-cold alcohol. The amino-acids in the filtrate are then converted into their ethyl esters. This was done originally by Fischer by dissolving the syrup in about three times the weight of absolute alcohol, saturating with gaseous hydrogen chloride and after allowing the mixture to stand over night, evaporating it down under diminished pressure on a water-bath. The residue is then redissolved in the same quantity of alcohol, the solution is again saturated with hydrogen chloride, and again evaporated under diminished pressure. The process is, if necessary, repeated a third time. The acids are thus converted into the *hydrochlorides of the esters*. A modification of this process, which in the hands of Osborne and his co-workers has yielded satisfactory results, has been suggested by Phelps and Tellotson. (*Amer. J. Sci.*, 1907, **24**, 194.) This consists in treating the syrup with alcoholic hydrogen chloride and zinc chloride, and distilling through it, when heated to 110°, alcohol containing 10% of anhydrous hydrogen chloride gas (see also Osborne and Breese Jones, *Amer. J. Physiol.*, 1910, **26**, 212). When *glycine* is present in appreciable quantities, the greater part of the hydrochloride of its ester separates out, when the concen-

trated solutions of the hydrochlorides of the esters are allowed to stand at 0° for 24 hours and can be filtered off at this low temperature in a nearly pure state.

The esters must then be set free from their hydrochlorides. This is a process requiring care and experience to be carried out in a satisfactory manner. The original process of Emil Fischer consisted in adding 33% sodium hydroxide to the syrup dissolved in an equal volume of water, covered with twice the volume of cold ether, and cooled to 0°, until the excess of free acid is neutralised, and then adding gradually an excess of granulated potassium carbonate with continual shaking, until a pasty mass is formed. During the operation, the supernatant ether is several times poured off and renewed. Some of the esters are so readily hydrolysed by acid that even with great care the conversion of the hydrochlorides into free esters is not quantitative. For this reason, the residue left after treating with alkali and ether should be again treated with alcohol and hydrogen chloride; and the greater part of the inorganic salt which separates should be filtered off. The hydrochlorides of the esters thus obtained should be again treated in the same way as before. This process is, if necessary, repeated a third or fourth time. Large quantities of ethereal extracts of the esters are thereby obtained, which are dried for about 15 minutes with potassium carbonate, and then for twenty-four hours with fused sodium sulphate. The residue after distilling off the ether in a water-bath is then ready for fractionation. It should be noted that some of the lower boiling esters (glycine and alanine) distil over with the ether. They can be recovered in the form of the hydrochlorides by extracting the ethereal distillate with hydrochloric acid. The fraction thus obtained can be treated in a similar way to the lowest boiling fraction got by distillation.

Various modifications of the above process have been suggested. Thus, the chlorine in the alcoholic solution of the hydrochlorides of the esters can be quantitatively estimated, and the exact quantity of sodium ethoxide in alcoholic solution necessary to combine with the hydrochloric acid is then added. Most of the sodium chloride separates out and the filtrate therefrom can then be distilled. Some of the lower esters pass over with the alcohol. They are recovered in the way described above (Abderhalden, see p. 28).

Another modification has been suggested by Levene (*J. Biol. Chem.*, 1905–6, **1**, 45). The excess of hydrochloric acid is first neutralised with concentrated barium hydroxide, and then freshly heated anhydrous barium oxide is added, till a pasty mass is formed. The same

precautions as to temperature and renewal of supernatant ether are employed as when sodium hydroxide and potassium carbonate are used. The advantages claimed for the method are that less heat is evolved on neutralisation, that the free esters are less readily hydrolysed by barium hydroxide than by sodium hydroxide, and that the barium oxide is an effective drying reagent.

Fractionation of the Esters of the Amino-acids and Examination of the Individual Fractions.—The esters prepared by the above-described methods are obtained in the form of a dark-brown oil, which is then subjected to fractional distillation. The lower fractions are distilled off under a pressure of 10–12 mm. which can be obtained by means of an ordinary water-pump; the higher fractions, on the other hand require a lower pressure, which can be best obtained with the use of a mechanically driven double Geryk pump, between which and the distillation apparatus is interposed a condensing tube cooled with liquid air. In the absence of such a tube, the vapours due to the slight decomposition taking place during distillation are apt to raise the pressure and contaminate the pump. Levene and van Slyke (*Biochem. Zeitsch.*, 1908, **10**, 214) have substituted for the liquid-air cooled condenser a wash-bottle containing sulphuric acid and cooled by a freezing mixture. Another method suggested for obtaining a low vacuum consists in the employment of cocoanut charcoal cooled in liquid air. The fraction collected will depend upon the amino-acids obtained, but the following may be separated in a typical hydrolysis. *Fraction I.*—Below 40° (vapour) under 10 mm. pressure, (esters of glycine and alanine). *Fraction II.*—40–60° under 10 mm. (esters of alanine, leucine and proline). *Fraction III.*—60–90° under 10 mm. (esters of valine, leucine and proline). *Fraction IV.*—100° (water-bath) under 0.5 mm. pressure (esters of leucine and proline). *Fraction V.*—100–130° (oil-bath) under 0.5 mm. pressure (esters of phenylalanine, aspartic acid, glutamic acid and serine). *Fraction VI.*—130–160° (oil-bath) under 0.5 mm. pressure (esters of phenylalanine, glutamic acid, aspartic acid and serine).

Each fraction is then hydrolysed separately, and the constituents as far as possible quantitatively separated. The lower fractions are hydrolysed by simply boiling with 5 to 6 times their volume of water until the alkaline reaction disappears, whereas the higher fractions which contain the di-amino-acids, must be hydrolysed with barium hydroxide solution, as hydrolysis with water alone is ineffective.

Separation of the Individual Amino-acids from the Various Fractions.—The number of acids to be separated is increased, owing

to the fact that some of them, which in their original state in the proteins are optically active, are partially racemised during the process of hydrolysis.

Glycine.—The greater part of this acid, when it is present in appreciable quantities, separates in the form of the hydrochloride of its ester, after esterification of the hydrolysis products (see above, page 22). It is, however, generally obtained in some quantity, even after this separation in the lower fractions, from which it can be obtained by reconversion into the ester hydrochloride and thus separated from the alanine. Levene (*J. Biol. Chem.*, 1905–6, **1**, 45) has suggested its separation in the form of a picrate. (See also Levene and Van Slyke, *J. Biol. Chem;* 1912, **12**, 285.)

Alanine.—From the lowest fraction this acid can be obtained after separation of the glycine ester hydrochloride by hydrolysis and separation of the hydrochloric acid by boiling with lead hydroxide, when it separates in crystalline form. When present with leucine, etc., in the higher fractions, it remains along with proline in the mother-liquor, when the acids are subjected to fractional crystallisation from water. The proline also remains in the mother-liquor, but can be readily separated from the alanine by extraction with alcohol, in which the former acid alone is readily soluble. It is not easy to obtain the alanine quantitatively from mixtures.

Proline.—This acid is readily obtained from the fractions containing it, owing to the fact that it alone is easily soluble in absolute alcohol. To obtain it, the dried mixture of acid is exracted with 6 times the weight of absolute alcohol. The mother-liquors after the separation of the greater part of the leucine, valine, etc., from aqueous solution, are treated in this way, as very little, if any proline separates in the earlier fractions of crystals. The alcoholic extract is then evaporated to dryness, and re-extracted with alcohol, and if necessary these processes are repeated so as to get a more complete separation of proline from the other acids, which dissolve to some extent in alcohol in the presence of proline. The proline thus obtained is a mixture of the optically active and racemic forms. The copper salt of the former only is soluble in absolute alcohol; the two isomers can therefore be separated from one another.[1]

Valine, Leucine and Isoleucine.—The separation of these acids from one another is a matter of some considerable difficulty. The proline-free mixture is employed for separation. By the method of F.

[1] The proline and hydroxyproline amounts in a given fraction can now be quantitatively estimated by reason of the fact that they alone of the acids in the fraction do not yield nitrogen on treatment with nitrous acid (see page 82).

Ehrlich and Wendel (*Biochem. Zeitsch.*, 1908, **8**, 399), the acids are converted into their copper salts and then extracted with methyl alcohol, which dissolves isoleucine and valine. These two acids are then liberated from the copper salts by acids, racemised by heating with barium hydroxide solution to 150° in an autoclave, and the racemised acids are then reconverted into copper salts. These are then extracted with cold methyl alcohol, or hot 96% ethyl alcohol, which removes the isoleucine. This method of separation is not, however, very complete.

Another method has been more recently suggested by Levene and van Slyke (*J. Biol. Chem.*, 1909, **6**, 391 and 419). This depends upon the fact that lead salts of leucine and isoleucine are less soluble than that of valine. In the presence of 3% of valine they can be quantitatively precipitated without any of this substance being carried down. To carry out the separation, the carbon is estimated in the mixture and a little more lead acetate solution is added than is necessary to precipitate the leucines only, which amount can be calculated from the carbon content of the mixture. The acids are dissolved in 7 times their weight of boiling water, to which solution is added 1.5 c.c. of concentrated ammonia for every gram of acid. Lead acetate in 1.1 molar solution is then added in the quantity of 4 c.c. for each gram of the leucines. The precipitate thus formed is washed with 90% alcohol. If the ratio of leucine to valine is greater than 2:1 (or the carbon content of the mixture exceeds 53.7), the leucine is not quantitatively precipitated. A smaller quantity of lead acetate than is necessary for complete precipitation is then added, and the filtrate from the lead salt is concentrated *in vacuo*, made alkaline with ammonia, and the remainder of the leucine is then precipitated by the requisite quantity of lead acetate, or as an alternative, the filtrate from the first lead precipitate can be evaporated to dryness, the carbon content determined in the mixture thus obtained, and the leucine is then precipitated from the solution by the requisite quantity of lead acetate in the presence of ammonia, (the amounts of water, lead acetate and ammonia being those given above). Valine is obtained from the mother-liquor of the lead salt by precipitating the slight excess of lead with hydrogen sulphide, evaporating the filtrate to dryness, and washing the residue with alcohol-ether mixture (3 parts alcohol to 1 of ether). To regenerate the leucines, the lead precipitate is dissolved in dilute acetic acid and treated with hydrogen sulphide. The filtrate from the lead sulphide is evaporated to dryness and washed with a mixture of equal parts of alcohol and ether. The ratio of leucine to isoleucine can be

determined polarimetrically, or the acids can be separated by means of the copper salts, that of isoleucine being soluble in methyl alcohol.

The process of separation of the acids leucine, isoleucine and valine is therefore somewhat complex, and will probably in the future be simplified.

Phenylalanine.—Phenylalanine ester is readily soluble in ether, and advantage is taken of this fact to separate it from the other esters of the fractions in which it occurs. The fraction is thrown into water and if phenylalanine ester be present in large quantities it separates in oily drops. In any case the mixture is extracted with ether and the ethereal extract after distilling off the ether is hydrolysed with hydrochloric acid and the phenylalanine can then be obtained in the form of its hydrochloride.

Glutamic and Aspartic Acids.—The greater part of glutamic acid is isolated as the hydrochloride before the esterification of the amino-acids (see above, page 22). The remainder, together with aspartic acid, is obtained in the fraction from which phenylalanine ester has been removed by ether by the above described method. This is then hydrolysed by barium hydroxide solution. Barium aspartate (in the racemic form) partially separates. From the filtrate from this, glutamic acid can be separated as hydrochloride, and from the filtrate of the glutamic acid hydrochloride, the remainder of the aspartic acid can be crystallised out, after the removal of the excess of hydrochloric acid by lead hydroxide (and removal of the excess of lead by hydrogen sulphide).

Serine.—The ester of this acid is not soluble in light petroleum. If the fractions containing it be treated with a little water, and the mixture then thrown into 5 or 6 times the volume of light petroleum, the serine ester is precipitated as an oil, which, after again washing with petroleum, is hydrolysed with barium hydroxide solution. After separating the excess of barium, the free acid separates out on concentration, and can be purified by washing with alcohol and recrystallisation from water.

Tyrosine, Tryptophan and Cystine.—These three acids cannot be isolated in the form of their esters in the processes described above. The amount of some of these products which can be obtained by hydrolysis can be estimated with a fair degree of accuracy when only small quantities of the protein are available for investigation. The quantitative estimation of these products will therefore be treated later on, when discussing the routine methods for protein examination.

Tryptophan is destroyed by acid hydrolysis and can be only isolated when the hydrolysis is brought about by enzymes.

A valuable critical examination of the sources of error involved in the quantitative examination of the hydrolysis products by the ester method has been made by T. B. Osborne and Breese Jones (*Amer. J. Physiol.* 1910, **26**, 305). They point out that the chief sources of error are due to the following factors: (*a*) Incomplete hydrolysis; (*b*) incomplete esterification; (*c*) loss in distillation, (i) in the distillation of the ether, and (ii) in destructive secondary decomposition; (*d*) loss in separation of the individual acids from the various fractions.

In the above sketch only the outlines are given of the methods employed and suggested for the isolation of the mono-amino-acids from the hydrolysis products. Numerous examples of the applications of the methods are to be found in the *Zeitsch. physiol. Chem.*, from 1903 onwards, by Fischer, Abderhalden and their pupils, and in the *Amer. J. Physiol.*, 1907 and onward, by Osborne and his co-workers.

B. The Separation of Diamino-acids (Hexone Bases).

The methods for the separation of these hydrolysis products are due chiefly to the investigations of Kossel and his pupils, the process chiefly in vogue being founded principally on the work of Kossel and Kutscher (*Zeitsch. physiol. Chem.*, 1900, **31**, 165) and Kossel and Patten (*Ibid.*, 1903, **38**, 39). This has been more recently modified in various details by Osborne, Leavensworth and Brautlecht (*Amer. J. Physiol.*, 1908–9, **23**, 180).

The principle of the process depends upon the facts that arginine and histidine can be precipitated as silver salts, the former in a solution made strongly alkaline with barium hydroxide, the latter in a nearly neutral solution, whereas the lysine is not so precipitated, but can be obtained from the mother-liquor after precipitation of the silver salts in the form of its phosphotungstate and finally of its picrate. 25–50 grm. are sufficient for the separation.

The protein is hydrolysed with a mixture of 3 times its weight of concentrated sulphuric acid, and 6 times the weight of water for at least 14 hours. The initial heating should be carried out on a water-bath till the foaming ceases, and the flask should be afterwards transferred to an oil-bath. The liquid is then diluted, and the total nitrogen is then estimated by Kjeldahl's process in an aliquot part (1/100). Concentrated barium hydroxide solution is then

added to the hot liquid until the mixture is nearly neutral. The barium sulphate which is precipitated carries down with it a certain amount of the so-called "humin" substance. It is washed several times with hot water, and the washings are added to the original filtrate, which is then evaporated down and diluted till the total volume is 1 litre. The nitrogen is then determined in 10 c.c. of this liquid, and the difference between the total nitrogen of the protein and the nitrogen in this liquid gives the amount of "humin nitrogen" I formed in the hydrolysis. Ammonia is also a product of hydrolysis, and this can be estimated by completely neutralising the liquid with barium hydroxide, adding about 5 grm. of barium carbonate and then distilling in steam, and collecting the distillate in excess of $N/10$ acid. By titration, the amount of ammonia which distils over can be readily estimated. The residue is then filtered, and the barium sulphate and carbonate are washed thoroughly with hot water, and the washings are added to the main filtrate, which after evaporation is made up to the volume of 1 litre. Nitrogen is estimated in this liquid, and from the result, after subtraction of the ammonia nitrogen, the amount of nitrogen carried down with the second barium precipitate (consisting of the barium sulphate and carbonate) can be ascertained. This is designated "humin nitrogen" II.

Arginine and Histidine.—These two bases are precipitated together as silver salts by means of silver sulphate. This can either be added in hot concentrated solution, or little by little, as a solid to the heated liquid containing the bases, which is stirred round after each addition of the silver salt until the latter is completely dissolved. Before addition of the silver salt, the liquid is diluted to 3 litres. Addition must be continued until a small sample of the liquid when mixed with barium hydroxide solution gives a dark brown, and not a white or yellow precipitate. When this condition has been attained, the liquid is cooled and saturated with finely powdered barium hydroxide. The precipitate of the silver arginine and histidine compounds is then filtered off and washed with barium hydroxide solution. The filtrate contains the lysine and is employed for the isolation and estimation of this substance.

Separation of the Arginine and Histidine.—The precipitate of the mixed silver salts is finely ground up in water containing sulpuric acid, the disintegration process being facilitated by the addition of silver sand. Enough sulphuric acid should be employed to make the liquid acid. Hydrogen sulphide is then led in, the liquid is warmed to expel the excess, and then filtered from the precipitated

silver sulphide and barium sulphate. The precipitate is thoroughly washed, the washings added to the main filtrate and the whole is then evaporated and made up to 1 litre. By estimating the nitrogen in 20 c.c. of this liquid, the amount of arginine and histidine nitrogen can be ascertained.

The liquid is then neutralised with barium hydroxide solution, barium nitrate in solution is added so long as a precipitate is formed, and the filtrate from the barium sulphate is then evaporated to 300 c.c., made acid with nitric acid, and silver nitrate in concentrated solution is added in excess, *i.e.*, until a sample of the solution gives a dark-coloured precipitate when mixed with excess of barium hydroxide solution. The liquid is then nearly neutralised with barium hydroxide solution, a paste of barium carbonate is added and the mixture is warmed, first on a water-bath, and then over a free flame on wire gauze, and allowed to cool, when the precipitate settles. This, the histidine silver compound, is then filtered off, washed with weak barium hydroxide solution (5 to 6 drops of 5% barium hydroxide solute to 100 c.c. of water) until no nitrate remains in the precipitate, and the washings added to the main filtrate which serves for the isolation and estimation of the arginine.

The histidine silver precipitate is suspended in excess of sulphuric acid, decomposed by hydrogen sulphide, and by estimating the nitrogen in an aliquot part of the filtrate from the silver sulphide, the amount of histidine nitrogen can be ascertained. Histidine can be obtained from the remainder by separating the excess of sulphuric acid by barium hydroxide, and the excess of barium hydroxide added for the purpose by carbon dioxide, and evaporating the filtrate to 10 c.c., adding if necessary a small amount of sulphuric acid to get rid of the last traces of barium. By adding picrolonic acid in slight excess in alcoholic solution (the amount theoretically necessary is known from the nitrogen estimation in an aliquot part of the histidine-containing fluid) histidine is precipitated as picrolonate, $C_{10}H_8O_5N_4 . C_6H_9O_2N_3$, and is filtered off after standing for 3 days, dried at 100° and weighed.

The arginine is obtained in the filtrate from the histidine silver precipitate, also in the form of the silver salt by saturating the liquid with solid barium hydroxide. The precipitate is filtered off, freed from nitrate by washing with barium hydroxide solution, then decomposed after suspension in acid solution with hydrogen sulphide. The nitrogen (arginine nitrogen) is estimated in an aliquot part of the liquid, and from the remainder the arginine is precipitated as picrolonate. The method of treatment of the arginine silver compound is, in fact,

almost exactly the same as that employed for the histidine compound. The picrolonate is dried at 110° before weighing.

Lysine.—The filtrate from the arginine and histidine is acidified with sulphuric acid, and the excess of silver precipitated by hydrogen sulphide. The filtrates and the washings are concentrated to about 500 c.c., sulphuric acid to the extent of about 4% of the total liquid is added, and then phosphotungstic acid (in 10% solution) until the filtered sample on addition of more of the reagent remains clear for 10 seconds. After standing for 24 hours the precipitate is filtered, washed with 4% sulphuric acid, and then decomposed with excess of barium hydroxide. This latter process is accomplished by making the precipitate into a paste with water, and throwing it into boiling water; concentrated barium hydroxide solution is then added in excess (until the liquid is strongly alkaline) and the barium phosphotungstate is filtered off. The excess of barium is then precipitated by carbon dioxide and the filtrate from the carbonate evaporated down. The residue is taken up with water, and the nitrogen (lysine nitrogen) estimated in an aliquot portion, and again after filtration, evaporated. The resinous-looking residue is then stirred round with an alcoholic solution of picric acid which is added in small quantities at a time. Care must be taken not to add an excess of picric acid in which the lysine picrate dissolves. The reaction is best carried out in a white porcelain basin. The precipitate, after standing for 24 hours is filtered, washed with a little alcohol, dissolved in a small quantity of hot water, the solution if necessary is filtered and then evaporated till the lysine picrate separates on cooling in needles. It is collected on a weighed filter, washed with alcohol, dried and weighed. Its formula is $C_6H_{14}O_2N_2 . C_6H_2(O_2N)_3OH$. From the first mother-liquor of the picrate a small quantity of lysine can still be obtained by adding sulphuric acid (to 4%), extraction of the picric acid by ether, and precipitation of the remaining base by phosphotungstic acid; from this the lysine is obtained as picrate by the method already described.

It is needless to add that throughout all the above processes for the separation of these bases, every care must be taken thoroughly to wash the precipitates, which are often very bulky. This is in many cases conveniently done by removing them from the filter and grinding them up in a mortar (sometimes with the addition of silver sand) with the liquid which is used for washing. Where an estimation of the amino-acids only is required, it will suffice to determine the nitrogen by Kjeldahl's method only in the various fractions, without troubling to isolate the acids themselves in a crystalline form.

Osborne, Leavensworth and Brautlecht (*loc. cit.*, page 28) have modified the above processes in various details.

I. The solution after filtration from the first barium sulphate precipitate is concentrated, while still slightly acid, under reduced pressure at 70°. The ammonia is removed by concentration in an open dish after the addition of barium carbonate.

II. The first silver precipitate is made with silver nitrate instead of the sulphate, as the latter is very slightly soluble in water.

III. The solution obtained from this first silver precipitate which contains the arginine and histidine is brought to about 250 c.c. volume and made to contain 5% sulphuric acid. The histidine is then precipitated by mercuric sulphate solution, converted into the silver compound and the nitrogen estimated in the solution obtained from the latter in the usual way. The filtrate from the mercury precipitate is freed from mercury by hydrogen sulphide, and from sulphuric acid by neutralising with barium hydroxide to litmus, and adding barium nitrate as long as a precipitate is formed. From this solution, the small quantity of histidine which is not precipitated by mercuric sulphate is separated by adding excess of silver nitrate, as shown by the brownish precipitate produced on adding a drop of the solution to barium hydroxide. Barium hydroxide is then added to the solution till it is *neutral* to litmus. A small quantity of histidine silver always separates at this point and the precipitation is completed by adding cold saturated solution of barium hydroxide. The filtered solution is then tested, and if a permanent precipitate forms on further addition of a drop of barium hydroxide solution to 10 c.c. of the solution filtered clear, 2 c.c. more of barium hydroxide are added to the main solution and the test is repeated. When 10 c.c. of the test solution remains clear after the addition of barium hydroxide, the small precipitate of the histidine silver is filtered and added to the main portion. By the transposition of the order of precipitation with silver and mercury salts, according to Kossel's method, it was found easier by the authors to get a complete separation. The arginine and histidine can be estimated by making a nitrogen determination in aliquot parts of their solutions. The lysine can be obtained as picrate.

More recently Van Slyke (*J. Biol. Chem.*, 1911, **10**, 15) has devised a new method for estimating the arginine, histidine and lysine nitrogen. As the operations entailed in these processes can be carried out when only a small quantity of material is available, the description of this process is given among the routine methods for examination of proteins (page 82). In no case has a quantitative yield of the mono-amino-

acids been obtained. Nor is it by any means certain that all the amino-acids coupled up in the polypeptide radicle have been as yet isolated.

Classification of the Proteins.[1]

No real systematic classification based on differences of chemical constitution is yet possible. The provisional system now adopted is based partly on physical differences, more especially differences in solubility, and partly also on their biological origins and chemical characters.

The known proteins can, however, be included in one of the following groups.

I. The Simple Proteins. (*a*) *The Protamines.*—These are the very basic substances obtainable from the spermatozoa of fish, which on hydrolysis yield relatively large amounts of diamino-acid. (See hydrolysis products of salmine in the table on page 20.) They are characterised also by the large amount of nitrogen in them (about 32%).

(*b*) *The Histones.*—These are also proteins of somewhat basic character, but yield smaller quantities of diamino-acids than the members of the previous class. They also contain less nitrogen in the molecule (usually 18 to 19%). They are intermediate in character between the members of the preceding and the succeeding classes. They are contained in certain fish roes, and the protein derived from the conjugated protein hæmoglobin is also generally placed in this class.

(*c*) *The Albumins.*—The proteins of this class are less basic than the histones, and are soluble in water. They are found largely in animal tissues, and the albumins of egg white and of blood serum may be regarded as typical examples. They yield only moderate amounts of diamino-acids on hydrolysis. They are not precipitated from solution by magnesium sulphate, but they are precipitated by saturation with ammonium sulphate.

(*d*) *The Globulins.*—This class of proteins is widely distributed both in the animal and vegetable kingdoms. The members are characterised by the fact that they are insoluble in water, but soluble in salt solutions, from which they can mostly be precipitated by saturation with magnesium sulphate and half-saturation with ammonium sulphate.[2]

[1] See note, page 17, also *J. Physiol.*, 1907, 35, 17, and *Amer. J. of Physiol.*, 1908, 21, 27.

[2] The writer has suggested that the difference between the globulins and the albumins depends upon the position of the reactive amino and carboxyl groups in the molecule. When these groups are so situated in the molecule that they can react with one another to form internal anhydrides, as is possible in the amino-acids, the proteins are water-soluble

Some of the members of this class can be obtained in crystalline form.

(*e*) *The Alcohol Soluble Proteins or Prolamines.*—These proteins are of vegetable origin, and the typical member of the class is gliadin from wheat-flour. They were at one time called the gliadins, but Osborne has substituted the name prolamine to indicate that the members on hydrolysis yield relatively large quantities of proline and amide nitrogen. They also yield large quantities of glutamic acid. They are almost insoluble in water and in absolute alcohol, but are easily soluble in alcohol of 70–90%.

(*f*) *The Glutenins.*—Only one or two proteins of this class have been definitely isolated and studied (glutenin of wheat and oryzenin from rice). They are insoluble in alcohol-water, salt solutions, or water, but are soluble in dilute alkalies, from which solution they can be precipitated by acids.

(*g*) *The Scleroproteins* (or, according to the American nomenclature, *the albuminoids*).—This is a somewhat heterogeneous class of protein substances, derived chiefly from the supporting and connective tissues of animals. The chief representatives of the class are gelatin, hair, horn, and keratin. Included in this class are also some proteins containing halogens which form the supporting structure of certain marine organisms.

II. The Conjugated Proteins. (*a*) *The Nucleoproteins.*—The proteins of this class are widely distributed, and from the fact that they were originally isolated from the fish spermatozoa, which consist chiefly of nucleus they are supposed to form the chief constituent of nuclei. The protein in this class is held in some form of combination with nucleic acid. Nucleoproteins are found both in plant and animal cells.

(*b*) *The Glycoproteins.*—The substances of this class yield on gentle treatment with acids or on hydrolysis substances of carbohydrate-like character. But little is yet known as to their chemical character. The chief members of the class are the mucins and mucoid substances,

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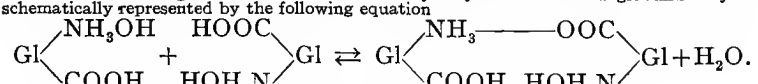
and belong to the class of the albumins. When, however, the amino and carboxyl groups are not so situated, the amino group of one molecule can react with the carboxyl group of another to form a salt-like compound of doubled molecular weight. This is the insoluble globulin, which in presence of water undergoes slight hydrolytic dissociation. The presence of salt molecules causes an apparent alteration in the hydrolysis equilibrium owing to the adsorption by the dissociated globulin molecules. In this way, the latter are sterically inhibited from reacting with one another. The hydrolysis of the solid globulin may be schematically represented by the following equation



(*Proc. Roy. Soc.*, 1910, **83**, 96, and *Kolloid Zeitsch.*, 1911, **8**, 233).

from which glucosamine, an amino-sugar derivative has been obtained, and cartilage, which on treatment with acid yields the peculiar substance known as chondroitin-sulphuric acid.

(*c*) *The Phosphoproteins.*—The chief members of this class are caseinogen of milk and vitellin of eggs. They yield phosphoric acid on gentle treatment with alkalies (1% sodium hydroxide at 37°).

(*d*) *The Hæmoglobins* and probably other allied substances, such as turicin, which split on treatment with cold dilute acid into a protein and a chromatogenic group.

(*e*) *The Lecithoproteins.*—This includes substances supposed to be compounds of lecithin-like substances with proteins. They are possibly only adsorption compounds.

III. Derived Proteins. (*a*) *Metaproteins.*—Acid and alkali albumins, substances obtained by gentle treatment of proteins by acid and alkali, which are soluble in excess of both and acid and alkali, but are precipitated from solution on neutralisation.

(*b*) *Proteoses.*

(*c*) *Peptones.*

(*d*) *Polypeptides.*

The members of the classes (*b*) and (*c*) are derived from the proteins by enzymatic digestion. They will be treated in detail in a separate section (See p. 467). The peptones are probably in most cases polypeptides, but this latter name is generally reserved for the synthetically obtained substances. Certain soluble proteins undergo change on heating, yielding insoluble so-called *coagulated proteins.* It is not perhaps convenient to place the substances thus produced in a separate class.

Qualitative Reactions of the Proteins.

The proteins as a class are recognised with tolerable facility. The following are the chief reactions of analytical value for the proteins.

1. On heating, proteins decompose with evolution of ammonia, and give an odour of burning animal matter. Smaller quantities may be recognised by the ammonia evolved when heated with soda-lime. Sulphur is present in all true proteins, and may be detected by igniting the substance with alkali-metal carbonate and nitre, dissolving the product in dilute hydrochloric acid, and testing the clear solution with barium chloride, when a white precipitate of barium sulphate will be produced if the substance tested contained sulphur. The sulphur of proteins may also be detected by boiling the substance with a strong

solution of sodium hydroxide and a few drops of lead acetate, when black lead sulphide will be produced.

The foregoing tests merely prove the presence of nitrogen and sulphur, and are not conclusive evidence of the presence of proteins. For the more certain recognition of proteins, the following tests may be employed.

2. The solutions of proteins have a lævo-rotatory action on polarised light. The optical activity is modified by free acids and by alkalies, but not by neutral salts.

According to Gamgee and Croft Hill, hæmoglobin is dextro-rotatory (*Ber.*, 1903, **36**, 913). The nucleoproteins are also dextro-rotatory (Gamgee and Jones, *Beitr. chem. Physiol. Path.*, 1903, **4**, 10).

3. The faintly acid solutions of most proteins (not peptones or proteoses) are coagulated when heated to boiling for a few minutes. Very small amounts of free alkali suffice to prevent the reaction, and it is retarded or prevented by any considerable quantity of free acid. Coagulation of proteins (except peptones and proteoses) may also be effected by addition of excess of strong alcohol. The conditions affecting the coagulation are described at length on pages 54 *et seq.*

4. Proteins are precipitated from solutions by various mineral acids. Nitric acid is most generally employed. If this acid is allowed to flow into a protein solution, a white ring forms at the junction of the liquids. This reaction is known under the name of *Heller's test.*

Acetic, tartaric, lactic and orthophosphoric acids do not precipitate proteins, but precipitation is caused by metaphosphoric acid.

5. Ferrocyanic acid is a good precipitant of the proteins. If the solution of a protein be rendered distinctly acid with acetic acid, and potassium ferrocyanide added, a white flocculent precipitate is produced. The reaction is delicate, and answers with all varieties of proteins except certain proteoses and peptones. These substances and gelatin are not precipitated. Recently-prepared hydroferrocyanic acid has been recommended in place of acetic acid and a ferrocyanide. A thiocyanate gives a similar reaction.

6. Trichloracetic acid and sulphonylsalicylic acid are good precipitants of the proteins. The precipitates of certain proteoses produced by trichloracetic acid dissolve in water on heating, but it must be remembered that the acid can cause a certain amount of hydrolysis. The peptones are not precipitated by trichloracetic acid.

7. Uranylacetate precipitates the proteins.

8. Proteins are precipitated by salts of the heavy metals. The precipitates thus obtained have no definite chemical composition, and

the phenomena involved in the precipitation are somewhat complex. There appears generally to be a certain amount of formation of a metallic salt of the protein, but complete reaction is inhibited by the adsorption of salt molecules from solution. Furthermore, when the salt reaches a sufficiently high concentration in the solution, ordinary salt precipitation occurs in the same way as when the salt of an alkali metal is employed. This salt action has been discussed in several papers. (See Galeotti, *Zeitsch. physiol. Chem.*, 1904, **40**, 492; Pauli, *Beitr. chem. Physiol. Path.*, 1905, **6**, 233; Schryver, *Proc. Roy. Soc.*, 1910, **82B.**, 96.)

9. Proteins are precipitated from solution by salts other than those of the heavy metals. The salt most frequently employed is ammonium sulphate. The concentrations at which precipitation commences and is completed are characteristic of the individual proteins. Salt precipitation is therefore employed for separating proteins from one another.

10. By virtue of their colloidal character, proteins are carried out of solutions by precipitation therein of other colloids. The substances chiefly employed are gum mastic in alcohol, and dialysed ferric hydroxide. These colloidal reactions have been extensively employed recently for the quantitative separation of proteins from solution, and are described in some detail below. Certain powders are also capable under favourable conditions of absorbing proteins from solution completely. The most effective adsorbents are precipitated silicic acid, meerschaum and iron oxide (Landsteiner and Uhlirz, *Zentralbl. Bak. u. Par.* [i], 1906, **40**, 265).

11. Proteins are precipitated from solutions by the ordinary alkaloidal reagents. (See Vol. 6, page 185.)

Those chiefly employed are:

(*a*) Phosphotungstic acid and phosphomolybdic acid (especially the former).

(*b*) Potassium-mercuric iodide and potassium-bismuth iodide.

(*c*) Tannic acid.

(*d*) Picric acid.

The tannic acid can be employed either in the form of *Almén's reagent*, which is prepared by dissolving 4 grm. of tannic acid in 190 c.c. of 50% alcohol, and adding 8 c.c. of acetic acid of 25% strength, or in the form recommended by *Hedin*, which is made up in the following way: 100 grm. of tannic acid, 25 grm. of sodium acetate, 25 grm. of sodium chloride and 50 c.c. of glacial acetic acid are dissolved in water, and the solution is then made up to 1 litre.

The picric acid is generally employed in the form known as *Esbach's solution* which is prepared by dissolving 10 grm. of picric acid and 10 grm. of citric acid in water and diluting to 1 litre.

The above alkaloidal reagents precipitate the majority of the proteins in acid solutions only; the strongly basic protamines, however, can be precipitated in alkaline solution. The peptones are not precipitated by picric acid or potassium mercuric iodide,[1] but are precipitated by tannic, phosphomolybdic, and phosphotungstic acids.

Colour Reactions of the Proteins.

The most characteristic reactions of the proteins are the colour reactions. These are not common to all proteins, as certain of the reactions are due to special groups, such as tryptophan, which are not found in all the members of the class.

The following are the chief reactions employed:

1. Solid proteins are coloured deep yellow by a solution of iodine.

2. Fuming nitric acid destroys solid proteins with evolution of nitrogen, but if treated in the solid state with somewhat weaker acid (*e.g.*, sp. gr. 1.2 to 1.25) they acquire a bright yellow colour. The same reaction is produced if the solution of a protein be boiled for some time with strong nitric acid. The colour is attributable to the formation of a yellow substance of indefinite composition known as xanthoproteic acid. It is soluble in ammonia and fixed alkali hydroxides with orange-red or brownish-red colour.

This reaction is probably due to the presence of a radical containing the benzene ring. With gelatin this reaction is negative or very faint.

3. When a solution of a protein is treated with Millon's reagent, a white precipitate is formed which turns brick-red on boiling, and the supernatant liquid also becomes red after a time. Solid proteins become red when boiled with Millon's reagent. The test is useful, but a similar reaction is yielded by gelatin, keratin, and allied substances containing an aromatic group in the molecule.

Millon's reagent is prepared by treating metallic mercury with an equal weight of nitric acid of 1.4 sp. gr. (or 1 c.c. of mercury to 10 c.c. of nitric acid). When the action slackens, a gentle heat may be applied till complete solution is effected. The solution is then diluted with twice its volume of cold water, allowed to stand for some hours, and decanted from the deposit which forms. The liquid thus prepared is a solution of mercurous nitrate, holding nitrous acid in solution, to the presence of which its action is partly due. Hence the reagent answers best when freshly prepared.

[1] See, however, page. 471 *et seq.*

Barfoed substitutes for Millon's reagent a neutral solution of mercuric nitrate, which gives a yellow colouration when heated with proteins; and on then adding a drop or two of yellow, fuming nitric acid, and again heating, a bright red or brownish-red colouration is produced.

Millon's reaction does not occur in presence of sodium chloride. Gelatin also yields the reaction faintly, or not at all.

4. If a few drops of a dilute solution of copper sulphate be added to the solution of an albumin or globulin, a precipitate of copper albuminate will be produced. On addition of excess of sodium or potassium hydroxide to the liquid, the precipitate will dissolve with production of a fine violet colouration. If ammonia be substituted for a fixed alkali, a blue solution will be obtained. The test may be varied by dissolving the solid protein in a strong solution of sodium hydroxide, or by adding the alkali to a previously-prepared solution of the protein. If to the strongly alkaline liquid obtained in either of these ways a few drops of dilute copper sulphate solution be added, the same violet colouration is produced. This does not undergo any change on heating the solution to boiling, unless dextrose or other reducing substance be simultaneously present. Fehling's solution may be substituted for cupric sulphate in applying the above test, but in either case an excess of the reagent must be carefully avoided, or the violet colouration due to the protein will be masked by the blue colour of the copper solution.

Certain proteoses differ from the albumins and globulins in giving a precipitate with copper sulphate which dissolves in alkali hydroxide with reddish-violet or rose-red colour. Deutero-albumose and peptones give no precipitate with copper sulphate alone, and on subsequently adding sodium hydroxide the colour produced is rose-red; while if ammonia be substituted a reddish-violet solution is obtained.

The foregoing reaction is due to Piotrowski, but is generally known as the *biuret test.* It is also produced by gelatin, and, according to H. Schiff, by all substances which contain two $CO.NH_2$ groups combined in the molecule with one or more CO.NH groups.

In practice, the biuret test has often to be applied in presence of neutral salts. Its indications are not affected by sodium chloride. In presence of magnesium sulphate a white precipitate of magnesium hydroxide is produced by the alkali, and should be filtered off before adding the copper solution. In presence of ammonium sulphate a large amount of fixed alkali must be added to obtain the violet colouration

tion. Hence A. Bömer substitutes zinc sulphate for ammonium sulphate as a precipitant of proteins other than peptones.

5. *Colour Reactions of Proteins due to the Presence of the Tryptophan Group.*—(a) The proteins which contain this group give certain definite colour reactions of which the most important is that due to *Adamkiewicz.* This consisted originally in treating the protein solution with one volume of concentrated sulphuric acid, and two volumes of glacial acetic acid, in the presence of which a reddish-violet colour is produced if the protein yields tryptophan on hydrolysis. Hopkins and Cole have shown, however, that the colour is due to the fact that glacial acetic acid often contains glyoxylic acid as an impurity, which substance is formed on the exposure of the acetic acid to sunlight. They therefore substitute glyoxylic acid for acetic acid in this reaction. The solution of this acid may be made, according to Benedict (*J. Biol. Chem.*, 1906, **6**, 51) in the following way: 10 grm. of magnesium powder are covered with water and 250 c.c. of saturated oxalic acid solution are added gradually, the flask being kept cool. The mixture is then filtered, slightly acidified with acetic acid and diluted to 1000 c.c. A few drops of this solution are added to the protein solution, and then gradually the concentrated sulphuric acid, when if tryptophan be present, a colour develops.

(*b*) *Reichl's test* consists in mixing a protein with an alcoholic solution of benzaldehyde and adding dilute sulphuric acid (equal volume of acid and water) and ferric sulphate. A blue colouration is produced.

(*c*) *Rhode's test.*—A weak solution of dimethylaminobenzaldehyde is mixed with the protein; on allowing concentrated sulphuric acid to run in, a reddish-violet colour is produced which changes to dark violet.

(*d*) According to Cole, tryptophan yields the Molisch-Udransky reaction (see below.)

6. *Reactions due to the Presence of a Carbohydrate Group.*—The glycoproteins contain a carbohydrate group, in consequence of which they yield certain colour reactions. Other proteins which are perhaps not glycoproteins are said to yield the same reactions. It must be remembered, however, that glycoproteins are often only separated with difficulty from other proteins (*e.g.*, in the case of egg-albumin), and conclusions as to the nature of the protein, drawn from the following reactions must be used with caution.

(*a*) *Molisch-Udransky Reaction.*—Concentrated sulphuric acid is added to a solution of a protein containing a few drops of an alcoholic

solution of α-naphthol. A violet colour is produced which turns yellow on the addition of alcohol, ether or sodium hydroxide. If thymol be substituted for α-naphthol, a carmine-red colour is produced.

(*b*) *Bial's modification* of the orcinol reaction: A small quantity of dried protein is added to 5 c.c. of fuming hydrochloric acid and the mixture is then warmed. When the protein is nearly all dissolved, a little solid orcinol is added and then a drop of ferric chloride solution. After warming for some time, a green colouration is produced, which is soluble in amyl alcohol.

7. Diazobenzene sulphonic acid in the presence of potassium hydroxide yields an orange to reddish-brown colour which on treatment with zinc dust changes to a fuchsine colour. Tyrosine and histidine yield the same reaction.

8. Ruhemann (*Trans. Chem. Soc.*, 1910, **97**, 2025) has recently shown that triketohydrindene hydrate is a delicate reagent for the proteins and Abderhalden and Schmidt (*Zeitsch. physiol. Chem.*, 1911, **72**, 37) have shown that the amino-acids and peptides also react. They dissolved 0.1 grm. of the reagent in 30–40 c.c. water, and found that 1–2 drops of this reagent, on boiling with the protein solution, yielded a more or less intense blue colour. To get a pure blue colour the solution of the protein must be accurately neutralised.

9. On mixing a dilute protein solution with a small quantity of 10% potassium hydroxide solution and adding a drop of a 1% solution of diacetyl a pink colour is produced. This is due to the presence in the protein of the group NH: $C(NH_2)$.NH.R. Complex proteins in addition to the pink colour give a fluorescence which is lost if the protein be allowed to hydrolyse before the diacetyl is added. (Harden, *Proc. Roy. Soc.*, 1906, 77B, 424, and Harden and Miss Norris, *J. Physiol.*, 1911, **42**, 332.)

THE GENERAL METHODS OF SEPARATION AND ISOLATION OF THE PROTEINS.

The Preliminary Preparation of Materials.

As proteins are derived chiefly from animal and vegetable tissues which exist in the form of cells, it is a matter of primary importance to secure a thorough disintegration of material before submitting the same to systematic investigation. Unless the cells are completely disintegrated, it is almost impossible to extract the proteins in a satisfactory manner. The difficulties are also increased by the pres-

ence of fats and other lipoid matter, which adhere to the proteins and prevent access to them of the solvents employed for extraction. Satisfactory results depend, therefore, to a large extent on the care which

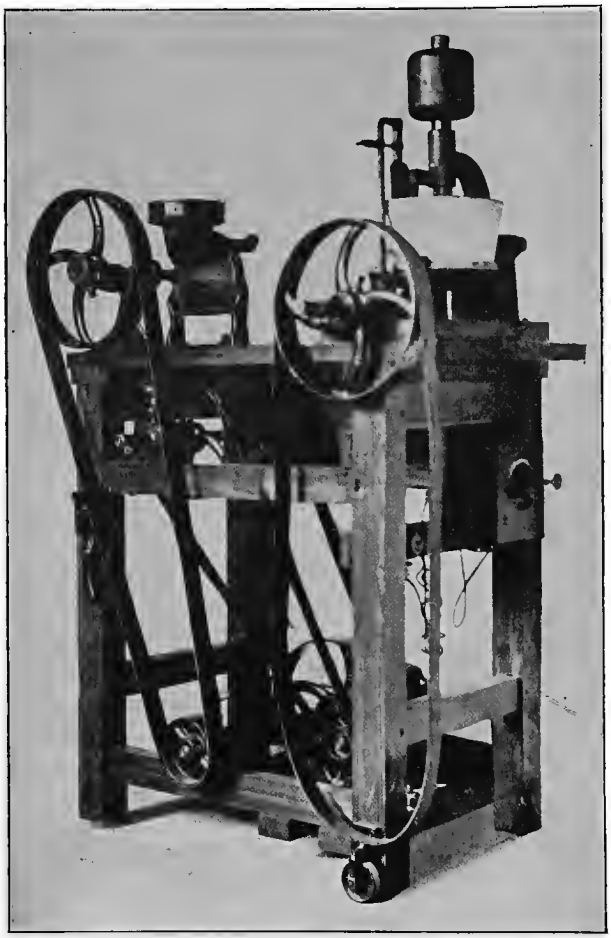


FIG. 1.—Grinding machinery for disintegrating raw material.

has been expended on the preliminary treatment of the materials, the importance of which can be hardly overestimated.

The method to be employed will depend largely on the nature of the material, and considerable experience and judgment are often

necessary to enable the operator to decide on the method to be adopted. For ordinary analytical operations, or for preparations on a small scale, the necessary preliminary processes can be carried out by hand; when a large number of operations have to be performed, or when it is necessary to prepare relatively large quantities of material (hundreds of grams or more) it is almost essential to work with mechanical power.

A. **Treatment of Material Containing Relatively Large Quantities of Moisture.**—Most animal tissues contain about 80% of moisture. Two alternative methods exist for treatment of these and similar materials, which can be submitted to investigation either (*a*) in the moist state, or (*b*) after freeing from water and fats and lipoids.

(*a*) *Treatment in the Moist State.*—The material is first freed from adhering fat (or connective tissue) by dissection, and then coarsely disintegrated by passing it two or three times through a mincing machine. Most of the ordinary domestic implements, such as sausage machines, are available for this purpose. This coarse disintegration leaves most of the cells intact, and these must be broken up before the material is available for investigation. The most usual method of accomplishing this purpose is to grind the material in a mortar with silver sand. When small quantities only are to be treated, this process can be easily performed by hand, especially if small quantities of toluene or toluene water (water saturated with toluene) are added. This hydrocarbon, on account of its capacity for dissolving lipoids, promotes cytolysis. When large quantities of material must be treated, the grinding process must be carried out with employment of mechanical power, and the latter is of advantage even when small quantities only are subjected to treatment. A convenient form of pestle and mortar for the purpose is that manufactured (in porcelain) by the End Runner Mill Co. of Ashbourne, (Eng.). An illustration of this apparatus as it is fitted up in the laboratory of the Research Institute of the Cancer Hospital is appended. In this case it is driven by an electric motor which also serves to drive a large coffee-machine (for grinding dry material) which is mounted on the same table as the mill. The grinding of the material is continued until a fine emulsion is formed, which can be readily pressed through muslin. In this condition it is available for extraction of the proteins.

Another method for breaking up the cells consists in alternately freezing and thawing the coarsely disintegrated material. The most effective agent for this purpose is liquid air.

(*b*) *Treatment by Desiccation.*—Various methods of desiccation have

been proposed in recent years, by means of which the material to be investigated can be obtained in the form of a fine dry powder, capable of passing through a fine sieve, and in which the proteins exist in an unaltered condition. As the albumins on heating undergo coagulation and other changes, it is necessary that the desiccating processes should be carried out at a relatively low temperature (not exceeding 35–38°).

A process for this purpose has been described in detail by Wiechowski (*Beitr, z. physiol u. pathol. Chem.*, 1907, **9**, 232) in which the manipulation is not difficult and which yields a very satisfactory product. For the purposes of disintegration for subsequent protein extraction, Wiechowski's method can be simplified in various details. The material is first passed through a mincing machine with the addition of toluene, and this process can be repeated two or three times. A quantity of water can be extracted from this minced material by suspending it in a linen bag, or metal basket in toluene in a vessel at the bottom of which is placed some fused calcium chloride; care must be taken that it does not come in contact with the latter substance. This preliminary drying is not, however, in all cases necessary. When the finely minced material is moist with toluene, the greater part of the moisture can be readily driven off at the temperature of 37°, especially when the mixture is exposed to a rapid current of air. With small quantities of soft material such as mucous membrane, pancreas gland, etc., this drying process can be carried out in an incubator, without the employment of a forced draught. In this case the toluene tissue emulsion is spread in a thin layer on a glass plate. As soon as the surface is dry, the plate is removed from the incubator and the material is scraped off from the plate, so that the still moist under-surface can be exposed. If necessary, a little more toluene is sprinkled over the moist surface. The material is then returned to the incubator, and the drying is continued until a product is obtained which can be eadily disintegrated on grinding in a mortar.

When dealing with harder material, such as muscular tissue, or with large quantities, it is necessary to dry the toluene emulsion of the substance under investigation in a good current of air. For this purpose a special apparatus is necessary, which can, however, be readily constructed by a tinsmith. This consists of a tinned iron chamber, either cylindrical or rectangular in shape, provided with an electrical fan at one end by means of which air can be either sucked or driven through the chamber from a large outlet at the other end. When air is sucked through (and the ventilating fan is placed in the front of the chamber), the air inlet should be covered with a thin sheet of cotton-

wool to filter off the dust; where air is driven through (and the ventilating-fan is placed at the back of the chamber), the air is filtered through a screen placed just in front of the fan. The toluene emulsion of the substance to be dried is introduced into the front of a chamber or perforated tinned trays, upon which it is spread in thin layers. The larger the perforations of the trays, the greater the surface of the mixture exposed to the current of warm air. The air is warmed by placing one or more lighted burners at the back part of the apparatus, some little way behind the trays containing the mixture to be dried. Where a warm (incubator) room is available, this heating is not necessary and the whole drying apparatus can be placed bodily in the warm chamber. When, however, an incubation room is not available, the cylindrical form of apparatus is preferable and the air is best drawn over by suction, the ventilating-fan being made so as to just fit inside the front part of the cylinder. A thermometer should be inserted just over the trays containing the material and the heating of the back part of the apparatus can then be so regulated that the temperature does not rise above 37°. The evaporation of the toluene and water serves, however, to automatically keep the temperature low, and it is only when the material is nearly dry that there is any danger of a large rise of temperature. Even then, the danger of injury to the material by heat is considerably diminished, as proteins have less tendency to undergo change when the amount of moisture present is small.

The accompanying illustration shows a form of drying chamber for use in an incubation room. Other forms are catalogued by various apparatus manufacturers but in most cases a simple and inexpensive chamber can be readily designed to meet the particular requirements for any given investigation.

When the drying process is sufficiently complete, the material can be readily disintegrated by grinding. With relatively small quantities of material, this process can be easily effected by an ordinary hand coffee-mill, but with larger quantities, it is again advisable to use motor power or a larger and stronger mill either hand- or power-driven. Various forms of paint-mills can be employed for this purpose, but the less expensive coffee-mill is generally quite satisfactory, unless large quantities of material are being continually manipulated, under which circumstances a machine of stronger construction is required. A coffee-mill mounted for grinding purposes is figured in the illustration (page 42).

The powder thus obtained generally contains appreciable quantities

of fats and lipoids, which can be extracted by an organic solvent either by percolation, extraction in a Soxhlet apparatus, or any other convenent method. After freeing from the solvent, the powder can be sieved. By this process muscular tissue can be separated from the greater part of the fibrous connective tissue, and can be obtained in a form which will pass through a fine-mesh sieve. The enzymes contained in the original tissue also remain intact. When in this form, furthermore, the proteins can generally be readily extracted, as water or other solvents have free access to the solid matter.

Another process for desiccation of tissues can also be conveniently employed in a large number of cases, which consists in the treatment of the material with an anhydrous salt, which by abstracting water is readily converted into a hydrated form. The salts usually employed

FIG. 2.—Drying chamber with fan for use in an incubation chamber.

for this purpose are sodium and calcium sulphates. The former is the more convenient as only a slight rise of temperature occurs when it is converted from the anhydrous to the hydrated form. The tissue or other material is ground up with a little more than the equal weight of the anhydrous salt. The mixture, after standing for some time, can be readily ground to a fine powder. If it appears moist after standing for an hour or so, and cannot be easily pulverised, a little more sodium sulphate should be added. Animal fluids such as blood or serum can also be treated in this way. Njegovan (*Biochem Zeitsch*), 1912, **43**, 203) recommends that in carrying out this process, the material warmed to 37° should be mixed well exactly the amount of anhydrous sodium sulphate necessary to combine with the water (to form $Na_2SO_4.10H_2O$), and the mixture should then be dried in a desiccator *in vacuo* over sulphuric acid. The total bulk can, by this

means be kept small. It must be remembered that any aqueous extracts of a powder obtained in this way will contain sodium sulphate in solution, and this fact must be taken into account when it is necessary to precipitate the dissolved proteins by the addition of salts. (See *salt precipitation of proteins*, pages 64 and 65.) The method has been found very convenient when it is required to separate the coagulable proteins by heat precipitation. (See page 54.)

As to the relative advantages of examining material in the moist and desiccated conditions, it must be remembered that proteins can be kept for an almost indefinite time without putrefying when in the latter condition. When, therefore, a large number of investigations have to be carried out, it is not always convenient to examine all the samples when fresh. As a large number can be readily desiccated at one time with the apparatus described, the desiccation method will be found to be of considerable service, especially as it is possible to reserve samples for future reference.

B. **Treatment of Material Containing Relatively Small Quantities of Moisture.**—In this category are included a large number of plant seeds, roots, etc., and also a large number of animal proteins belonging to the group of the scleroproteins. In this case the preliminary treatment is comparatively simple. The material is ground in a convenient form of mill (either of the edge-runner or paint or coffee-mill variety) to a fine powder. The form of mill to be employed can be determined by experiment. Small seeds and such like material can be best ground in a mill of the coffee-mill type, whereas large seeds, with husks that are readily broken are easily ground in the edge-runner type of mill or by pestle and mortar. Very hard material such as horn must be pulverised by some form of apparatus such as is employed for rock crushing. When the ground material contains oils or fats, the latter should be extracted after grinding, by light petroleum or ether. After extraction, the material is filtered off from the solvent (conveniently on a Buchner funnel, or by means of a porcelain centrifugal machine), and freed from the last traces of solvent by air-drying. It can then be sieved. In the case of many materials, the greater part of the husks can be separated by the last-named process.

C. **Material Requiring Special Treatment.**—Certain material which undergoes rapid change requires special treatment. Included in this category is muscular tissue which rapidly undergoes change after removal from the living organisms, with the onset of rigor. In investigating the protein changes in this case (which have as a rule already taken place in the ordinary butcher's meat) it is necessary to

remove the tissue as quickly as possible from the animal after death, and to carry out the disintegration process at a very low temperature. Investigations involving treatment of this description are, however, of a very special character and need no prolonged discussion in this place. Special apparatus has been devised for the purpose. (See for example, Kossel, *Zeitsch. physiol. Chem.*, 1901, **33**, 5.)

Quantitative Estimation of Total Proteins.

A. **Estimation of Materials in Which the Only Nitrogenous Products are Proteins.**—Where the only nitrogenous products are proteins, the amount of the latter substances present can be estimated to a fair degree of accuracy by ascertaining the amount of nitrogen present in the material. Where nitrogenous substances other than proteins are also present, the latter must be separated from the mixture by some method of precipitation; the quantity of protein present can in this case be ascertained, either by direct estimation of the nitrogen in the precipitate, or by estimation of the total nitrogen in the material, and of the nitrogen in the filtrate from the protein precipitate, the difference between these two numbers giving the amount of nitrogen in the proteins; the method to be chosen will depend upon the relative amounts of the various nitrogenous substances present, or the manipulative details of the experiment.

The nitrogen in the majority of proteins varies within comparatively narrow limits. (See page 18.) An approximately accurate estimation of the protein can as a rule be obtained by multiplying the amount of nitrogen found by the factor 6.25. If, however, a greater degree of accuracy is required, the pure proteins can be isolated by one of the various methods described in the succeeding sections, and the amount of nitrogen in the product thus obtained can be directly estimated. From this result the correct factor can be calculated.

The Estimation of Nitrogen by Kjeldahl's Method.—Nitrogen in proteins is now almost always estimated by the method originally devised by Kjehldahl. This consists in destroying the material by means of concentrated sulphuric acid. The carbon is oxidised to carbon dioxide, and the nitrogen is converted (except in the case of nitro-derivatives and a few other substances) into ammonia. By making the incineration mixture alkaline, after the destruction of the organic substances, and distilling off the ammonia into a known volume of standard acid, the amount formed in the process can be ascertained; from which result, the quantity of nitrogen present in the material can be directly calculated.

Many modifications of the original Kjeldahl method have been described. A large number of these are of quite unessential character, and it is unnecessary in this place to refer in detail to the literature of the subject. The method described below gives accurate results, and affords no difficulties in the manipulation. (For the official American (A. O. A. C.) method see Vol. I.)

A quantity of substance varying in weight from 0.5 to 5 grm. according to the amount of nitrogen present, is introduced into a round-bottomed flask of hard Jena glass, and some water, 10–20 c.c. of concentrated sulphuric acid and a crystal of copper sulphate are then introduced. The flask is then adjusted obliquely over a gas flame, and the excess of water is then gently evaporated off. The object of starting the process with dilute instead of concentrated acid is that the dilute acid hydrolyses the proteins present; the hydrolysis products are more readily destroyed by the concentrated acid than are the unhydrolysed proteins. The copper sulphate promotes catalytically the oxidation process. Mercury can also be employed for the same purpose. As soon as the acid is concentrated, the mouth of the incineration flask is closed by a loosely fitting balloon stopper, made by blowing a bulb or a piece of tubing, and drawing out and sealing the stem. The heating at first should be gentle, and so regulated that the incineration mixture does not froth up too much. When the first more violent action is over and the mixture is thoroughly charred, the size of the flame can be increased until the liquid in the flask boils briskly. The heating is continued until the contents of the flask are colourless. Care should be taken to have sufficient sulphuric acid present. If the contents of the flask show a tendency to become dry, more concentrated acid should be added. A modification of this process is sometimes useful when the material is refractory and is not easily incinerated by the acid which is often the case when it contains large quantities of fat. This consists in adding an amount of potassium sulphate equal in weight to that of the material, instead of a copper sulphate crystal. When the excess of water has been evaporated off, a higher temperature can be attained than when potassium sulphate is absent. When the material is very resistant to complete destruction the addition of potassium persulphate (Milbauer, *Zeitsch. analyt. Chem.*, 1903, **42**, 725; Dakin, *J. Soc. Chem. Ind.*, 1902, **21**, 848) or of fuming sulphuric acid (Budde and Schon, *Zeitsch. analyt. Chem.*, 1899, **38**, 344) and other oxidising substances has been recommended.

When the incineration is complete, the mixture is diluted with water, and 40% sodium hydroxide solution is carefully added until the

mixture is strongly alkaline. When copper sulphate has been added, this point will be indicated by the deep blue colour of the liquid. The mixture is then distilled until the distillate is no longer alkaline. The latter should be collected in a known volume of standard ($N/10$) sulphuric acid, which should be more than sufficient to neutralise all the ammonia which distils over. This amount can be ascertained by titrating the excess of acid when the distillation is complete.

If the incineration is carried out in a flask sufficiently large (700 c.c.), the ammonia can be distilled off from the same vessel. In conducting the distillation, it is advisable to cool the distillate before receiving the acid, as the hot vapours act upon the glass, and too high results are obtained. Various forms of apparatus appear in the catalogues of the manufacturers; most of them are in the form of batteries, so devised that several estimations of nitrogen can be carried out at one time. The form devised by Dr. Horace Brown is convenient, and is so constructed that the vapours exert the minimum action on the glass.

For titrating the excess of acid which has not been neutralised by the ammonia various indicators can be employed. Dialysed litmus and sodium alizarinsulphonate give satisfactory results.

Modifications of Kjeldahl's method have been devised to meet the cases where nitro-derivatives or other substances in which the nitrogen is not converted by sulphuric acid into the form of ammonia have been devised. Such substances are, however, rarely present in the material in which proteins have to be estimated, and it will suffice in this place to indicate the most commonly employed process used in these cases, viz., the method of Jodblaur. The substance, if not in solution, is intimately mixed with calcium sulphate (plaster of Paris), and introduced into the incineration flask. 25 c.c. of a mixture made by dissolving 40 grm. of phenol in 1 litre of concentrated sulphuric acid are then introduced and shaken round for a few minutes with the mixture of the substance with plaster of Paris. After standing for 5 minutes and cooling, 2–3 grm. of well-washed zinc dust and one or two drops of mercury are introduced. Heat is then gradually applied and the heating continued until the mixture is colourless. The ammonia is then distilled off in the usual way. In these operations the phenol is converted into a nitro-derivative, which is reduced to an amino-derivative by the zinc. From the amino-derivative ammonia is formed by the subsequent action of the sulphuric acid.

B. **Estimation of Proteins in Materials in which Nitrogenous Substances other than Proteins are Present.**—A very large num-

ber of methods have been proposed for estimating proteins when these substances are present with other nitrogenous compounds. The following are the chief processes employed: (*a*) Precipitation by compounds of heavy metals; (*b*) precipitation by organic solvents, especially alcohol; (*c*) precipitation by metallic salts; (*d*) precipitation of coagulable proteins in the form of a coagulum; (*e*) precipitation by or absorption from solution by colloids; (*f*) precipitation by alkaloidal reagents. The choice of method to be adopted will depend upon various circumstances. Thus, for example, all proteins are not coagulable; if therefore a coagulation method be applied to materials containing substances of this character the total proteins will not be precipitated. Various tissue extracts and animal fluids contain in addition to coagulable proteins such substances as mucins and nucleoproteins; these latter compounds will not be carried down quantitatively in the coagulum. In this case an alcohol precipitation method can be employed. It is possible, however, that the coagulation method may in this case be used to separate the coagulable from the non-coagulable proteins. (See, however, the discussion of the coagulation method given below, page 56.)

Where proteins occur mixed with their more complex digestion products, their separation from these latter substances is a matter of some difficulty. Certain of the proteoses (albumoses) are precipitated by the ordinary protein precipitants, such as alcohol, salts, etc., whereas others are not. In these cases, where possible, the proteins proper should be separated by a coagulation method. Animal tissues, as a rule, are free from proteoses, but certain natural vegetable products contain them. It is doubtful whether in such cases a perfectly satisfactory method exists for the estimation of the total proteins apart from the digestion products.

(*a*) **Precipitation with Compounds of Heavy Metals.** *The Process of Stutzer.*—This method consists in precipitating the protein by copper hydroxide. The reagent is prepared, according to Stutzer, by the following method: 100 grm. of copper sulphate are dissolved in 5 litres of water to which 2.5 grm. of glycerol are added. From this solution the copper hydroxide is precipitated by dilute sodium hydroxide solution which is added until the liquid is strongly alkaline. The copper hydroxide is filtered off and rubbed up with water containing 5% glycerol, and the supernatant liquid is then filtered off. This washing is repeated until the liquid is freed from the excess of alkali. The residue is then made up to a paste with water containing 10% glycerol, and so diluted that a paste suspension is formed, which can

be readily sucked up into a measuring pipette. The reagent should be preserved in flasks (preferably not too large; Sulzer recommends those of 150 c.c capacity) filled to the top and tightly corked, and kept in a cool dark place. The content in copper hydroxide of the reagent should be determined before use by estimating the total solids. The precipitation of the protein is carried out in the following way: 1 to 2 grm. of the finely sieved material are placed in a beaker with 100 c.c. of water, and heated to boiling. Where starch is present, the heating should be continued for 10 minutes, for which purpose the mixture is placed on a water-bath. 0.3 to 0.4 grm. copper hydroxide is then added and, after cooling, the precipitate is poured onto a Swedish filter paper and thoroughly washed with water. The filter with contents is then transferred to a round-bottomed flask, and the nitrogen estimated by Kjeldahl's method.

This process has been largely applied to the estimation of proteins in plant materials.

Where phosphates are present these should be precipitated by the addition of a few cubic centimetres of an alum solution before the addition of the cupric oxide. Where insoluble alkaloids are present, they can be removed by heating the material to boiling, first with alcohol containing 1% acetic acid and then pouring off the supernatant liquid onto a filter, which is afterward used for filtering the protein precipitate. The residual solid is then treated with water and copper hydroxide in the way already described.

A modification of Stutzer's process has been suggested by Barnstein, which is an application of a method which was originally employed by Ritthausen for the estimation of the proteins in milk. 1 to 2 grm. of the material under investigation are heated with 50 c.c. of water to the b. p. When starch is present, the heating is continued for 10 minutes in a water-bath. 25 c.c. of a copper sulphate solution containing 60 grm. of the crystalline sulphate in a litre are added, and then 25 c.c. of sodium hydroxide solution containing 12.5 grm. of the hydroxide in a litre (with constant stirring). The precipitate is allowed to settle, washed by decantation, the wash waters being poured through a filter and then brought onto the same filter and washed with warm water until the filtrate is free from copper (no reaction with potassium ferrocyanide). The nitrogen is then determined by Kjeldahl's method in the precipitate. By using the quantities of copper sulphate and sodium hydroxide given above, a green basic cupric sulphate is precipitated. Modifications as regards these quantities have been suggested, but it does not appear that the analytical results are more satisfactory.

Proteoses are precipitated by the above processes. As peptones do not occur (or only very rarely) in plants, the Stutzer and Barnstein methods can in most cases be applied to the estimation of the total proteins in material of vegetable origin.

Other compounds of heavy metals have been suggested for protein precipitation. Schjerning has employed uranium acetate (*Zeitsch. aualyt. Chem.*, 1900, **39**, 545 and 633)

(*b*) **Precipitation by Organic Solvents.**—This method is especially valuable for estimating the total proteins in animal tissues, which contain, in addition to coagulable proteins, substances such as mucin or nucleoproteins, which do not coagulate on heating. Alcohol is the solvent most generally employed, but acetone can also be used. The solution of the proteins is thrown into from 5 to 10 times the volume of alcohol or acetone with continual stirring, and after the mixture has stood for a short time, the proteins generally separate in a flocculent form, and can be readily filtered off, and washed with alcohol of the strength in which the precipitation was carried out (*e. g.*, 5 to 10 times the volume of alcohol to 1 volume of water). By this method, not only the natural proteins, but also some of the more complex digestion products (proteoses) are also precipitated. The coagulable proteins which are soluble in water, gradually become insoluble after long contact with alcohol or acetone. Hence, if it is necessary to redissolve the precipitate, the filtration should be carried out as soon as possible after filtration.

Hardy and Mrs. Stanley Gardiner have recently employed this process for separating the total proteins from blood-serum and plasma. For the purpose they cooled the serum or plasma to 0°, and then threw it into several times the volume of alcohol or acetone, cooled to −8°. The filtrate was then filtered off at 0°, washed with cooled ether (0°) until free from alcohol and acetone, and finally extracted with warm ether in a Soxhlet apparatus. When in this form, the warm ether did not coagulate the proteins. The white powder was finally dried *in vacuo* over sulphuric acid, and the total proteins were thus obtained free from adhering lipoids. The advantage of this method is that the proteins can be obtained in unchanged form and weighed, and used for subsequent investigations (*Proc. Physiol. Soc., J. Physiol.*, 1910, **40**, 68).

(*c*) **Precipitation by Salts.**—Proteins are precipitated from solution by various salts, of which the chief employed for the purpose are sodium chloride, magnesum sulphate, ammonium sulphate, zinc sulphate and sodium sulphate. The proteins differ from one another in that they are precipitated, either completely or partially, at different

degrees of saturation of the solution, by the salt. The differential salt precipitation is the only known method by means of which soluble proteins can be separated from one another. As, therefore, salt precipitation is employed more usually as a method for separation of the individual proteins from one another, rather than as a general method for precipitation of the total proteins, it may be more suitably discussed later under the heading of the Systematic Separation of the Proteins (page 64). With certain limitations, it may be employed for estimation of individual proteins, such as the albumins and globulins of blood serum.

(*d*) **Precipitation of Coagulable Proteins in the Form of a Coagulum.**—The actual changes involved in the process of coagulation are not yet fully understood. Recent researches have, however, clearly indicated that two distinct sets of phenomena are involved in the reaction, viz., (*a*) a chemical change caused by the interaction of the protein and the water, and (*b*) the agglutination of the chemically changed protein particles to form a coagulum which can be readily filtered. The former of these phenomena has been investigated by Chick and Martin (*J. Physiol.*, 1910, **40**, 405), who have confirmed isolated observations of earlier investigators in showing that dried proteins can be heated for some hours to temperatures of 110 to 130° without losing their solubilities in water. These authors have also shown that in some cases (*e.g.*, of hæmoglobin) coagulation is a chemical process of the first order with a very high temperature coefficient. In the case of the coagulation of egg-albumin, the conditions of the coagulation are somewhat more complex.

The second phenomenon, viz., that of agglutination, depends upon the fact that proteins, as will be clear from the chemical introduction to this article, can contain excess of either reactive amino or carboxyl groups, some members of the series yielding large quantities of dicarboxylic acids, others large quantities of diamino-acids on hydrolysis. Some will have, therefore, a predominating acid character, whereas others will possess a predominating basic character.[1] According, therefore, to the chemical (and stereochemical) character of the proteins, they will carry different electrical charges when suspended in an electrically neutral medium. Now according to the Helmholtz-Bredig hypothesis on the relationship between surface tension and electrical charge, particles suspended in a medium (the liquid in which

[1] The "reactive" basic or acid groups will depend not only on the yield of diamino or dicarboxylic acids yielded on hydrolysis, but probably also on the stereochemical configuration of the groups, some of these groups being conceivably so situated in the complex molecule that they are incapable of reaction. For a general discussion on this point see Schryver, "The General Characters of the Proteins," London, 1909, page 4.

the coagulation has been carried out) will possess their maximum surface tension (and the maximum tendency to agglutinate and fall out from the medium in which they are suspended) when their electrical charge is zero, that is to say, when they are suspended in a medium of definite hydrogen ion concentration known as the isoelectric point, which is characteristic for such individual protein. For the majority of the coagulable proteins this point is attained in a slightly acid medium. (Michaelis and Mostynski, *Biochem. Zeitsch.*, 1910, **24**, 79; Michaelis and Rona, *Ibid.*, 1910, **27**, 38, and 1910, **28**, 193, and other subsequent papers in the same journal by Michaelis and his collaborators). When the medium in which coagulation is carried out is appreciably more acid or more alkaline than the isoelectric point, the coagulation of the protein is inhibited. The isoelectric point shows, however, a certain range, *i.e.*, the acidity can be increased or diminished within certain limits, without materially affecting the coagulation. (See Michaelis, *Biochem. Zeitsch.*, 1911, **33**, 182, on the dissociation of amphoteric electrolytes.)

With regard to the conditions of the medium in which the chemical coagulative change takes place, it must be remembered that acids and alkalies hydrolyse proteins, forming as a first hydrolysis product the acid albumins, and alkali albumins. It has been found by Chick and Martin that the chemical change of coagulation in the proteins so far investigated proceeds best in slightly acid media. In media up to the strength of $N/50$ acetic acid, proteins can be heated without hydrolysis.

As a practical result of the investigations just mentioned, it has been found that proteins can be coagulated in such a form that they can be readily filtered off, when the process has been carried out in a not too acid medium. They have obtained the best results when the liquid under investigation has been made acid before heat-coagulation by means of a weak acid such as butyric acid. This should be added to the mixture until it is very distinctly acid to litmus paper. As the addition of a slight excess of the weak acid does not largely increase the hydrogen ion concentration (beyond the range for the optimum condition), such weak acids as butyric give particularly favorable results.

There is also one other factor which exerts a marked influence on the separation of a coagulum. This is the presence of salts and other dissolved substances in the medium in which the coagulation is carried out. Numerous researches have been undertaken with the object of determining the influence of this factor. (See especially Pauli, *Beitr. chem. Physiol. Path.*, 1907, **10**, 53, and Pauli and Handovsky, *Ibid.*, 1908, **11**, 415.) In view, however, of the recent investigations of Martin, Michaelis and others, and the light they throw upon the

phenomena involved in coagulation, these earlier researches require revision. In some cases the presence of salts accelerates agglutination (egg-albumin) whereas in others is impedes it (serum-albumin). In the case of egg-albumin, the accelerating capacity of sulphates is markedly greater than that of the chlorides.

In the present state of our knowledge, it is not possible to generalise on the influence of salts on the coagulative process. It would seem advisable where salts are present which impede coagulation, to dialyse the liquid first and then to heat after acidifying with butyric acid.

Researches are also required on the influence of substances other than salts. It would be of interest, for example, to know how such substances, such as nucleoproteins and mucins, which are non-coagulable proteins, affect the coagulation of the coagulable substances, as mixtures of these two classes of substances are often met with in tissues. An accurate quantitative method for their separation is a desideratum, although they can be quantitatively separated together by the already described alcohol method. An interesting observation of Michaelis and Rona (*Biochem. Zeitsch.*, 1910, **29**, 494) that an intermediate coagulation product of serum-albumin, which by solution is converted into what is apparently the natural albumin, is worthy of recording in this place.

Various modifications of the method of simple coagulation in acid solution have been suggested, of which two are given below.

Devoto's Method for Estimation of Proteins in Urine.—100 c.c. of the urine are employed to which 50 c.c. of ammonium sulphate crystals are added with continual stirring. The mixture is slightly warmed to promote the solution of the crystals. The mixture is then heated for 1/2 hour in a closed vessel in a boiling water-bath. The nitrogen is estimated in the coagulum.

Hopkins has modified Devoto's method by filtering the coagulum onto a hardened paper, and then transferring it back to a beaker with water, and heating the suspension for 1/2 hour in a water-bath. The precipitate is then filtered again onto the same hardened filter. The large amount of salt appears to interfere with the agglutination of the coagulated protein. (See remarks in preceding section.) (*J. Physiol*, 1890, **25**, 306.)

Coagulation by Alcohol.—A convenient method for estimating proteins in tissues, serum, etc., consists in treating the liquid or solid material in a fine state of subdivision with somewhat more than an equal weight of anhydrous sodium sulphate, which combines with the water forming hydrated sodium sulphate. After drying in this

manner, the mixture can be powdered, and in this form boiled for half an hour under reflex condensor with absolute alcohol. The alcohol is then filtered off through a nitrogen-free filter paper, and the coagulated protein is then washed onto this paper by hot water, and the washing continued until the washings are free from sulphate. The filter paper with the coagulum is then transferred to a flask and the nitrogen is estimated by Kjeldahl's method. In the case of tissues and serum about 5 grm. are sufficient. The tissue or serum are introduced into a glass mortar, the sulphate is added, and after standing for some time, until the material is dry on the surface, the mixture is stirred with the pestle, and, if necessary, more sulphate is added. The addition of this salt should be continued until the whole mass can be readily powdered. It is then transferred to a flask into which also the small amounts of powder remaining in the mortar are quantitatively removed by rubbing them up with small additional quantities of sodium sulphate. After heating with alcohol, the latter is decanted off through the filter and the residual coagulum is gradually transferred to the filter by means of hot water. The nitrogen can if necessary be estimated in the filtrate instead of the coagulum. The former method is, however, the more convenient as it is free from salt and can be incinerated more readily with smaller quantities of sulphuric acid. Mucins and nucleoproteins will be found in the filtrate from the coagulum, but no experiments have been carried out to determine whether the method is available for separating quantitatively in all cases such substances from coagulable proteins. It is, however, applicable to the estimation of total proteins in a large number of materials, and has furthermore the advantage in that where a large number of analyses have to be carried out with material that readily putrefies, the latter can be kept after treatment with sodium sulphate for a long time without undergoing change, and the analyses can be performed when convenient. The amount of non-protein nitrogen can be ascertained either in the filtrate of the coagulum, or, better still, by estimating the total nitrogen in a fresh portion of the material and deducting therefrom the amount of nitrogen found in the coagulum. (Schryver, *Biochem. J.*, 1906, **1**, 129.)

(*e*) **Separation of Proteins from Solution by Precipitation with, or Adsorption by Colloids.**—Certain colloidal substances, such as kaolin, adsorb proteins from solution (Landsteiner and Uhlirz, *Centralbl. Bakt. u. Parasitenk., Abth.* 1, 1905, **40**, 265). This method has been employed by Michaelis and Rona (*Biochem. Zeitsch.*, 1907, **5**, 365) for quantitatively separating proteins from solution. These

same investigators have, however, introduced other methods depending upon the capacity of colloidal substances for removing proteins from solution, in one of which gum-mastic was employed, and in the other dialysed colloidal ferric hydroxide.

Gum-mastic Method (*Biochem. Zeitsch.*, 1906, **2**, 219; 1907, **3**, 109; 1907, **4**, 11; 1907, **5**, 365; 1907, **6**, 1).—When an alcoholic solution of gum-mastic is added to water, an emulsion is formed, which can be de-emulsified on the addition of electrolytes, when the mastic separates in the form of a flocculent precipitate. If proteins be present at the same time, these will be carried down with the mastic precipitate, if the latter be present in sufficient excess. Certain of the proteoses are also precipitated in this way. The method has been chiefly applied to blood-serum, and the method of employment is illustrated by the following example: One volume of serum is mixed with three volumes of alcohol, whereby a part of the protein is precipitated. To the filtrate is then added one volume of a 50% solution of mastic in alcohol. The mixture is then diluted till the alcohol does not form more than 30% of the total fluid. The liquid is then just acidified with acetic acid, and 10% magnesium sulphate in the proportion of 10 to 15 c.c. for each litre of liquid is added. The mastic will carry down the last traces of protein. If the total nitrogen in the serum be estimated and also the nitrogen in the filtrate, the amount of protein can be calculated. It is advisable to partly separate the proteins by alcohol before addition of the mastic solution, as the latter must be in large excess.

Dialysed Ferric Hydroxide Method (*Biochem. Zeitsch.*, 1908, **7**, 329).—The principle of this method is similar to that of the mastic method and has also been applied by the discoverers chiefly to the investigation of serum and plasma, although it is probably applicable to other fluids. As an example of the method the following illustration is given: 50 c.c. of serum are diluted with 10–12 times the bulk of water. 40 c.c. of *liquor ferri dialysatus* are then added, drop by drop, with constant shaking. The protein is precipitated, the non-protein substances remaining in the filtrate, the presence of the salts causing the iron hydroxide to separate from solution, carrying with it the proteins.

(*f*) **Precipitation by Alkaloidal Reagents.**—Proteins can be precipitated by potassio-mercuric iodide, tannic acid, phosphotungstic acid, and other alkaloidal precipitants. As these reagents precipitate so many substances other than proteins, there is not much object in employing them. One special method deserves mention owing to the frequency with which it is employed for one special purpose.

Esbach's Method for Estimating the Urinary Proteins.—This is only

a rough method, but it is sufficiently accurate for many clinical purposes, and, owing to the simplicity of manipulation, is well adapted to the purpose for which it was devised. It consists in adding a picric acid solution to urine and *measuring* in a special tube the amount of precipitate. The picric acid reagent is made by dissolving 10 grm. of picric acid and 10 grm. of citric acid in water and diluting to a litre. The urine is acidified with acetic acid, if not already acid, and diluted till the sp. gr. is 1.006–1.008. This is poured into the tube (Fig. 3). to the mark *U*. The reagent is then added to the mark *R*. After standing for 24 hours, the height of the deposit in the tube which is graduated in amounts of protein in grm. per litre, is read off. The method can also be employed for approximate estimations of protein from other sources.

FIG. 3. Esbach's tube.

The Isolation of the Individual Proteins from the Prepared Material and Their Quantitative Estimation.

In the classification of the proteins given on page 33 it will be noticed that the various classes differ from one another in their behaviour toward solvents. Some, such as the albumins, are soluble in pure water; others, such as the globulins, are almost insoluble in pure water, but soluble in aqueous solutions of salts. Another class is distinguished by the fact that its members are insoluble in water or salt solutions, but soluble in aqueous alcohol, while the members of a fourth class are insoluble in all the above solvents, but dissolve in alkaline solutions, from which they can be precipitated by subsequent addition of acids. In addition to the proteins of these classes, a large number are known, belonging chiefly to the group designated as sclero-proteins, which form to a great extent the supporting and connective tissue of organisms, which are insoluble in all known solvents. In discussing the systematic investigation of protein-containing material, it will be convenient, therefore, to treat separately the examination of those proteins which can be obtained in solutions, and those proteins which are insoluble[1] in all known solvents.

A. The Systematic Examination of Proteins Which can be Obtained in Solution.[2]

When the material contains soluble proteins belonging to several classes, the individuals can be separately extracted and separated from

[1] *I.e.*, insoluble without decomposition, *e.g.*, hydrolysis.
[2] The conjugated proteins are dealt with later (pages 70 *et seq.*).

one another by treatment of the prepared (fat-free and disintegrated) material by a series of solvents in the following order:

(i) Pure water, which dissolve the *albumins*.
(ii) Saline solutions, which dissolve the *globulins*.
(iii) Aqueous alcohol, which dissolves the *prolamines*.
(iv) Dilute alkaline solution, which dissolves the *glutelins*.

The prolamines and glutelins have so far been isolated only from material of vegetable origin. Animal tissues are therefore extracted only by the first two named solvents. Certain plant material contains, however, all the above classes. In addition, plant and vegetable tissues contain in a large number of cases *conjugated proteins*, especially *nucleoproteins*. These are often extracted by water in the form of a soluble salt, and can be precipitated from solution by the careful addition of acetic acid, either before or after separation of other proteins by coagulation. In certain cases they are extracted by sodium carbonate solution. They contain phosphorous, and some idea as to the amount present can be formed by estimating the phosphorus in organic combination in the solution. The details of their preparation, and their properties will be considered later. It must be remembered that these substances, and other conjugated proteins can be extracted by water from protein-containing material, some of which is relatively rich in these substances, and their presence in aqueous extracts must not be overlooked.

Notes on the Methods of Manipulation.—Although the processes involved in the examination of protein-containing material appear at first sight very simple, the actual manipulation is often a matter of some considerable difficulty, necessitating a certain amount of experience for the successful accomplishment. The chief difficulty is connected with the filtration of the extracts.

(*a*) *The Solvents and the Methods of Extraction.*—When water is employed for extraction, it must be remembered that the material often contains salts. The aqueous extract may in these cases contain, therefore, the globulins, which are practically insoluble in pure water, though soluble in saline solutions. If much water be employed for extraction, the saline solution will be so dilute that relatively little globulin will be dissolved. If the extract be submitted to dialysis in a parchment membrane, first against tap water, and then against distilled water until the solution in the dialysing membrane is practically free from the salts, the globulins will be precipitated and can then be filtered off; the filtrate will contain the albumins and water-soluble conjugated proteins.

The process of aqueous extraction is often facilitated by agitating the material under examination with the solvent in a shaking machine.

Plant globulins are usually extracted with warm (60°) 10% sodium chloride solution. Sodium salicylate in $N/4$ solution is also a very good solvent, and extracts most plant globulins when cold. (Schryver, *Proc Roy. Soc.*, 1911 **83B**, 96). In this case, far smaller quantities of solvent can be used for extraction than when sodium chloride is employed. The amount of solvent to be used in each case will depend on the globulin content, and can be readily ascertained by preliminary experiment with small quantities of material. Five or six times the weight of sodium chloride solution is usually ample, and when $N/4$ sodium salicylate is used, an equal bulk of the solution will often suffice. (For methods of filtering the pasty material thus obtained, see notes on filtration, page 63, below.) The animal globulins are soluble in salt solutions of lower concentration. (See Mellanby, *J. Physiol.*, 1905–6, **33**, 338.)

The alcohol soluble proteins are practically insoluble in pure alcohol and water. The optimum concentration for the solvent is 70% alcohol. The glutelins can be obtained from the residue of the material after extraction with water, salt solutions and alcohol by treatment with 0.5% potassium hydroxide solution. It is advisable to submit the *dried* residue to this extraction by alkali. The drying is accomplished by treating the residue after exhaustive extraction with 70% alcohol with gradually increasing strengths of alcohol, and finally with ether, and removing the last traces *in vacuo*. The material can then be readily powdered.

In all cases, when a quantitative estimation of the various proteins is required, the material should be extracted to exhaustion by the above-mentioned solvents in the order named. The first extract can be made up to a definite volume after the solvent and material under examination have been a sufficiently long time in contact (with, when necessary, mechanical agitation), and the protein dissolved can be estimated in an aliquot part of the extract. To determine whether a particular protein has been entirely removed, the ordinary protein tests (pages 35 *et seq.*) can be applied to the successive extracts.

(*b*) *On the Methods of Filtration.*—The filtration of protein solutions is often a process of considerable difficulty owing to the slowness with which they pass through ordinary filter paper. Precautions must in all cases be taken to avoid putrefaction during the process. This can be generally avoided by the addition to the solutions of various antiseptics, of which the chief employed are toluene, menthol, thymol and

sodium fluoride. (Note: The commercial preparation of this substance is often markedly alkaline.) Antiseptics, which act chemically on proteins, such as mercuric chloride or formaldehyde, should be avoided. Even, however, when the danger of putrefaction can be avoided, the process of filtration by ordinary methods, especially of large quantities of liquid, is so prolonged and tedious as to be impracticable. In working with small quantities of material, enough solution can be generally obtained by filtration through folded filter papers. When carrying out quantitative investigations, an aliquot portion of the total liquid used for extraction can be filtered through and used for subsequent work.

Various modifications of the ordinary filtration methods have been suggested when working with protein solutions. Mucilaginous matter can often be separated in a satisfactory manner by filtration through muslin or gauze. Egg-white solutions can be filtered in this manner. Some solutions, which will not filter through paper sheets, can be filtered through paper pulp. This is made by shredding filter paper and rubbing up the pieces to a thick paste with water, or the solvent employed for extraction (*e.g.*, saline solution) and then throwing the paste onto a Buchner funnel, and sucking off excess of the solvent by means of a filter pump. The thickness of the layer convenient for the process can be ascertained by preliminary experiment. This will depend upon the fineness of the suspended particles and the amount to be filtered. As the filter becomes clogged, the solid particles can be removed from the surface of the pulp by scraping.

In many cases this method of filtration is hardly more satisfactory than the employment of the ordinary filter, and for dealing with these a special process has been devised by Osborne. This consists in adding shredded filter paper to the mixture of the solvent and the material undergoing extraction until an apparently solid mass is obtained. This mass is then subjected to high pressure in a Buchner press (which is made by the *Vereinigte Fabriken für Laboratoriums bedarf* of Berlin) by means of which a pressure as high as 300 kilo per square centimetre can be attained, and continuing the pressure until most of the added liquid has been squeezed out. The slightly turbid filtrate obtained in this manner can, as a rule, be readily filtered through the ordinary filter, or through filter pulp. It is advisable that the amount of liquid should be small in comparison with the solid, otherwise very large quantities of filter paper are necessary to produce the apparently dry mass. When the quantities are large, as much as possible of the liquid should be separated either by decantation or filtration through a folded filter. Osborne himself has applied it to

the residues left on large filters, after the filtration has continued for three or four hours. It is often, however, possible to use quantities of solvent which are small in comparison with the material to be extracted (as in the case mentioned above, where sodium salicylate solution is employed for the extraction of plant globulins. In this case Osborne's method can be directly applied to the mixture.)

In other cases, filtration can be entirely avoided by centrifugalisation. The solid particles then separate at the bottom of the containing vessels, and the supernatant liquid can be pipetted off. To get quite clear supernatant fluids, prolonged centrifugalisation at high speeds is often necessary and several makers supply machines capable of rotating at the speed of 5,000 revolutions a minute, or even more rapidly still.

The Separation of the Proteins from the Solutions in Various Solvents.

(i) **The Separation from Aqueous Solutions.**—Where the material has been subjected to aqueous extraction, substances which are insoluble in pure water, are often extracted owing to the presence of salts. Attention has been already called to this point. This same remark also applies to certain fluids such as blood serum, which, owing to the presence of salts, contains globulins, which are practically insoluble in pure water. When such substances are present, the aqueous extract or other fluid should be subjected to dialysis until it is practically salt free. Care must be taken to prevent putrefaction during this process; and disinfectants such as toluene, chloroform or thymol should be added. During the process of dialysis, the globulins separate and can be filtered off. The filtrate will then contain the albumins, nucleoproteins, or other water soluble proteins. Various methods exist for separating these materials. In any case where a preparation free from salts or other simple substances is required, the solution should be submitted to preliminary dialysis.

(*a*) *The Method of Desiccation.*—The water can be directly evaporated off and the proteins obtained as a dry residue. Care must be taken that during this process the temperature does not rise too high, otherwise albumins undergo coagulative change. It is inadvisable that the temperature should exceed 37°. Where the protein solution is dilute, it can be best treated by first concentrating in an ordinary vacuum distillation apparatus. The concentrated solution should then be evaporated down further in shallow layers *in vacuo*. The "Petri" dishes, which are employed in bacteriology, are very conven-

ient for this purpose. The proteins are then often obtained in the form of scales, which can be readily scraped off from the bottom of the dish. When the solutions are sufficiently concentrated, or when the quantities of liquid to be manipulated are small, the solution can be evaporated directly in thin layers, without previous evaporation in a distillation apparatus. The final evaporation can be carried out either in a stream of air in an incubator (37°) or in a desiccator, either at ordinary temperatures or at 37°. In any case, evaporation must be sufficiently rapid to prevent putrefaction. If the liquid be placed in shallow dishes in sufficiently thin layers, desiccation will generally follow after leaving it for one night at 37°, without forced draught. An apparatus for working with forced draught is figured on page 46.

(*b*) *Method of Precipitation by Organic Solvents.*—The proteins are precipitated from aqueous solution by throwing the latter into 5–10 times the volume of alcohol or acetone. If the precipitate be allowed to remain too long in contact with the solvent, coagulation is apt to take place. A convenient method of precipitation by ice-cold alcohol has been already described (page 53). By this method, unchanged natural proteins can be obtained.

(*c*) *Method of Precipitation by Salts.*—This is one of the most important methods for the preparation of proteins—in fact, the only method available for the separation of various water-soluble proteins from one another. The proteins differ from one another in that they are separated from solution by different concentrations of various salts. Thus, the serum-globulins are precipitated (from dilute salt-solutions) by saturation with sodium chloride and magnesium sulphate, and by half-saturation with ammonium sulphate, whereas, the serum-albumins are not precipitated at all by the first two-named salts, and are completely precipitated by ammonium sulphate only when this salt is added in such quantities as to nearly completely saturate the solution. The degrees of saturation at which precipitation begins or ends for any particular salt are characteristics for each individual protein; they vary slightly with the concentration of the protein solution, a convenient strength for determining these factors being about 1%. The method of separation of proteins by the salting-out process was systematically investigated by Dénis (*Nouvelles études chimiques, Paris*, 1856, and *Mémoire sur le sang*, Paris, 1859) and was the subject of many subsequent investigations by Hofmeister and his pupils and by Kühne who applied the process to the separation of the digestion products of the proteins. The property of precipitating proteins by a salt is an additive one, depending upon both the acid

and the metal. This will be seen by reference to the following table, in which the figures indicate the number of grams in 100 c.c. of solution when precipitation of globulin commences from a solution of egg-albumin, where the protein is present to the extent of 2 grm. in 100 c.c. after admixture of the salt solution. In this table it will be noticed that the metals show a decreasing power of precipitation, passing from left to right, whereas the acids show a decreasing power, passing from the top to the bottom of the table.[1]

	Lithium	Sodium	Potassium	Ammonium	Magnesium
Sulphate.....	8.61	11.39	No pp.	13.39	15.93
Phosphate....	Not investigated	11.69	13.99	16.57	.....
Acetate.....	Not investigated	13.83	16.38	No pp.	No pp.
Citrate.....	Not investigated	14.42	17.07	21.99	Not investigated
Tartrate.....	Not investigated	15.11	17.08	25.05	Not investigated
Bicarbonate..	Not investigated	No pp.	25.37	Not investigated	Not investigated
Chromate.....	Not investigated	21.22	25.59	No pp.	Not investigated
Chloride.....	Changes proteins	21.21	26.28	No pp.	No pp.
Nitrate.....	Not investigated	46.10	No pp.	No pp.	Not investigated
Chlorate.....	Not investigated	58.82	No pp.	Not investigated	Not investigated

The actual physical interpretation of the "salting-out" phenomenon has not yet been determined. In actual practice, only a limited number of salts have been employed for the process, these being sodium chloride and the sulphates of sodium, ammonium, magnesium and zinc. Some salts, such as potassium sulphate, are unavailable for the purpose, owing to the fact that they reach their limit of solubility in water before they can precipitate.

The limits of precipitability of a protein by salt solutions may be ascertained in the following way: To 2 c.c. of the protein solution contained in a series of test-tubes are added 8 c.c. of liquid containing varying quantities of water and a saturated solution of the salt under investigation. The lower limit denoting incipient precipitation is observed. The precipitate in the test-tubes containing larger concentrations of the salt is filtered off and one or two drops of the salt solution are added to the filtrate. As soon as these fail to produce a precipitate, the higher limit of precipitation is reached. If more than one protein be present, a second precipitation will be observed on addition of further quantities of salt after the higher precipitation limit has been reached if the second protein has different precipitation limits to the first. The lower and higher precipitation limits of the second protein can be determined in a series of test-tubes to which salt solution of higher concentrations has been added. In the case of the digestion products of the proteins which will be discussed in detail later (page 473), several frac-

[1] (Hofmeister, *Arch. exp. Path. Pharm.*, 1887, 24, 247, and 1888, 25, 1.)

tions with different precipitation limits have been isolated; the isolation of such products has been one of the most important applications of the "salting-out" method.

In separating proteins by this process, two alternative methods are applicable; the fractions can be separated either by different concentrations of the same salt, or different salts can be employed. Thus in the case of the serum proteins, the globulin fraction can be precipitated either by saturation with sodium chloride or magnesium sulphate, which do not precipitate the albumin fractions, or by half-saturation with ammonium sulphate; the albumin fraction is completely precipitated only when the solution is nearly fully saturated by the last-named salt.

It must not be supposed, however, that the fractions obtained by the salting-out method are definite chemical entities. A quantitative separation of different proteins can no more be accomplished by fractional salt precipitation, than can the separation of several liquids with not far removed boiling-points by the method of fractional distillation. A precipitate produced by, for example, half-saturation with ammonium sulphate, will contain a certain amount of the protein which is precipitated by full saturation, and *vice versa*, the precipitation of a substance which, for the most part, comes down on half-saturation, is not complete. (For an experimental investigation of this subject, see Haslam, *J. Physiol.*, 1905, **32**, 267, and 1907, **36**, 164.) Purer fractions can be obtained by repeated re-solution of the precipitates in water, and re-precipitation at a given volume in a definite degree of saturation with a given salt. For this reason, the salting-out method can only be used quantitatively in a quite empirical way; thus, for example, it is possible to estimate the amount of protein precipitated from a given dilution of serum on half-saturation (globulin fraction) and complete saturation (albumin fraction) with ammonium sulphate, and to determine the changes in the relative amounts of these fractions under different circumstances, such as in samples of serum removed from the same animal in varying conditions of nutrition.[1] In a similar way, it is possible empirically to separate digestion products of proteins into fractions; in all cases it must be remembered that fractions thus obtained are not chemical entities. With pure proteins, on the other hand, the precipitation limits for any salt may be regarded more or less as definite physical constants for any given concentration of the protein.

The method can often be conveniently employed for separating a

[1] The more dilute the protein solution, the more completely is a separation by salt precipitation accomplished. See recent paper on separation of globulin and albumin in serum by Wiener, *Zeitsch physiol. Chem.*, 1911, 24, 29.

protein from a large bulk of solution. The precipitate obtained by salting-out can be dissolved in a small amount of water, after washing with salt-solution of the same degree of saturation in which the precipitation was carried out. The solution thus obtained can be freed from salt by dialysis, and the protein obtained therefrom by the method of desiccation described above.

(*d*) **The Separation of Proteins from Solution in a Crystalline Form.**—Several proteins can be obtained from solution in a crystalline form. From aqueous solution, egg- and serum-albumins and hæmoglobin can be prepared in the form of crystals, and from saline solutions various globulins. (See below, page 68.) It will suffice here to indicate the method for preparing crystalline egg-albumin (Hopkins and Pinkus, *J. Physiol.*, 1898, **23**, 130). Egg-white is beaten to a froth (to break up the membranes) with exactly its own bulk of saturated ammonium sulphate solution. The mixture after standing overnight, or at least for a few hours, is filtered from the precipitated protein. The filtrate is now measured. 10% acetic acid is then very gradually added from a burette until a definite precipitate is formed, and not a mere opalescence due to the liberated gas bubbles. The amount of acid necessary to produce this will vary (chiefly because of the varying loss of ammonia when the liquid has been allowed to stand in open vessels). This point corresponds to the point of incipient acidity to litmus. When this stage is reached 1 c.c. of the diluted acetic acid for each 100 c.c. of liquid is added in addition to that already present. The precipitate thus produced is at first amorphous, but on shaking occasionally and allowing to stand for 24 hours, the full yield of crystals will be obtained. The product can be recrystallised by dissolving in water and adding half-saturated ammonium sulphate containing acetic acid in the proportion of 1–1,000 till a permanent precipitate forms, and then adding 2 c.c. of the acid ammonium sulphate mixture for each 100 c.c. of liquid in excess of this.

Serum-albumin can be obtained crystalline in a similar way, if the liquid be made acid with sulphuric instead of acetic acid. Crystallisation takes place more readily at somewhat higher temperatures (35–40°) (Inagaki, *Verh. phys. med. Gesellsch. Würzburg*, 1906, **38**, 17).

The relationship of these crystalline proteins to the natural proteins is not accurately known. They differ in certain physical properties. It is generally thought that they are the salts of the proteins.

(ii) **The Separation of Proteins from Saline Solutions.**—When a protein is insoluble in water but soluble in saline solutions, it can be separated from the latter either by dilution with water or by dialysing

away the salt, or by "salting-out." Certain proteins of this class can also be obtained in crystalline form.

When the protein is separated by salt precipitation, it is sometimes possible to employ the same salt as that from which the saline solution is made. Thus, if the globulin be dissolved in ammonium sulphate solution, it can be precipitated by increasing the salt concentration until the precipitation limit is reached. In other cases, when precipitation in a given saline solution does not take place even when the solution is saturated, another salt must be employed.

Many of the plant globulins can be obtained from the saline solutions in crystalline form, and as an example of the method, Osborne's modification of the earlier methods for preparing crystalline edestin may be quoted. The hemp seeds are first milled and the oil extracted with light petroleum and sieved. 1 kilo of the powder thus obtained is then treated with 2 litres of 10% sodium chloride solution. To this mixture is then added 800 c.c. of 10% sodium chloride solution to which has been added half the amount of concentrated barium hydroxide solution necessary to produce an alkaline reaction to phenolphthaleïn. This quantity is determined by estimating the amount of barium hydroxide necessary to produce a pink colouration with an aliquot portion of the saline-edestin mixture. The object of the addition of the barium hydroxide is to set free edestin from its combination with acid, as it exists in the saline solution partly in the form of salt. The whole is then thoroughly stirred and thrown onto three or four large folded filter papers. After three hours, about 1,500 c.c. of liquid should filter through. The residue and filters are then mixed with filter paper so as to form an apparently solid mixture, which is then pressed in a Buchner press (see method already described, page 62). The liquids thus obtained are filtered through a paper-pulp filter (page 62) and from the slightly opalescent solution thus obtained, the edestin can be separated in crystalline form by one of two methods. Either the solution can be placed in large dialysing membranes and the salt dialysed away when the edestin after 3 or 4 days separates, and can be filtered off on a hardened filter paper and then washed with alcohol and ether and dried over sulphuric acid *in vacuo*, or the opalescent solution is diluted with water heated to 70° until the sodium chloride concentration is reduced to 3%, and then allowed to stand for some hours at 5°, when the greater part of the edestin separates. It can be recrystallised by making an 8% solution in a 10% saline solution, warming the solution thus obtained, after filtering it through paper pulp, to 50° and diluting with twice the

volume of water of the same temperature. On cooling this mixture to 5° a very pure edestin is obtained. This should be washed after filtration with 0.5% saline, then with 50% alcohol, till free from chloride and finally with increasing strengths of alcohol and ether and then dried *in vacuo*. If allowed to dry in the presence of moisture, horny masses are obtained which will not dissolve in salt solutions.

Edestin may also be extracted with $N/4$ sodium salicylate from which solution it can be precipitated by diluting with 10 times the volume of water (see page 61). The precipitate thus obtained can be recrystallised from sodium chloride solution by the method described above.

Many other plant proteins can be prepared in a similar way. (See article on plant-proteins, this volume, pages 94 and 95.)

(iii) **Separation of Proteins from a Solution in Aqueous Alcohol.** 70% alcohol is a convenient solvent for gliadin and other alcohol soluble proteins. The extract is evaporated *in vacuo* (temperature of water-bath not to exceed 70°) while the liquid is turbid. If the protein separates out in an oily form, more strong alcohol must be added to bring it into solution. The thick syrup thus obtained is then thrown into 6 to 8 times the volume of ice-cold distilled water to which a small quantity of salt has been added. The protein then separates and is redissolved in strong alcohol, with the addition of water, if necessary, *i.e.*, if sufficient is not present in the original precipitate to make the alcohol sufficiently dilute to dissolve it. The solution is then thrown into cold water and the process of solution and precipitation again repeated if necessary. By this means the protein is freed from carbohydrates and other non-protein substances. The precipitate is then taken up with alcohol, the extract evaporated and absolute alcohol added from time to time, so long as the solution remains clear. The evaporation and addition of alcohol is continued until a thick syrupy solution in concentrated alcohol is obtained. This is then thrown in a fine stream into 8 to 10 times the volume of strong alcohol. The protein then separates as a sticky mass, which if kept under absolute alcohol gradually becomes hard and can be powdered. It is disintegrated under alcohol, and rapidly washed with exclusion of the air moisture as much as possible, with alcohol and ether, and finally dried *in vacuo* over sulphuric acid. If moisture gains excess to the mass, it is re-converted into a pasty condition.

(iv) **Separation of Proteins from Solutions in Alkalies.**—The proteins which are insoluble in water, salt solutions and alcohol are also, as far as they have yet been described, plant proteins. The

typical example, glutenin, is obtained from wheat meal or gluten after the extraction of the alcohol-soluble protein (gliadin) from the same, by treatment with 0.5% potassium hydroxide solution. This solution is filtered through pulp, and the protein precipitated therefrom by neutralising with very dilute hydrochloric acid solution. It can be purified after separating the last traces of gliadin which it is apt to contain, with 70% alcohol, by redissolving in alkali and reprecipitation by acid.

B. The Preparation of Proteins Which Cannot be Obtained in Solution.

The general method of preparing the proteins of this class consists in separating all the other constituents of the material under investigation, which can be obtained in solution. The treatment will consequently vary in each individual case, according to the nature of the other constituents. Water will remove the soluble salts and carbohydrates; ether or petroleum the fats and other lipoid constituents. Insoluble carbohydrates such as starch can be removed by digestion with diastase, by means of which they are converted into soluble carbohydrates, and certain digestible protein constituents can be removed by digesting the material with a proteoclastic enzyme; in this way certain scleroproteins such as horn can be prepared, as these are not acted upon by the digestive enzymes. References to the methods of preparing the proteins included under the above heading are given in the table containing the lists of known proteins on pages 91 and 92.

The Properties and Methods of Preparation of the Conjugated Proteins.

A. **The Nucleoproteins.**—This important class of proteins is widely distributed in both the animal and vegetable kingdoms and is distinguished by the fact that the protein is in combination with a nucleic acid. These proteins are supposed to form a constituent of the nuclei of cells.

Nucleic Acid.—Although nucleoproteins are so widely distributed, they only occur in the majority of tissues in very small quantities—in fact only very few materials contain nucleoproteins in sufficient amounts for it to be possible to isolate and identify the nucleic acid without employing very large amounts of material. The tissues which contain relatively large quantities of nucleoproteins and which are generally employed for the preparation of nucleic acid are the mature spermatozoa of fish, the thymus gland, testicular substance, and yeast cells.

Nucleic acid and nucleoproteins are characterised by the fact that they contain phosphorus in organic combination, and nucleoproteins are to be distinguished from another class of conjugated proteins, viz., the phosphoproteins, by the fact that the latter substances on treatment with 1% sodium hydroxide solution at 37° undergo hydrolysis and yield their phosphorus in the form of phosphoric acid which can be directly precipitated by ammonium magnesium citrate, whereas the phosphorus remains in organic combination in the case of the nucleoproteins when these substances are submitted to the same treatment (Aders Plimmer and Scott, *Trans.*, 1908, **93**, 1699). The existence of a nucleoprotein in tissues may be surmised therefore, when it has been ascertained that the material contains phosphorus in organic combination in a substance insoluble in alcohol (to exclude certain phosphorus-containing lipoids, which are soluble in alcohol) and which is not set free as phosphoric acid when the material is treated with 1% sodium hydroxide solution at 37°. The most certain method, however, of determining whether a nucleoprotein is present consists in directly preparing the nucleic acid itself, but as this is, owing to the small quantities present, only possible in a very limited number of cases, the nucleoprotein should be isolated by the method given below, and the mode of combination of the phosphorus present ascertained by the process already described.

The method generally employed now for isolating nucleic acid is that originally suggested by Neumann (*Arch f. Anat. u. Physiol.*, 1899, *Supplementband*, 552), who showed that nucleic acid is relatively very stable toward alkalies. This consists in treating the finely minced or otherwise disintegrated material with hot alkaline solutions containing sodium acetate, neutralising the extract thus obtained with acetic acid, and throwing the product into alcohol. Nucleic acid is thereby precipitated in the form of its sodium salt. In preparing the substance from thymus gland, the tissue is first hardened by boiling with alcohol, after which it can be readily minced in a mincing machine. For 1 kilo of substance, 2 litres of water, 100 c.c. of 30% sodium hydroxide and 200 grm. sodium acetate are employed. The filtered extract is neutralised with 150 c.c. of 50% acetic acid, evaporated to 500 c.c. and the solution thus obtained is cooled to 40° and thrown into an equal volume of 96% alcohol. The preparation can be purified by dissolving in water, adding a small quantity of concentrated sodium acetate solution and throwing the solution into alcohol; without the presence of sodium acetate; precipitation does not take place. The free acid can be obtained from the purified sodium salt by throwing a solution of the latter into 3 times

the volume of alcohol to which 2 c.c. of concentrated hydrochloric acid has been added to every 100 c.c. In the presence of mineral acids, nucleic acid is unstable and undergoes hydrolysis. Nucleic acids are amorphous substances, which are characterised by the fact that they yield on hydrolysis by acids the following groups of substances: I. The alloxuric bases, guanine, adenine, xanthine, and hypoxanthine; II. the pyrimidine substances, cytosine, uracil, thymine; III. a carbohydrate and derivatives of carbohydrates produced by the action of acids on these substances, viz., formic and lævulinic acids; IV. phosphoric acid. In addition to these, ammonia is also produced by hydrolysis, but this, like lævulinic and formic acid, is probably only a secondary product produced by the action of acids on the primary hydrolysis products. According to Steudel, a true nucleic acid is a tetrametaphosphoric acid, to each phosphorus atom of which is combined a carbohydrate group (in a similar way glycerol is combined in glycerophosphoric acid) and a molecule of either guanine, adenine, cytosine or thymine.[1]

Steudel, as the result of a quantitative estimation of the hydrolysis products of the thymus nucleic acid, has formulated its hydrolysis in the following manner:

$$C_{43}H_{57}O_{30}N_{15}P_4 + 8H_2O + O = C_5H_5ON_5 \text{ (guanine)} + C_5H_5N_5 \text{ (adenine)} + C_5H_6O_2N_2 \text{ (thymine)} + C_4H_5ON_3 \text{ (cytosine)} + 4C_6H_{12}O_6 \text{ (hexose)} + 4HPO_3 \text{ (metaphosphoric acid)}.$$

(See Steudel, *Zeitsch. physiol. Chem.*, 1904, **42**, 165; 1904, **43**, 402; 1905, **46**, 332; 1908, **56**, 215.) The constitution of nucleic acids cannot be considered as yet as definitely settled, especially as regards the carbohydrate groups in the molecule. According to the more recent investigations of Levene and Jacobs, a nucleic acid is built up by the condensation of a number of complexes, called *nucleotides*, which are formed by the combination of phosphoric acid with a carbohydrate and a base such as guanine. Nucleic acid is therefore a polynucleotide. The nucleotides can themselves, according to the method of hydrolysis, undergo scission into two kinds of complexes, either into a complex made up from the carbohydrate and phosphoric acid, or into one composed of the carbohydrate and the base. The latter class of substances have been designated *nucleosides*. To the

[1] It has been asserted that the pyrimidine bases are secondary decomposition products derived from the alloxuric bases. This is, however, not the view of the more recent investigators. (See Steudel, *Zeitsch. physiol. Chem.*, 1907, 53, 508.)

class of nucleotides belong guanylic acid, and certain substances such as inosinic acid, which has been isolated from meat extract. To the nucleosides belong inosin which has been obtained by the hydrolysis of inosinic acid and is also found in meat extracts, and guanosin, which has been obtained by Levene and Jacobs (*Ber.*, 1909, **42**, 2469, 2474, 2703, 3247) by the hydrolysis of guanylic acid (an intermediate hydrolysis product of pancreas nucleic acid) and of yeast nucleic acid and which is identical with the so-called vernin obtained from various plants (Schulze and Trier, *Zeitsch. physiol. Chem.*, 1910, **70**, 143). Other intermediate products allied to nucleic acid, such as thyminic acid are known. (For investigations on nucleotides, see various papers by Levene and Jacobs on inosinic acid, etc., in the last few volumes (1909 onward) of the *Berichte.*)

The Nucleoproteins.—Much work remains to be accomplished before the characters of these substances can be satisfactorily determined. This statement refers more especially to the protein part of the molecule with which the nucleic acid is combined. By extraction of nucleoprotein-containing material with cold solvents, the so-called *α-nucleoproteins* are obtained. If these substances be heated, or if the material be extracted with hot solvents, a certain amount of protein is coagulated, and there remains in solution the so-called *β-nucleoproteins;* these are the substances which are generally referred to when the term nucleoprotein is employed. Furthermore, if nucleoproteins be submitted to hydrolysis by proteoclastic enzymes, part of the protein undergoes degradation, and a residue is left to which the term nuclein is applied. From these remarks it will be obvious that a quantitative separation of simple proteins from nucleoproteins, is in the present state of knowledge, not feasible (see page 56); the only criterion as to the amount of nucleoprotein in a mixture is the amount of nucleic acid present (when sufficient material is available for isolating such a substance) or the amount of phosphorus in organic combination which cannot be set free as phosphoric acid by hydrolysis of the material with 1% sodium hydroxide solution at 37°. (See introductory remarks to this section on the nucleoproteins.) In only a few cases can a definite protein be isolated, in which cases basic substances of the histone or protamine classes can be obtained. The materials which have been chiefly employed for the preparation of histones are the blood corpuscles of birds and snakes, the thymus gland, and the testicles of the fish gadus and lota. The protamines have been prepared from the matured testicles of salmon, herring and other fish. The general method of preparation consists in extracting the material with acid,

and precipitating the protein as a salt of the acid by alcohol. They can also be precipitated in *neutral* solution with the ordinary alkaloidal reagents. The protamines are generally purified by precipitation as picrates.

The general method of preparing nucleoproteins consists in extracting the tissues with either water or dilute alkali, and precipitating the nucleoprotein from the solution with dilute acid. The protein can be purified by repeated solution in weak alkali and reprecipitation by weak acids. It is often advisable to prepare the material before extraction by treating it with alcohol (to extract lipoids, etc.) and ether and then grinding to a fine powder. In the majority of cases the yield obtained is only very small.

B. **The Phosphoproteins.**—These substances are distinguished from others by the fact that on treatment with 1% sodium hydroxide solution at 37° for 24–48 hours, the whole of the phosphoric acid is set free from organic combination. These substances are not widely distributed, and occur chiefly in the milk, egg-yolk and ova of fishes, that is, in the substances which constitute the food-stuffs of the embryo bird and fish, and the young mammal. There is also a small quantity present in the pancreas (Plimmer and Scott, *Loc. cit.*, page 71). The two proteins which have been chiefly investigated are caseinogen from milk and vitellin from egg-yolk. The substances are fairly strong acids. (See below, page 88.) Caseinogen can be prepared by the following modification of the original method of Hammarsten (Danilewski and Radenhausen; *Maly's Jahresber.*, 1880, **10**, 186). 2 litres of partly separated milk are diluted with 8 litres of water and 10 c.c. of glacial acetic acid are then added. The caseinogen is thereby precipitated and the supernatant liquid is decanted off. The precipitate is washed with 2–4 litres of slightly acid water, and then squeezed in a cloth. It is then rubbed up to a thick paste with 1–2% ammonia solution, which is afterward diluted to 1,000–1,500 c.c. with ammonia solution of the same strength. The fat separates at the surface of the solution, as the caseinogen is dissolved. The liquid is separated from the fat, and then 10–12 c.c. of glacial acetic acid are added. The caseinogen is thereby precipitated and is purified by redissolving in ammonia and reprecipitating by acids. This process can be repeated until a pure product is obtained. (Compare section on milk proteins.)

Vitellin can be prepared in the following manner (Levene and Alsberg, *Zeitsch. physiol. chem.*, 1901, **31**, 543): Egg-yolks are mixed with an equal volume of 10% sodium chloride solution and the mixture

is extracted three times with ether, the mixture being allowed to stand each time for 24 hours in contact with the ether. A liquid is then obtained which can be readily filtered and from which the pigment can be extracted with ether. It is diluted with 20 times the volume of water. The precipitate thus formed is allowed to settle, then redissolved in sodium chloride solution, which is extracted afterwards with ether, and the vitellin is reprecipitated by dilution with water. The precipitate thus obtained is extracted first by alcohol and afterwards with ether, until nothing more is given up to this solvent.

C. The Glycoproteins.

The chemical knowledge of this group of substances is still very small. The members of the class are characterised generally by the fact that their solutions in alkalies yield mucilaginous solutions, and that they yield on hydrolysis substances which reduce Fehling's solution. The principal members of the class are the mucins and the cartilaginous substances, which latter substances, from the fact that they yield on hydrolysis the so-called chondroitin sulphuric acid, of which the constitution is as yet unknown, are sometimes designated the chondro-proteins. Of the mucins, some are precipitable by acetic acid and insoluble in excess of the acid (*true mucins*); others are soluble in excess of acid (*mucoids*), whereas others again are not precipitable by acetic acid (*pseudomucins*). According to their properties they are precipitated from solutions by either acetic acid or alcohol and their separation from other substances is largely a matter of mechanical treatment; they are precipitated often in the form of sticky strands of substance which can be collected on a glass rod. The homogeneity of the substances which have been obtained by the various methods of preparation is in most cases very doubtful. For details, reference must be made to the list of proteins given at the end of the section where the original papers are quoted.

Certain of the glycoproteins yield on hydrolysis an amino-derivative of a sugar, viz., glucosamine.

METHODS OF IDENTIFICATION OF THE INDIVIDUAL PROTEINS.[1]

A. Physical Constants.

The establishment of physical constants of the individual proteins is a matter of considerable difficulty and is beset with many pitfalls. In the first place, attention has been already repeatedly called to the

[1] For the meaning of the asterisk at the commencement of certain sections see page 76.

difficulty of quantitatively separating the proteins from one another; in the second place, these colloidal substances possess the property of adsorbing salts and other simpler substances from solution, and these adsorbed substances profoundly affect the physical constants; in the third place, the proteins are substances both basic and acidic in character, and capable of forming salts both with acids and bases. As the molecular weights of the proteins are high, the presence of minute quantities of acids and bases may also profoundly affect the physical constants. It is only necessary to instance here the great differences in the optical rotation of any substance and its salt; leucine, *e.g*, has an optical rotation $[\alpha]_D^{20} = -10.42$, whereas that of its hydrochloride is $[\alpha]_D^{20} = +15.33$ in 20% hydrochloric acid. It may be easily understood from this example that a minute quantity of acid in a protein solution may easily cause a great change in any physical constant. Furthermore, there is a great difficulty in determining whether any inorganic substance is in chemical combination with a protein, or whether it is merely physically adsorbed, and on this subject a very extensive literature exists. From these introductory remarks, it will be obvious that a physical constant of a protein cannot be determined without submitting the substance to an extremely careful purification, such as by prolonged dialysis (lasting perhaps for some weeks) or by other available methods. Few physical constants, therefore, can be employed in the ordinary routine of fixing the identity of a given protein; for this purpose, certain chemical methods described in detail later, are simpler and give far more reliable results. It will suffice therefore, in the sequel, to refer in most cases only very shortly to the physical methods of identifying proteins, and to quote some of the more important references to the literature bearing on the subject. Those processes which seem applicable to a routine examination are indicated by an asterisk.

(i) **Solubilities of Proteins in Various Solvents.**—The differences of the solubilities of proteins in various solvents has already been repeatedly referred to in the discussions on the methods of preparation. When they are soluble in water, or dilute alcohol, thick syrupy solutions can be obtained, when small quantities of the solvent are employed. The proteins are soluble in these solvents in all proportions. The solubility of the globulins in salt solutions, on the other hand, vary very considerably, and the degree of solubility can, in many cases, be accurately measured. (See Hardy, *J. Physiol.*, 1905, **33**, 254; Mellanby, *J. Physiol.*, 1905, **33**, 338; Osborne and Harris, *Amer. J. Physiol.*, 1905, **14**, 151; Schryver, *Proc. Roy. Soc.*, 1910, **83B**, 96.)

*(ii) **Precipitability of Proteins by Salts.**—The method of determining these constants has been already given. The precipitation limits are important in the case of the digestion products of proteins, which are discussed in a separate article. (See p. 467.) The determinations have been made with various salts on animal proteins (see *loc cit., p.* 65 and Lewith, *Arch. Exp. Path. Pharm.*, 1887, **24**, 1) and with ammonium sulphate in the case of various vegetable proteins (Osborne and Harris, *J. Amer. Chem. Soc.*, 1903, **25**, 848).

(iii) **Coagulation Temperature and Clotting Point of Proteins.**—The coagulation temperature will depend upon the reaction of the medium, the presence of salts or other substances, and upon the rate at which the mixture is heated. This subject has been already discussed in detail on pages 54 and 55, and is mentioned again below (page 88). Hitherto, the coagulation point of proteins has been regarded as a physical constant, but in view of the more recent investigations, this is no longer possible for the reasons already given. The subject requires, therefore, no farther discussion in this place.

(iv) **Optical Rotation of Proteins.**—With the exception of hæmoglobin for which $[\alpha]_c = +10.4$ (Gamgee and Croft Hill, *Ber.*, 1903, **36**, 913) and some nucleoproteins (Gamgee and Jones, *Beit. physiol. path. Chem.*, 1903, **4**, 10) the proteins are lævo-rotatory. The number of reliable estimations of the optical activity is small. They have been made with a certain number of carefully purified plant proteins by Osborne and Harris (*J. Amer. Chem. Soc.*, 1903, **25**, 842).

(v) **The Molecular Weight of Proteins.**—The molecular weight of proteins may be determined by means of Moore's osmometer (Moore and Parker, *Amer. J. Physiol.*, 1902, **7**, 261, and Moore and Roaf, *Biochem, Journ.*, 1907, **2**, 34) or by Roaf's modification of the same. The presence of salts and other substances influences to a great extent the state of aggregation of the proteins. The subject of the molecular weight of proteins still requires a considerable amount of investigation, and although the experimental method is a comparatively simple one, the interpretation of the results is uncertain until more is known about this subject. (See Lillie, *Amer. J. Physiol.*, 1907, **20**, 127 and Roaf, *Quart. Journ. Physiol.*, 1910, **3**, 75.)

* (vi) **"The Gold Number."**—Certain colloids, especially those of the class known as the "suspension colloids," are readily precipitated from solution by the addition of electrolytes. The presence of other colloids inhibits the precipitation, and the quantity which causes this inhibition is characteristic for any particular colloid. Zsig-

mondy (*Zeitsch. anal. Chem.*, 1901, **40**, 697, and Zsigmondy and Schulz, *Beitr. Chem. Physiol. Path.*, 1903, **3**, 137) have proposed to employ this principle for discriminating between the various proteins and other colloids, using for the purpose a certain colloidal gold solution prepared by a standard method. The number of milligrams of a colloidal substance which are just insufficient to prevent 10 c.c. of a bright red gold solution, prepared by the standard method, from changing into violet on addition of 1 c.c. of a 10% sodium chloride solution, is designated as the "gold number" of that colloid. The gold solution is prepared in the following way: 120 c.c. of water, distilled through a silver condensing tube into a beaker of Jena glass of 300–500 c.c. capacity, are heated and during the warming 2.5 c.c. of a 0.6% solution of hydrogen gold chloride solution and 3–3.5 c.c. of 0.18 *N* potassium carbonate solution made from the purest obtainable material are added. After boiling and while the mixture is still hot, 3–5 c.c. of dilute formaldehyde solution (0.3 c.c. commercial formalin to 100 c.c. water) are added. After a short time, a bright red colour is produced. Only Jena glass rods should be used for stirring. To determine the "gold number" small quantities of solution of the colloid under investigation are introduced into a series of 50 c.c. Jena beakers. The colloid solution is delivered from a 0.2 c.c. pipette graduated in thousandths of a cubic centimeter, the quantities used being generally 0.005, 0.01, 0.02, 0.05 0.5 c.c. of the solution. 0.5 c.c. of the gold solution are then introduced into each beaker, and the mixture is stirred. After 3 to 5 minutes 0.5 c.c. of sodium chloride solution (100 grm. sodium chloride to 900 c.c. water) are introduced. A higher limit at which no change takes place and a lower limit at which the red solution becomes violet, are then observed. The number of milligrams in each of these solutions multiplied by 2 gives the interval designated as the "gold number."

The following are the "gold numbers" of certain proteins:

Substance	Gold Number
Gelatin	0.005–0.1
Russian glue	0.005–0.01
Isinglass	0.01–0.02
Caseinogen	0.01
Glycoprotein	0.05–0.1
Deutero-albumose	∞
Egg-globulin	0.02–0.05
Ovomucoid	0.04–0.08
Crystallised egg-albumin	2–8
Amorphous egg-albumin	0.03–0.06
Fresh egg-white	0.08–0.15

Some colloids have much higher numbers than these (dextrin and potato starch, 10–500), whereas others (silicic acid, soluble glass, mucin)

are quite inactive. The method has been suggested for distinguishing between various commercial preparations.

(vii) **Refractive Indices of Protein Solution.**—The refractive index of protein solutions may be expressed by the formula $n-n_1 = \alpha c$, when n = refractive index of the solution, n_1 = refractive index of the solvent, c = concentration, α = constant.

Protein	α
Ovomucoid	.00160
Ovovitellin	.00130
Caseinogen	.00152

(Brailsford Robertson, *J. Biol. Chem.*, 1909–1910, 7, 359.)

B. Chemical Constants.

Several chemical constants have been suggested for the identification of individual proteins, some of which, such as the determination of the acidic or basic functions, are only applicable to materials which have been submitted to very careful purification, and which are known to be free proteins and not salts of the same. In other cases, such careful preparation of the material is not necessary, and in these instances the determinations of the chemical constants may be employed as routine methods for fixing the identity of any given protein; and the methods employed for the purpose are described in some detail below (paragraphs marked by an asterisk). Of these the two principal are the estimation of the sulphur content of the protein, and the determination of the so-called "Hausmann numbers," and a third method has been quite recently introduced by Van Slyke, for estimating the nitrogen in the various individual diamino-acids.

*(i) **The Empirical Composition of Proteins and Their Sulphur Content.**—The carbon, hydrogen, nitrogen and oxygen content in proteins do not as a rule vary within very wide limits, and the numbers obtained by an ordinary combustion are not particularly characteristic for the individual proteins. In the case of nitrogen, it must be remarked, there are occasionally wide variations, and certain rare basic proteins, belonging to the class of histones and protamines contain relatively large percentages of this element, but in the vast majority of cases, the variations from a mean are too small to afford any numbers that may serve to characterise a protein. This is not, however, the case with the element sulphur, as the amount of cystine yielded by hydrolysis varies within wide limits, as the following table, giving the sulphur content of some characteristic proteins, shows.

Sulphur Content of Proteins.	%
Globin (from hæmoglobin)	0.42
Serum albumin	1.90
Egg-albumin	1.62
Blood globulin	1.11
Edestin (hemp-seed)	0.91
Egg-globulin	0.12
Zein	0.60
Gliadin	1.14
Glutenin	1.08
Caseinogen	0.76
Fibrinogen	1.25
Gelatin (commercial)	0.7
Hair	5.0
Horn	3.20

As the sulphur is readily estimated with a considerable degree of accuracy and as the variations of the amount are great in different proteins, the determination of sulphur content affords a valuable chemical constant for fixing the identity of proteins. Some proteins contain in addition to the above elements phosphorus (phosphoproteins and nucleoproteins) and halogens (thyroid gland which contains iodine and certain sponges which contain iodine or bromine or both). Where these elements are present, their amounts should be estimated quantitatively.

For the estimation of sulphur, the dry oxidation process with sodium peroxide is a convenient one (Pringsheim, *Ber.*, 1903, **36**, 4244, 3904, **37**, 2155, Pringsheim and Gibson, *Ber.*, 1905, **38**, 2459). More recently wet oxidation processes have been suggested for this purpose, notably by Benedict (*J. Biol. Chem.*, 1906, **1**, 363), the modification of which by Wolf and Osterberg (*Biochem. Zeitsch.*, 1910, **29**, 429) is stated to give good results, and is rapid. According to this method, the organic material is first oxidised with fuming nitric acid, until the solid matter has all passed into solution and nitrous fumes are no longer given off, and the oxidation is finally completed by heating with Benedict's reagent, which is made by dissolving 200 grm. of copper nitrate and 50 grm. of potassium or sodium chlorate in 1,000 c.c. of water. The mixture of the solution of the organic substance in nitric acid with the reagent is first evaporated to dryness and then heated over a free flame. The sulphur is thereby oxidised to sulphate which is estimated as the barium salt in the usual way. Phosphorus is best estimated by Neumann's wet ashing method (*Zeitsch. physiol. Chem.*, 1902, **37**, 115) as modified by Plimmer and Bayliss (*J. Physiol.*, 1906, **33**, 441). The estimation of sulphur, and phosphorus in the same sample of material can be carried out by the method of Wolf and Osterberg (*loc. cit.*).

(ii) **Estimation of Hydrolysis Products.**

*(*a*) **The Determination of the "Hausmann" Numbers.**—The chemical factors which are most characteristic of the individual proteins

are the quantities of the various amino-acids which are yielded by hydrolysis, the methods for estimating which have been discussed in some detail in the earlier pages of this article. It has there been repeatedly stated that the methods are complex and large quantities of material are necessary for investigation; although the separate estimation of the diamino-acids is often feasible (pages 28 *et seq.*). A series of very simple chemical constants which are dependant on the hydrolysis products are the so-called "Hausmann" numbers. Although much complicated manipulation is necessary to separate the individual amino-acids, the diamino-acids as a group are, owing to their more basic character, readily separated from the mono-amino-acids by the fact that they are precipitable by alkaloidal reagents. Furthermore, most proteins yield on hydrolysis certain quantities of ammonia, which, in the case of some of the vegetable proteins, are relatively large ("amide"-nitrogen). In addition, a small amount of nitrogen is contained in the insoluble pigmented substance which is separated during hydrolysis and known as "humin" nitrogen. The distribution of the nitrogen among these groups of hydrolysis products is expressed as percentages of the total nitrogen contained in the proteins, and the figures thus obtained are known as "Hausmann's" numbers. The method usually employed for determining these factors is the original process of Hausmann, as modified by Osborne and Harris (*J. Amer. Chem. Soc.*, 1903, **25**, 323) and Gümbel (*Beitr. Chem. Physiol. Path.*, 1904, **5**, 297).

About 1 grm. of protein is boiled with 20% hydrochloric acid, usually from 7–10 hours, until the solution no longer gives the biuret reaction. It is then evaporated at 40° to 2 or 3 c.c. and the residue is transferred to a flask with about 350 c.c. of water, and cream of magnesia is added in slight excess. The latter must be freed from every trace of ammonia by prolonged boiling before addition to the hydrolysis products. The mixture is then freed from the ammonia formed by hydrolysis by heating *in vacuo* to 40°, and passing the distillate through a known excess of $N/10$ sulphuric acid. The amount of this acid neutralised indicates the amount of ammonia formed by hydrolysis. The residue left in the flask is then filtered through nitrogen-free paper, which is then washed thoroughly with water, the washings being added to the original filtrate. The nitrogen ("humin" nitrogen)[1] in the dark-coloured mass left on the filter is then estimated by Kjeldahl's method. The filtrate and washings are then concentrated to 100 c.c., cooled to 20°, and 5 grm. of sulphuric acid are

[1] Sometimes called melanin nitrogen.

then added. To this mixture is then added 30 c.c. of a solution made by dissolving 20 grm. of phosphotungstic acid and 5 grm. concentrated sulphuric acid in 100 c.c. of water. The precipitate thus formed is filtered off after standing for 24 hours, and is then washed with a solution containing 5 grm. of sulphuric acid and 2.5 grm. of phosphotungstic acid in 100 c.c. of water. The washing is effected by rinsing the precipitate from the filter into a beaker and returning to the paper three successive times, each portion of the wash being allowed to run out completely before the next is applied. About 200 c.c. of washings are usually obtained. The nitrogen in the precipitate (diamino-acid or "basic" nitrogen is estimated by transferring precipitate and filter paper to a Kjeldahl flask and digesting with 35 c.c. of concentrated sulphuric acid for 7–8 hours. Crystals of potassium permanganate are occasionally added during this incineration to promote the oxidation processes. Care must be taken to avoid "bumping" during the process. The Kjeldahl estimation is completed in the usual way. The mono-amino-acid nitrogen is determined by difference, *i.e.*, by subtracting the amount of "amide" "humin" and "basic" nitrogen from the total nitrogen contained in the protein, which is determined by a separate Kjeldahl estimation. A few typical "Hausmann" numbers are given in the accompanying table.

Protein	*N* %	Amine *N*	Amino *N*	Basic *N*	Humin *N*
Egg-albumin	15.51	8.64	68.13	21.27	1.87
Caseinogen	15.62	10.36	66.00	22.34	1.34
Salmine		0	.....	87.8	.....
Edestin	18.64	10.08	57.83	31.70	0.64
Glutenin	17.49	18.86	68.31	11.72	1.08
Gliadin	17.66	23.78	70.27	5.54	0.79
Gelatin (commercial)		1.61	62.56	35.83	

(For other determinations, see J. H. Millar, *Transactions of the Guiness Research Laboratory*, Vol. 1, Pt. 2.)

An extension of this method, whereby the nitrogen in the individual diamino-acids can be estimated, has been recently suggested by Van Slyke. Although the method is so recent that it has up to the present received but little application except at the hands of its discoverer, it seems so rational, and requires so little material, that there can be little question of its utility. It is therefore described in some detail in the following pages.

***Van Slyke's Method for the Estimation of the Arginine, Histidine and Lysine Nitrogen.** (Compare Vol. 7, p. 263).—The three bases are precipitated together as phosphotungstates. The principle of the method depends chiefly on two facts, viz., (*a*) that when the protein hydrolysis products are treated with nitrous acid, only the nitrogen contained in the form of amino groups (NH_2) reacts with the acid with evolution of nitrogen gas. If reference be made

to the formulæ of the diamino-acids, it will be evident that arginine contains three-fourths of its nitrogen in a form which will not react with nitrous acid, whereas histidine contains two-thirds in this form; lysine on the other hand contains all its nitrogen in the form of amino-groups. (*b*) Arginine on hydrolysis with potassium hydroxide yields ornithine and urea, and the latter decomposes further into ammonia and carbon dioxide: $C_6H_{14}O_2N_4 + 2H_2O =$

Arginine.

$C_5H_{12}O_2N_2 + 2NH_3 + CO_2$.

Ornithine.

Under the correct conditions of experiment, arginine is the only one of the bases precipitated by phosphotungstic acid, which evolves ammonia on boiling with alkali, half its nitrogen being given off in this form. From this reaction, the amount of arginine in the phosphotungstic acid precipitate can be readily ascertained.

If now the total nitrogen be known, and the amount of nitrogen present in the amino-form (as estimated by treatment with nitrous acid), then if D = non-amino nitrogen (*i.e.*, difference between total and amino-nitrogen) in the phosphotungstic acid precipitate, the amount of histidine nitrogen can be calculated from the formula.

Histidine N = $\frac{3}{2}$ (D − $\frac{3}{4}$ Arg.), where Arg. = arginine nitrogen. The lysine nitrogen = total N − (Arginine + Cystine[1] + Histidine)N. In carrying out the process, the first stages are practically the same as those employed in determining the Hausmann numbers, viz., the hydrolysis products are separated into ammonia, melanine, diamino-acids and mono-amino acids. Certain modifications in the details have been introduced by van Slyke.[2]

For hydrolysis, 1–3 grm. of protein are employed, and 10–20 parts of 20% hydrochloric acid. In order to ensure complete hydrolysis, the flask in which the hydrolysis takes place is weighed. At intervals the heating is stopped, and the flask cooled and weighed, and about 1–2 c.c. of the fluid removed and weighed and the amino-nitrogen is estimated in this sample by treatment with nitrous acid. The method for this estimation is given in detail on pages 486 and 487. As there is ammonia nitrogen in the liquid, it is advisable to carry out these estimations of amino-nitrogen under standard conditions, (*i.e.*, at nearly constant room temperature, and to allow the nitrous acid to act for 6 minutes). The hydrolysis is continued till complete,

[1] Part of cystine from the hydrolysis products is also precipitated by phosphotungstic acid. Winterstein, *Zeitsch. physiol. Chem.*, 1901, 34, 153.

[2] The method has been rendered feasible by the elaboration by van Slyke of the method for estimating amino-nitrogen, which is described in detail on page 486. Reference should be made to this description before proceeding further. See also Vol. 7, p. 263.

generally for 6–8 hours, that is, until the amount of amino-nitrogen estimated in a weighed sample of the known total weight of hydrolysis mixture, is constant. The "amide"-nitrogen is then estimated in the hydrolysis products, which are transferred to a large flask, diluted and distilled under diminished pressure, after making just alkaline with a 10% suspension of calcium hydroxide. The total nitrogen should be estimated in an aliquot part of the diluted liquid. 100 c.c. of alcohol should be added before distillation to prevent frothing. The ammonia which is evolved is estimated by collecting in excess of $N/10$ sulphuric acid.

The "melanine" nitrogen is carried down with the calcium hydroxide and is estimated by the method described above in the Osborne and Harris process for the determination of the Hausmann numbers.

Modifications have been introduced, however, into the methods for precipitating the bases. The filtrate from the melanine is neutralised with hydrochloric acid, returned to the vacuum distilling flask, and concentrated to about 100 c.c. It is then washed into a 200 c.c. Erlenmayer flask, 18 c.c. of concentrated hydrochloric acid and 15 grm. of phosphotungstic acid are added, and the mixture is diluted to 200 c.c., and heated on a water-bath till the precipitate has entirely or nearly dissolved. The phosphotungstates of the bases then separate on standing as a granular or crystalline precipitate, which can be readily washed and filtered. The mixture is allowed to stand for 48 hours to complete the precipitation. As the precipitate is not entirely insoluble in acidified water, the manipulation is so regulated that the washing can be effectively carried out with the smallest possible volume of liquid. For this purpose, a hardened filter paper is cut to fit accurately into a 3-in. Buchner funnel so as to be in contact both with the bottom and the side walls. The part which touches the side walls is folded into about 20 plaits, so that it fits tightly. The precipitate is poured into the pocket thus formed, and filtered from the liquid as completely as possible, and the filtrate is transferred from the suction flask to a beaker. The precipitate on the funnel is washed by pouring onto it 10–12 c.c. of a washing solution containing 2.5% phosphotungstic acid, and 3.5% hydrochloric acid; the precipitate and washings are stirred together so as to form a smooth suspension, care being taken that all the lumps are broken up in the process. The precipitate is then sucked dry, and the washings are repeated (8–15 times) until the filtrate is free from calcium. The first washings are used to dislodge the granules from the flask in which the precipitation of the phosphotungstate is carried

out. To test for calcium in the washings, a solution of oxalic acid in 3% sodium hydroxide is employed. In case the washings are turbid, they should be filtered through a folded filter paper and the precipitate is subsequently dissolved in alkali and added to the solution of the main bulk of the precipitate. This latter solution is made by transferring the precipitate from the filter paper as completely as possible by mechanical means to a large beaker (of more than 1000 c.c. capacity), and the small quantity still remaining on the hardened filter paper (and the folded filter paper through which the turbid washings have been poured) is dissolved out by spreading out the paper in a basin containing water made alkaline by the addition of a few drops of 20% sodium hydroxide. These washings are added to the suspension in water of the main bulk of the precipitate, to which is then added a few drops of phenolphthalein solution and, drop by drop, 50% sodium hydroxide solution until the solution becomes red. The solution at the end should be distinctly red, but should only contain 3 or 4 drops of alkali in excess. A large excess must be avoided, otherwise the cystine and arginine will be destroyed. In this way the phosphotungstates are brought into solution. The solution is then diluted to 800 c.c. and the phosphotungstic acid is precipitated with 20% barium chloride solution, the latter being added in small quantities at a time, until it is in *slight* excess. If the solution loses its red colour during the precipitation, a few drops more of alkali should be added.

The barium phosphotungstate is then filtered off, and washed until the washings are free from chloride, and the filtrate is then concentrated *in vacuo*. During this process, a small amount of phosphotungstate separates, and the distillation is interrupted when the liquid is concentrated to about 50 c.c. in order to filter this off. The filtrate and washings are then further concentrated (conveniently in a smaller flask) and the residue is then made up to 50 c.c. in a measuring flask.

Estimation of Arginine.—25 c.c. of this liquid are employed for the estimation of arginine. These are transferred to a Kjeldahl flask of Jena glass of 200 c.c. capacity, which is connected with a reflex condensor, at the top of which is a trap containing 15 c.c. of $N/10$ sulphuric acid and a drop of sodium alizarin sulphonate to collect the ammonia evolved. 12.5 grm. of solid potassium hydroxide are added to the liquid, which is then boiled for exactly six hours. At the end of this time, 100 c.c. of water are added, and the ammonia remaining in the flask is distilled off by connecting it with the condensor of an

ordinary Kjeldahl still, the distillate being collected in the $N/10$ acid washed out from the trap. Care must be taken that no more than 100 c.c. of liquid are distilled off, otherwise secondary decomposition of products other than arginine will take place in the concentrated alkali. The ammonia derived from the arginine is then estimated by titrating back the excess of alkali in the usual manner. Each c.c. of acid neutralised corresponds to 0.0028 grm. of arginine nitrogen in the solution decomposed, or 0.0056 grm. of the total arginine nitrogen. When cystine is treated under these conditions, about 18% of the nitrogen is evolved as ammonia. With the exception of the sulphur-rich keratins, however, this amount is generally negligible, as the majority of proteins only yield small amounts of cystine. The 200 c.c. Kjeldahl flask cannot as a rule be used for more than two estimations, owing to the action of alkali on the glass, and copper flasks cannot be employed owing to the oxidising action of the metal on cystine.

The Estimation of Total Nitrogen.—For this purpose, the residue left after the arginine decomposition can be employed. 35 grm. of concentrated sulphuric acid are gradually (and with cooling) added to this together with 0.25 grm. of copper sulphate. The Kjeldahl estimation is completed in the usual way.

Estimating Cystine in the Phosphotungstic Acid Precipitate.—10 c.c. of the solution of the bases are employed for this purpose. The amount of cystine is estimated by determining the amount of sulphur present. This can be carried out with one of the modifications of Benedict's method of incineration with copper nitrate (see page 80). Each milligram of barium sulphate corresponds to 0.06 mgrm. of cystine nitrogen. Blank control analyses should be carried out with the reagents, and the necessary corrections made.

Estimation of Amino-Nitrogen of the Bases.—For this purpose, 10 c.c. of the solution of the bases are employed. The method for carrying out the decomposition with nitrous acid is described in detail on page 486. Owing to the slowness with which the terminal NH_2 group acts, the reaction should be allowed to continue for half an hour at 20°. A blank determination under the same conditions should be made with the reagents. Cystine gives off gas equivalent to 107% of the theoretical, but no correction need be made for this except in the proteins which are very rich in cystine (keratins). Blank analyses should be carried out with the chemicals used, and corrections should also be made for the solubilities of the phosphotungstates in the solutions in which they are precipitated. These may be calculated from the following table:

SOLUBILITIES OF BASES IN 200 C.C. OF SOLUTION IN WHICH THEY ARE PRECIPITATED.

	Total N. Add to individual bases	Amino N	Non-amino N
Arginine N.	0.0032	0.0008	0.0024
Histidine N.	0.0038	0.0013	0.0025
Lysine N.	0.0005	0.0005	0.0000
Cystine N.	0.0026	0.0026	0.0000
Sum (subtracted from figures for filtrate)	.....	0.0052	0.0049

The amino- and non-amino-nitrogen may also be estimated in the filtrate from the phosphotungstic acid precipitate. The non-amino nitrogen gives a measure of the proline, hydroxyproline and tryptophan. The last-named is, however, generally absent, as it appears to be destroyed on acid hydrolysis.

(*b*) **Estimation of Certain Individual Hydrolysis Products.**—Although the majority of the hydrolysis products can be estimated only approximately and then by methods involving difficult manipulation, a few can be estimated with comparative ease, and by the determination of the amounts yielded by different proteins, a valuable factor is obtained for their characterisation. The amount of cystine yielded by hydrolysis can be ascertained by the estimation of the total sulphur in the protein (page 80). Miller has suggested a method for the estimation of tyrosine, which forms a definite compound with bromine. By estimating the amount of bromine taken up by a mixture of hydrolysis products containing tyrosine the amount of the latter substance present can be ascertained. The solution to which potassium bromide and hydrochloric acid has been added is titrated with $N/5$ potassium bromate solution until potassium iodide-starch indicator denotes the presence of free bromine. The reactions can be represented by the following equations:

$$NaBrO_3+5KBr+6HCl=5KCl+3Br_2+3H_2OC_6H_4(OH)CH_2.$$
$$CH(NH_2)COOH+4Br=C_6H_2Br_2(OH).CH_2.CH.(NH_2).COOH.$$

According to these, about 18.8 $N/5$ bromate solution are equivalent to 1 grm. tyrosine (*Trans. Guiness Lab.*, 1903, 1, 40, and Brown and Millar, *Trans. Chem. Soc.*, 1906, **89**, 145). Brown and Millar have applied the method to the estimation of tyrosine set free by a short pancreatic digestion.

A method has also been suggested for the quantitative estimation of tryptophan (Levene and Bouiller, *J. Biol. Chem.*, 1906–7,

2, 481). See also Fasal (*Biochem. Zeitsch.*, 1912, **44**, 392, for a colourimetric method.)

(iii) **Determinations of the Acid and Basic Functions of a Protein.**—As the proteins are built up by the conjugation of mono-, amino- and diamino mono-carboxylic acids and dicarboxylic acids, they can contain according to the groups from which they are formed varying numbers of free amino- or carboxylic acid groups, and consequently combine with varying quantities of acids or bases to form salts. The simultaneous presence of both these groups tends to confer on the majority of the proteins an amphoteric character so that they act neither as strong acids nor as strong bases. In only a few cases is the acid or basic character therefore strongly marked, and the acid or basic functions can be directly ascertained by simple titration with bases and acids. Caseinogen, which is a phosphoprotein, can be directly titrated with alkalis (Lacquer and Sackur, *Beitr. chem. Physiol. Path.*, 1902, **3**, 193). The protamines, furthermore, are strong bases. The difficulty of determining the acid and basic functions is also increased by the fact that the proteins have a high molecular weight and in the presence of only a few free amino- or carboxylic acid groups only relatively very small amounts of acid or base are necessary to convert a protein into a salt. Furthermore, owing to the high molecular weights, solutions containing quite large quantities of protein, (*e. g.*, 5% solutions) are, technically, dilute, and, except in the presence of excess of acid or base, the protein salt will undergo hydrolytic dissociation. Owing to these facts there is often a great difficulty in preparing proteins sufficiently pure for determination of the physical constants, a circumstance to which attention has been repeatedly drawn in the foregoing pages. It is also difficult to prepare the majority of the proteins sufficiently pure for estimating their acidic or basic functions. It will also be obvious, from these remarks, that these functions cannot be readily ascertained by direct titration of protein solutions by normal solutions of acids and bases in the presence of indicators. For the purpose, physical methods have been generally adopted, such as the measurement of the changes in electrical conductivity (Sjöquist, *Skand. Archiv f. Physiol.*, 1895, **5**, 277) or the changes in the hydrogen ion concentration by the electric potential method (Liebermann and Bagarsky, *Pflüger's Archiv*, 1898, **72**, 51, and other investigators) when a protein is added to a solution of an acid or a base. Two other methods have been recently employed. It has already been mentioned that agglutination of a coagulated protein takes place most readily when suspended in an isoelectric

solution (Michaelis and his co-workers, *loc. cit.*, page 55), and this "isoelectric point" is characteristic for each individual protein. Furthermore, when a protein is suspended in an electric field, it moves neither to the cathode nor the anode when suspended in an isoelectric solution. By determining the hydrogen ion concentration of the medium in which the protein is electrically neutral, its acidic or basic functions can be ascertained. This is known as the method of electrocataphoresis.

By methods such as the above, the following constants have been determined:

Protein	Isoelectric point	Relative acidity constant
Serum albumin (mammal)	0.31×10^{-5}	$1.6 \times 10^{+3}$
Serum globulin (mammal)	0.36×10^{-5}	$2.2 \times 10^{+3}$
Caseinogen (cow's milk)	1.8×10^{-5}	$5.4 \times 10^{+4}$
Gliadin	6.0×10^{-10}	6.0×10^{-5}
Edestin	1.3×10^{-7}	2.8×10^{-0}

(Rona and Michaelis, *Biochem. Zeitsch.*, 1910, **28**, 193.)

(For description of apparatus for electrocataphoresis experiments, see Michaelis, *Biochem. Zeitsch.*, 1909, **16**, 81. See also Victor Henri, *Soc. de biologie*, 1907, page 475, and *Biochem. Zeitsch.*, 1909, **16**, 473.)

(iv) **Estimation of the Reactive Amino Groups.**—The reactive amino groups in a protein can be estimated by ascertaining the amount of nitrogen set free when the protein is treated with nitrous acid. The percentage of the total of the nitrogen existing in this form is a distinguishing factor for each individual protein. The method of estimation is somewhat difficult in the majority of the proteins, when the factor is represented only by a small number. As, however, the number of reactive amino groups in a protein increases rapidly when it is submitted to digestion, the method is a useful one for following the course of a protein degradation, and is described in detail in dealing with the digestive products of the proteins (page 486).

(v) **Estimation of the Reactive Carboxylic Acid Groups.**—In a substance of amphoteric character such as glycine, $NH_2 \cdot CH_2 \cdot COOH$, the amount of carboxylic acid groups cannot be estimated by direct titration in the presence of indicators. If, however, it be treated with formaldehyde in excess, a methyleneimino derivative, $CH_2(N=CH)COOH$ is formed, in which the basic amino group has been converted into a group, which does not possess strong basic characters. In such substances, the carboxylic groups present can be quantitatively estimated by titration with acids in the presence of indicators. This

method, again, is more useful when applied to the study of the degradation of proteins, during which amino and carboxylic acid group are set free from conjugation in peptide form, and is also described in greater detail in the article dealing with the digestion of proteins. It has, however, been applied to the proteins themselves (Henriques and Gjalbäk, *Zeitsch. physiol. Chem.*, 1911, **71**, 511).

Biological Differentiation of Proteins.

If a protein be injected into an animal, the serum of that animal acquires the property of precipitating that protein ("antigen") forming a so-called precipitin. This precipitin is yielded only when the serum is treated with a protein derived from the animal from which the "antigen" was obtained, or from nearly allied species. The reaction is therefore characteristic of the species from which the protein was obtained rather than for the protein itself, and has been used for medico-legal investigations, and for determining the origin of meat contained in sausages and other products. It is described in detail elsewhere (page 271).

A List of the Chief Animal Proteins.

The following does not profess to be an exhaustive list of all the animal proteins which have been described; nor is it feasible in this place to give an exhaustive bibliography of the subject. As, however, the methods for preparing these substances vary very considerably in details, according to the materials employed, it has been thought advisable to give the references to some of the principal papers bearing on the subject. It must be borne in mind that the chemical nature of several of the substances mentioned in the list is by no means definitely established and their assignment to any given group must in many cases be regarded as purely provisional. This remark refers more especially to the many substances classed as glycoproteins and scleroproteins. It is hoped that the selected references to original work given below will be sufficient to act as a guide to any worker who requires more details of manipulative methods than could be given in the foregoing text, in which only the most general aspects of a large subject could be treated.

Protein	Origin	Class	References
Albumin	Egg (ovalbumin) crystalline	Albumin	Hopkins and Pinkus, *J. Physiol.*, 1898, **23**, 130.
	Milk (lactalbumin)	Albumin	
	Serum (crystalline)	Albumin	Gürber, *Verh. phys. med. Gesellsch. Würzburg*, 1894, **1**, 143, and Inagaki, *Ibid.*, 1906, **38**, 17.
Albumose	Milk	Proteose	Fuld, *Biochem. Zeitsch.*, 1907, **4**, 488.
Amyloid	Pathological tissue	Glycoprotein (?)	Krawkow, *Arch. exp. Path. Pharm.*, 1898, 40, 195.
			Hanssen, *Biochem. Zeitsch.*, 1908, **13**, 185.
Arbacin	Testes of sea-urchin	Histone	Matthews, *Zeitsch. physiol. Chem.*, 1897, **23**, 399.
Bence-Jones protein	Pathological urine	Globulin (?)	Hopkins and Savory, *J. Physiol.*, 1911, **42**, 189.
Caseinogen	Milk	Phosphoprotein	Hammarsten, *Maly's Jahresber.*, 1872, 118; 1874, 135 and 1877, 158.
			Danilewsky and Radenhausen, *Ibid.*, 1880, 186.
Chondroproteins	Bone (osseomucoid)	Glycoproteins (?)	Hawk and Gies, *Amer. J. Physiol.*, 1901, **5**, 387.
	Trachea		Mörner, *Skand. Archiv. f. Physiol.*, 1889, **1**, 210.
	Tendon (tendomucoid)		Cutter and Gies, *Amer. J. Physiol.*, 1901, **6**, 185.
Clupeine	Testes of	Protamine	(See protamines.)
Collagen	Connective tissues	Scleroprotein	Numerous papers on collagens from various sources by Gies and his co-workers, *Amer. J. Physiol.*, 1901, and onward. Also Sadikoff, *Zeitsch. physiol. Chem.*, 1906, **48**, 130.
Conalbumin	Eggs	Albumin	Osborne and Campbell, *J. Amer. Chem. Soc.*, 1900, **22**, 422. Langstein, *Beit. physiol. path. Chem.*, 1902, **1**, 82.
Conchiolin	Shells of lamelli branchs	Scleroprotein	Wetzel, *Zeitsch. physiol. Chem.*, 1900, **29**, 388.
Cornein	Coral (*Ripidgorgia flabellum*)	Scleroprotein	Krukenberg, *Ber.*, 1884, **17**, 1843.
Crystallins (α and β)	Lens of eye	Globulins	Mörner, *Zeitsch. physiol. Chem.*, 1894, **18**, 61.
Cyclopterin	Testes of cyclops	Protamine	(See protamines.)
Elastin	Ligamentum nucæ	Scleroprotein	Richards and Gies, *Amer. J. Physiol.*, 1902, **7**, 93.
Fibrinogen	Blood	Globulin	Huiscamp, *Zeitsch. physiol. Chem.*, 1905, **46**, 273.
Fibrinoglobulin	Blood	Globulin	Hammarsten, *Zeitsch. physiol. Chem.*, 1899, **28**, 98.
Gadus histone	Testes of *Gadus*	Histone	Kossel and Kutscher, *Zeitsch. physiol. Chem.*, 1900, **31**, 188.
Gelatin	Closely allied to collagen	Scleroprotein	Mörner, *Zeitsch. physiol. Chem.*, 1899, **28**, 471.
			Sadikoff, *Ibid.*, 1906, **48**, 130.
Globin	Chromoprotein of blood	Globulin (?)	Schulz, *Zeitsch. physiol. Chem.*, 1898, **24**, 449.
Globulins	Animal cells	Globulins	Halliburton, *J. Physiol.*, 1880, **13**, 808.
			Pohl, *Beitr. physiol. path. Chem.*, 1906, **7**, 381.
	Egg		Langstein, *Beitr. physiol. path. Chem.*, 1902, **1**, 82. Osborne and Campbell, *J. Amer. Chem. Soc.*, 1900, **22**, 422. Sebelien, *Zeitsch. physiol. Chem.*, 1885, **9**, 453.
	Milk		
	Serum		Various fractions of, Freund and Joachim, *Zeitsch. physiol. Chem.*, 1902, **36**, 407, and Spiro and Fuld, *Ibid.*, 1902, **31**, 407. Wiener, *Ibid.*, 1911, **74**, 29.
Gorgonin	Skeletal tissue—*Gorgonia cavollisa* (coral).	Scleroprotein	Henze, *Zeitsch. physiol. Chem.*, 1903, **38**, 60; 1907, **51**, 64.
Hæmoglobin	Blood	Chromoprotein	Zinnoffsky, *Zeitsch. physiol. Chem.*, 1885, **10**, 16.

Protein	Origin	Class	References
Histone	Bird's blood	Histone	Kossel, *Zeitsch. physiol. Chem.*, 1884, **8**, 511.
	Thymus		Kossel and Kutscher, *Zeit. physiol. Chem.*, 1900, **31**, 188.
Ichthulin	Certain fish eggs	Phosphoprotein	Walter, *Zeitsch. physiol. Chem.*, 1891, **15**, 477.
			Levene, *Ibid.*, 1901, **32**, 281.
Keratins[1]	Egg-shells	Scleroprotein	Abderhalden and Strauss, *Zeitsch. physiol. Chem.*, 1906, **48**, 525.
Livettin	Egg-yolk		Plimmer, *Trans. Chem. Soc.*, 1908, **93**, 1500.
Lotahistone	Testicles of *lota*	Histone	Ehrström, *Zeitsch. physiol. Chem.*, 1901, **32**, 351.
Myosin (paramyosinogen)	Striated muscular tissue	Globulin	Halliburton, *J. Physiol.*, 1887, **8**, 331.
Myogen (myosinogen)	Striated muscular tissue	Albumin	
Mucins, mucoids and pseudomucins	Bile (?)[2]	Glycoproteins	Paÿkul, *Zeitsch. physiol. Chem.*, 1887, **12**, 196.
	Egg		von Fürth, *Beit. physiol. path. Chem.*, 1901, **1**, 252.
	Eye (hyalomucin)		Mörner, *Zeitsch. physiol. Chem.*, 1894, **18**, 244.
	Cystic fluids		Hammarsten, *Zeitsch. physiol. Chem.*, 1882, **6**, 194.
	Saliva		Hammarsten, *Zeitsch. physiol. Chem.*, 1901, **31**, 395.
	Snail (helix pomata)		Hammarsten, *Pflüger's Archiv*, 1885, **36**, 373.
	Synovial fluid		von Holst, *Zeitsch. physiol. Chem.*, 1904, **43**, 145.
	Trachæa and sputum		F. Müller, *Zeitsch. f. Biologie*, 1901, **42**, 468.
	Urine		Mörner, *Skand. Archiv f. Physiol.*, 1895, **6**, 332.
	Blood		Liebermeister, *Beitr. physiol. path. Chem.*, 1906, **8**, 439.
	Liver		Wohlgemuth, *Zeitsch. physiol. Chem.*, 1903, **37**, 475.
	Lymphatic organs		Bang, *Beit. physiol. path. Chem.*, 1904, **4**, 362.
	Meat (striated muscle)		Pekelharing, *Zeitsch. physiol. Chem.*, 1896, **22**, 245.
	Mucous membrane of stomach		Pekelharing, *Zeitsch. physiol. Chem.*, 1896, **22**, 233.
	Pancreas		
Nucleoproteins		Nucleoproteins	α-nucleoprotein: Umber, *Zeitsch. f. klin. Med.*, 1900, **40**, 464. Gamgee and Jones, *Beitr. physiol. path. Chem.*, 1904, **4**, 10. β-nucleoprotein: Hammarsten, *Zeitsch. physiol. Chem.*, 1894, **19**, 191 1902, **35**, 111.
	Serum		Bywaters, *Biochem. Zeitsch.*, 1905, **15**, 322.
	Spleen		Levene and Mandel, *Zeitsch. physiol. Chem.*, 1906, **47**, 151
	Suprarenals		Jones and Whipple, *Amer. J. Physiol.*, 1902, **7**, 423.
	Thymus		Huiscamp *Zeitsch. physiol. Chem.*, 1901, **32**, 145.
Onuphin	*Onuphis tubicola* (worm)	Scleroprotein	Schmiedeberg, *Reports Naples Zool-Station*, 1882, **3**, 373
Parahistone	Thymus gland	Histone	Fleroff, *Zeitsch. physiol. Chem.*, 1899, **28**, 307.
Protamines	Testicles of fish	Protamines	(General) Kossel, *Zeitsch. physiol. Chem.*, 1896, **22**, 178.
Reticulin	Mucosa (small intestine of pig)	Scleroprotein	Siegfried, *J. Physiol.*, 1893, **28**, 319.
Salmine	Testis of salmon	Protamine	(See protamines.)
Scombrine	Testis of mackerel	Protamine	(See protamines.)
Silk gelatin (sericin)	Silk	Scleroprotein	Bondi, *Zeitsch. physiol. Chem.*, 1902, **34**, 481.
Silk fibroin	Silk	Scleroprotein	E. Fischer and Skita, *Zeitsch. physiol. Chem.*, 1901, **33**, 177; 1902, **35**, 221.
Spongin	Sponges	Scleroprotein	Abderhalden and Strauss, *Zeitsch. physiol. Chem.*, 1906, **48**, 49.
Sturine	Testes of salmon	Protamine	(See protamines.)
Thyreoglobulin	Thyroid gland	Globulin	Oswald, *Zeitsch. physiol. Chem.*, 1899, **27**, 64; 1901, **32**, 121
Vitellin	Egg-yolk	Phosphoprotein	Levene and Alsberg, *Zeitsch. physiol. Chem.*, 1901, **31**, 543

[1] The numerous proteins included under this heading, such as hair, horn, tortoise shell, are all easily prepared by separating other constituents of tissues by water, alcohol, ether, peptic and tryptic digestion, etc. No special reference is necessary.

[2] It is doubtful whether this substance, prepared from the bile, is a true mucin.

PROTEINS OF PLANTS.[1]

By E. FRANKLAND ARMSTRONG, Ph.D., D.Sc., F. C. G. I.

The proteins of plants have the same general properties as those of animals, yielding on hydrolysis a mixture of the same amino-acids. Some of these amino-acids coexist in plants with the proteins. Although a large number of plant proteins exist, only those of wheat and barley are of outstanding commercial importance at present, though as the knowledge of the subject increases, it is probable that considerable attention will be directed to the study of the proteins of fodders from the point of view of their respective value in animal nutrition.

In the case of fodder plants the first requisite is the determination of total nitrogen which is expressed as protein after multiplication by a factor.[2] A second stage is the study of the distribution of nitrogen as (1) ammonia, (2) basic, (3) non-basic and (4) nitrogen precipitated by magnesium oxide. A future step may prove to be the identification of albumins as well as globulins in the plant material and the proof that it will yield on hydrolysis minimal quantities of all the necessary constructional units before it can be classed as a perfect food. At present, little more than the determination of total nitrogen is attempted in examining either animal fodders or foods used for human consumption though there are signs that the subject will receive greater attention in the future.

Analyses (*i.e.*, water, oil, proteins (N×6.25), digestible carbohydrates, fibre and ash) of some 300 feeding stuffs are given by Smetham (*J. Roy. Lancashire Agric. Soc.*, 1909; *J. Soc. Chem. Ind.*, 1910, 39). Their relative values as cattle foods are calculated on the basis that oil and proteins are of equal value and 2.5 times as valuable as carbohydrates.

[1] Authorities: T. B. Osborne, *Die Pflanzenproteine*, Wiesbaden, 1910; The Vegetable Proteins, London, 1909. Hutchison, *Food and the Principles of Dietetics*, London, 1911.
[2] This method takes no account of the presence of nitrogenous substances other than proteins.

When it is remembered that vegetable proteins are broken down to their constituent amino-acids in the animal organism and that these are used to build up the various tissue and other animal proteins, the importance of a knowledge of the composition of the vegetable proteins becomes obvious.

Proteins are classified according to their properties as albumins, globulins, glutelins, prolamins, albuminoids, histones, and protamines (see pages 33 and 34). Vegetable proteins belong to the first four groups, no representative of the remaining groups having been found in plants. Plants contain more than one kind of protein and plant proteins are conveniently grouped as cereal proteins, leguminous proteins and oil-seed proteins. Seeds naturally contain both the reserve proteins of the endosperm and in smaller quantities the proteins of the embryo. Consequently a mixture of proteins is obtained on extraction.

The vegetable *albumins* are slightly soluble in water and coagulated by heat; the best known are *leucosin* of cereal seeds, *legumelin* found in vetch and bean seeds, and *ricin* found in castor bean seeds.

Plant *globulins* are soluble in neutral 10% solutions of sodium chloride but insoluble in water. They are not as a rule precipitated by saturating their solutions with magnesium sulphate but they are all precipitated on saturation with ammonium sulphate. Most of them are only imperfectly coagulated on boiling their solutions. Several globulins have been obtained by dialysis in well formed crystals; crystals can generally be obtained by diluting the solution in sodium chloride with water at 50 or 60° until a turbidity forms and then allowing it to cool slowly. Crystalline globulins are *edestin*, *excelsin* and those of flax-seed and the castor bean. Other representatives of the class are *legumin*, *amandin*, *phaseolin*, *juglansin*.

Vegetable *glutelins* are insoluble in water, saline solutions or alcohol. In consequence their purification is a matter of difficulty. The best known is *glutenin* of wheat—similar proteins exist in rice and maize.

The *prolamins* or alcohol soluble proteins are very characteristic of cereals. They are nearly insoluble in water but dissolve freely in presence of traces of acids or alkalies, that is to say, as salts. On hydrolysis they yield a very large proportion of glutamic acid, proline and ammonia. *Gliadin* is present in wheat and rye, *hordein* in barley and *zein* in maize.

The methods of determining the products of hydrolysis of proteins

do not account at present for more than 50–60% of the total; the values for the various amino-acids obtained are therefore to be regarded only as provisional and no useful purpose is to be gained by quoting them here. (Compare Osborne and Jones, *J. Biol. Chem.*, 1910, I, viii–ix; Aberhalden and Weil, *Zeits. physiol. chem.*, 1911, **74**, 445.) The latter authors have shown that the existing values for aspartic acid should be at least doubled.

Extraction and Separation of Vegetable Proteins.

Water extracts albumin and proteoses and, in addition, a certain amount of globulin owing to the soluble mineral salts rendering the water equivalent to a weak salt solution. It is thus often preferable to extract immediately with 10% salt solution shaking the finely ground material with twice its weight of the solution. By dialysis of this extract, the globulins are precipitated and the albumins remain in solution. The albumins are separated by fractional coagulation effected by heating at different temperatures, 65° and 85° being those commonly chosen. The precipitated globulins may be fractionated by using salt solutions of varying strengths as solvents.

The residue after extraction with salt solution is extracted with 70 or 75% alcohol. The filtrates yield the prolamins on evaporation. The final residue contains glutelins; it may be extracted with very weak acids or alkalies.

The proteins of the vegetative parts of plants are present only in small quantities and they are very difficult to isolate. Hence they have not been studied to any extent.

In analysing vegetables, fruits, etc., it is customary to determine total nitrogen by the Kjeldahl method in the absence of nitrates and multiply by the conventional factor 6.25 (see page 48). Osborne's researches have shown that the factor varies with the different cereals as follows: wheat 5.7, rye 5.62, oats 6.31, maize 6.39, barley 5.82. In consequence the factor 5.7 is frequently used for cereals. The factor used should be indicated in returning the analysis.

Atwater and Bryant have made full analyses of the common vegetables and fruits, berries and nuts, both as purchased and of the edible portion only. (U. S. Department of Agriculture, *Bulletin* 28.) With the exception of beans, the edible portion of which contains 7% of protein, most vegetables contain less than 1.5% of protein and most fruits less than 1%.

The protein in the edible portion of nuts is as follows:

Nut	% Protein
Almonds	20.0
Beechnuts	21.9
Brazilnuts	17.0
Butternuts	27.9
Chestnuts (fresh)	6.2
Cocoanuts	5.7
Filberts	15.6
Hickorynuts	15.4
Peanuts	25.8
Pecans	11.0
Pistachios	22.3
Walnuts (Californian)	18.4

The leguminous foods, often called the "pulses," are comparatively high in nitrogen content. Analyses have been published by Balland (*Compt. Rend.*, 1897, **125**, 119). In dried material containing about 10% moisture, he finds in haricot beans from 14–25%, lentils, 21–24%, peas, 19–23% of protein. The proteins of the pulses are particularly rich in sulphur. Potatoes contain 1.4–2.8% normally and from 6–13% when dried. This consists almost entirely of a globulin (Osborne, *J. Amer. Chem. Soc.*, 1896, **18**, 575).

Roots such as turnips and carrots contain practically no protein, the nitrogen being entirely present in the form of amides, etc. Beets contain about one-third of their total nitrogen in the form of protein; in other vegetables only one-half of the nitrogen represents protein.

Banana flour contains about 4% protein, the fruit has about 1.5%. (Much information relating to the protein of food materials will be found in Hutchison, *Food and Dietetics*, Chap. XIII.)

Proteins of Cereals.

Wheat.—Wheat contains in all about 10% of protein comprising 5 different substances, viz.:

Class	Protein	Proportion present
Prolamin	Gliadin	4.25
Glutelin	Glutenin	4.0–4.5
Albumin	Leucosin	0.3–0.4
Globulin		0.6–0.7
Proteose		0.3

These all contain carbon, hydrogen, nitrogen and sulphur. The first two constitute over 90% of the protein of flour and together make what is termed gluten. The others are technically of but small importance and are mostly contained in the germ. The knowledge of wheat proteins is almost entirely derived from the researches of Osborne with Voorhees (*Amer. Chem. J.*, 1893, **15**, 392), Harris

(*J. Amer. Chem. Soc.*, 1903, **25**, 323, 842) and Clapp (*Amer. J. Physiol.*, 1906, **17**, 231) on which the following summary is based.

Gliadin is probably the sole alcohol-soluble protein of wheat though this has been in the past a point of controversy. It is most soluble in alcohol of approximately 70%, insoluble in absolute alcohol. It is very soluble in distilled water, forming opalescent solutions, sparingly so in water containing salts. It readily dissolves in very dilute acid or alkali but is precipitated unchanged on neutralisation. It also dissolves in methyl and propyl alcohols and in some aromatic alcohols. The same gliadin is obtained from strong and weak wheats; it is indifferent also whether it is extracted from flour or from gluten.

The most recent determination of the products of hydrolysis of gliadin is given in the table on page 98. It is characterised by giving an exceptionally large proportion of glutamic acid and ammonia, no lysine and very little basic amino-acid. It differs very considerably in composition from the other proteins used as foods. The distribution of the nitrogen is given on page 98. The specific rotation $[\alpha]_D^{20°} = -92°$ in 80% alcohol.

Glutenin readily dissolves in very dilute acids and alkalies and is precipitated on neutralisation, the precipitate consisting of an insoluble glutenin salt. The suspension in water coagulates on heating. Glutenin has almost exactly the same composition as gliadin but as the tables on page 98 show, the products of hydrolysis and the distribution of nitrogen are different. To prepare glutenin, crude gluten is extracted several times with alcohol and the residue dissolved in 0.2% potassium hydroxide. The protein is precipitated by exact neutralisation with acetic acid; it is then treated with alcohol and ether to remove fat, etc., redissolved in alkali, the solution filtered and the protein reprecipitated by acetic acid.

Leucosin is present in flour only in small quantity, but the germ contains about 10%. It is obtained from flour by extraction with water and precipitated by saturation with ammonium sulphate; this precipitate is dissolved in sodium chloride and the globulin precipitated by dialysis. Leucosin is coagulated in this solution by heating at 61° and filtered. When extracted from the germ it is at first obtained in combination with nucleic acids. The figures in the tables on page 98 show that the products of hydrolysis and distribution of nitrogen are very different to those given by gliadin and glutenin.

The **globulin** of wheat is obtained crystalline in minute spheroids by extracting with sodium chloride and dialyzing. It is precipitated

from solution in sodium chloride by saturation with magnesium or ammonium sulphate but not by saturation with sodium chloride. Though partly precipitated by boiling it is not coagulated below 100°.

It is contained in germ to the extent of 5% and may be extracted by sodium chloride solution at 70° at which temperature the albumin is coagulated. The product is apparently a globulin nucleate.

The **proteose** is possibly formed from the other proteins during the process of extraction. It is obtained from the sodium chloride extract, after removing globulin and leucosin as described, by adding sodium chloride to the extent of 20% and a little acetic acid, or by saturating with sodium chloride. It has been claimed that gluten does not preexist in flour but is a product of the action of an enzyme in presence of water, on the other proteins of the grain. This view is now generally discredited.

COMPOSITION OF WHEAT PROTEINS.

Protein	C	H	N	S	O
Gliadin	52.72	6.86	17.66	1.02	21.74
Glutenin	52.34	6.83	17.49	1.08	22.26
Leucosin	53.01	6.83	16.93	1.30	21.93
Globulin	51.03	6.85	18.39	0.69	23.04
Proteose	49.94	6.80	17.08	1.24	24.94

DISTRIBUTION OF NITROGEN IN WHEAT PROTEINS.

Protein	Total %	As ammonia	Basic	Non-basic	In MgO precipitate
Gliadin	17.66	4.30	1.09	12.25	0.14
Glutenin	17.49	3.30	2.05	11.95	0.19
Leucosin	16.93	1.16	3.50	11.83	0.43
Globulin	18.39	1.42	6.83	9.82	0.28

CONSTITUENTS OF WHEAT PROTEINS.

Constituent	Gliadin	Glutenin	Leucosin
Glycine	0	0.89	0.94
Alanine	2.00	4.65	4.45
Valine	3.34	0.24	0.18
Leucine	6.62	5.95	11.34
Proline	13.22	4.23	3.18
Phenylalanine	2.35	1.97	3.83
Aspartic acid	0.58	0.91	3.35
Glutamic acid	43.66	23.42	6.73
Serine	0.13	0.74	....
Tyrosine	1.20	4.25	3.34
Cystine	0.45	0.02	....
Lysine	....	1.92	2.75
Histidine	0.61	1.76	2.83
Arginine	3.16	4.72	5.94
Tryptophane	1.00	+	+
Ammonia	5.22	4.01	1.41
Total	83.54%	59.66%	50.32%

Gluten is composed of gliadin and glutenin in approximately equal quantities. The gliadin forms a sticky medium with water which binds together the particles of glutenin to a coherent mass possessed of considerable tenacity and elasticity. The properties of gluten and its power of holding water are due almost entirely to the proportion of inorganic salts present, in particular to phosphates. Both gliadin and glutenin are emulsion colloids. Crude gluten, as obtained from flour by washing out the starch, is a sticky elastic mass varying in colour from grey to light yellow; when dried it becomes brittle like glue and of a yellow-brown colour. In addition to proteins and mineral matter it contains small quantities of lecithin, fat, fibre and starch.

Norton (*J. Amer. Chem. Soc.*, 1906, **28**, 8) gives the following analysis of crude gluten from *durum* flour:

Fats, etc.	4.20%
Carbohydrates	9.44
Fibre	2.02
Mineral matter	2.48
Gliadin (alcohol soluble)	39.09
Glutenin	35.07
Globulin (soluble in 10% NaCl)	6.75
	99.05%

Crude gluten apparently contains about 75% of true gluten and 7% of non-gluten protein. The total amount of crude gluten corresponds very closely to the total amount of protein (N × 5.7) in flour, the loss in nitrogenous constituents being balanced by the retention of other substances.

The quality known as "strength" in flour, which may be defined as the capacity for making bold, well-risen loaves, depends mainly on the quality and quantity of gluten. Accordingly gluten determinations form the most usual way of ascertaining the strength of flours. (For the full discussion of the strength of flour, Jago, *Technology of Bread Making*, Chapter XV, should be consulted.)

(1) *Total Protein.*—Nitrogen is determined by Kjeldahl's method and multiplied by 5.7.

(2) *Crude Gluten.*—30 grm. of flour are made into a dough with 12–15 c.c. of water. Distilled water may be used but under factory conditions it is preferable to use the local water. The dough is allowed to stand an hour. It is then washed by kneading between the fingers in a gentle stream of tap water, the dough being held over a muslin filter so that any particles which are dropped can be recovered. Washing is continued until starch can no longer be removed, when the remaining dough has the characteristic consistency of gluten. It is

then placed under water for an hour. During this period the gluten may be tested between the fingers to gain some idea of the tenacity and elasticity. It is next dried by rubbing on the palms of the hands and mechanical removal of the water until the moment when the gluten begins to stick, when it is transferred to 2-in. squares of tared parchment or other stout paper and the wet weight determined. The dry weight is obtained after heating for 24 hours or until constant in a water oven. The method is crude but gives remarkably concordant results when performed always by the same person in precisely the same manner. A refinement consists in kneading the dough in a basin of water at a known temperature, instead of in running water.

To ascertain true gluten a nitrogen determination may be made in the dry gluten. The dry gluten varies in flours from 8–15%, according to their origin.

Some of the more common types of flour are indicated in the following table taken from the data given by Jago.

Flours from single wheats	Crude gluten		
	Wet	Dry	Ratio
English	22.0%	7.8%	2.9%
American spring patent	40.0	13.4	2.9
American winter patent	24.0	8.2	2.9
Californian straight grade	21.0	7.1	2.9
Manitoba hard patent	30.0	10.8	2.7
Hungarian	33.0	11.35	3.0
Bombay	26.0	9.05	2.9
Calcutta	28.1	9.68	2.9
Australian	42.0	14.2	3.0
Russian–Kubanka	48.0	15.8	3.0
Russian–Azima	39.0	12.7	3.0

The ratio of wet to dry gluten gives an indication of the water-holding capacity of a flour. The strongest glutens hold least water and the ratio varies usually from 2.7 to 3.1 under the experimental conditions described. With experience, a great deal as to the character of flour is learned from the feel of the gluten. A strong gluten is tough and elastic and not sticky; weaker gluten is soft and sticky and possesses but little toughness. It can be drawn out into long strands.

It was formerly customary to determine the amount of expansion of gluten under standard conditions in an instrument termed the aleurometer, but the results are of little value and the instrument has fallen into disuse.

The **non-gluten nitrogen** in flour is determined (Teller's method) by extracting 2 grm. with 15 c.c. of 1% sodium chloride in a 25

c.c. flask for an hour, shaking every 10 minutes. The flask is then filled up to the mark and allowed to stand 2 hours. The liquid is filtered clear and 50 c.c. taken for analysis by the Kjeldahl method. From the per cent. of nitrogen obtained 0.27 is deducted for the amount of gliadin soluble under these conditions.

Amino Nitrogen.—In addition to the proteins, flour also contains certain nitrogenous bases—asparagine, choline, betaine, etc. The proteins in 100 c.c. of the salt extract, obtained as described, are precipitated by 10 c.c. of a 10% solution of phosphotungstic acid, the precipitate being allowed to settle overnight. Nitrogen is then estimated in the filtrate.

Gliadin.—It has been suggested that the ratio of gliadin to glutenin or to the total protein of the flour influences the quality of gluten and affords a measure of strength. While the last statement is disputed, the estimation of gliadin is still of importance. It may be made either on the washed out gluten or on the flour directly. In either case the procedure consists in extraction with 70% alcohol. It is empirical in the sense that the proportion of solvent, the time and the temperature of extraction all influence the result.

Chamberlain (*J. Amer. Chem. Soc.*, 1906, **28**, 1657) takes 2 grm. of flour with 100 c.c. of alcohol (70%) and extracts at room temperature for 24 hours with occasional shaking. Nitrogen is determined in an aliquot portion of the filtrate and multiplied by 5.7. He finds that hot alcohol dissolves less protein than cold. This is due to proteins of the albumin type becoming coagulated and less soluble.

Jago uses hot 70% alcohol for this reason, Teller advocates a weaker alcohol (57%, sp. gr. 0.90). Greaves (*J. Biol. Chem.*, 1911, **9**, 271) finds that if more than 2 grm. of flour are used the extraction of gliadin is not complete. 65% (by volume) alcohol extracts the greatest amount of protein, the amount decreasing as the strength of the alcohol increases. 74% (by volume) alcohol extracts more nearly pure gliadin than does alcohol of other strengths.

The results obtained by the polarimetric determination of gliadin in the extract are fairly accurate but less reliable than those obtained by the Kjeldahl method.[1]

The extraction of gliadin from gluten is rendered difficult by the physical nature of gluten. Jago recommends the following process. 2.2 grm. of wet gluten, 11 grm. of spirit washed starch and 400 c.c. of 70% alcohol are taken. The gluten and half the starch are ground up in a mortar with a few drops of alcohol and starch and alcohol are

[1] This subject is fully discussed by Jago.

added alternately and the grinding continued. Ultimately a perfectly smooth dough is obtained. This is transferred to a bottle, the rest of the alcohol added hot and the whole agitated for 18 hours in a shaking machine.

To illustrate the results obtained we may quote from Jago (*loc. cit.*) the figures for three typical flours.

Percentages on flour	American spring	French, grown in England	Karachi
Total protein	12.95	10.19	8.14
Wet gluten	42.3	29.9	23.5
Dry gluten	15.02	9.75	6.77
Ratio	2.8	3.0	3.4
True gluten	10.77	7.8	5.37
Gliadin *ex* gluten	7.36	4.98	3.75
Gliadin *ex* flour	6.43	5.25	3.82
Percentages on dry gluten:			
Non-protein in gluten	28.3	20.0	20.7
Gliadin	49	51	55

A large number of similar tests are quoted and discussed by Jago who shows that while the protein and gliadin determinations on the flour direct fail to measure minor differences in character and quality, the gluten tests afford much information about the baking properties.

Edible Pastes.—The coarse ground meal from "durum" wheats known as semolina which is high in gluten is used as the basis for making macaroni and other edible pastes. The wheat is specially prepared and the semolina obtained as small round glazed granules. It is kneaded with warm water, sometimes admixed with vegetable juices or colouring matter, moulded into shape by pressure through holes and dried. *Macaroni* is the larger of the tube-like products; *spaghetti* is smaller and more cord like; *vermicelli* is the worm-shaped variety; *noodles* are a strap-shaped form made in Germany, they should contain a certain percentage of eggs. These products contain on the average 10% water, 12% protein and 75% carbohydrate. Egg-noodles according to the German requirements should contain at least 0.045% of lecithin phosphoric acid and 2% of ether extract corresponding to 4 eggs per kilo of flour (see Leach, *Food Inspection and Analysis*, page 348).

Gluten Flour and Bread.—Gluten flour and bread are used as substitutes for ordinary flour and bread in cases of diabetes. Much of the gluten flour which is sold commercially is quite unfit for this purpose as it contains a very large proportion of starch, and even the best brands usually contain as much as 5 or 6%.

Starch in gluten cannot be estimated by boiling with acid and even the diastase method gives unreliable results. It is preferable to adopt a modification of Pfluger's method for estimating glycogen. This method is generally applicable for the estimation of starch in presence of much protein, as for example in sausages.

Ten grams of material are boiled for half an hour with 80 c.c. of 5% potassium hydroxide under a reflux condenser. The mixture tends to froth; this may be partially overcome by shaking well when heating is commenced or by the addition of a few drops of amyl alcohol. The liquid is cooled and poured into 200 c.c. of 80% alcohol. The precipitate is allowed to settle and stirred slightly to cause it to agglomerate. It is filtered, washed and transferred into a 300 c.c. flask where it is boiled with 200 c.c. of 7.5% (by volume) hydrochloric acid for 2.5–3 hours. The filtrate is made up to 250 c.c. and dextrose determined in an aliquot portion by one of the methods described in Vol. 1.

Gluten flour tends to retain fat very firmly so that it is not extracted by ether.

Very few of the diabetic and obesity foods have the composition claimed for them. The estimation of total carbohydrates in them is generally of more importance to the analyst than that of protein.

Barley and Malt.

Barley contains 5 proteins. 10% sodium chloride solution extracts a globulin, an albumin and a proteose, all three being probably the same as those in wheat and oats. Alcohol extracts a distinctive prolamin known as *hordein* and the residue consists of a glutelin which has not been obtained in a state of purity owing to the large amount of gummy matter in the seeds.

The proportions are as follows:

Total protein	10.76%
Globulin, albumin and proteose	2.24%
Hordein	4.04%
Glutelin	4.48%

Hordein contains more carbon than gliadin, the percentage composition of the two being

	C	H	N	S	O
Hordein	54.29	6.80	17.21	0.83	20.87
Gliadin	52.72	6.86	17.66	1.02	21.74

The products of hydrolysis have been determined by Osborne and Clapp (*Amer. J. Physiol.*, 1907, **19**, 177) and by Kleinschmitt (*Zeitsch. Physiol. Chem.*, 1907, **54**, 110). It contains more glutamic acid (43.2%) and proline (13.7%) than gliadin.

The nitrogen distribution is as follows: as ammonia 4.01%, basic 0.77, non-basic 12.04, in magnesium precipitate 0.23; total 17.21% (Osborne and Harris, *J. Amer. Chem. Soc.*, 1903, **25**, 323). The basic nitrogen is very low, the ammonia nitrogen unusually high.

Specific rotation, $[\alpha]_D^{20} = 122.9°$ in neutral 70% alcohol solution.

The **leucosin** is the same as in wheat; it is closely connected with the diastatic power of barley. Osborne states that the most active preparations of diastase which he obtained consisted of albumin (53%) and proteose. Leucosin comprises about 0.3% of the barley corn.

On germination the proteins of barley are changed. Osborne and Cambell (*J. Amer. Chem. Soc.*, 1896, **18**, 542) conclude that they do not acquire the properties of proteoses; that hordein disappears, a protein (*bynin*), soluble in alcohol, of entirely different composition, taking its place. The globulin also disappears and is replaced by a new globulin—*bynedestin;* the albumin remains unaltered, but very much increases in quantity. Three or more proteoses are also formed.

1. **Bynin** is readily soluble in dilute alcohol, insoluble in water and salt solutions. It has C 55.03, H 6.68, N 16.26, S 0.84, O 21.2—that is, more carbon and less nitrogen than hordein.

2. **Bynedestin** is more soluble in very dilute salt solution than the globulin of barley. In 10% sodium chloride solution it gives a turbidity at 65° and a flocculent precipitate at 84°, but coagulation is incomplete even on protracted heating at 100°. It is partly precipitated by saturation with magnesium sulphate. It has more carbon and less nitrogen than barley globulin.

	C	H	N	S	O
Barley globulin.....	50.88	6.65	18.1	24.37	
Bynedestin.....	53.19	6.69	15.68	1.25	23.19

The following are the remaining proteins of malt.

3. **Leucosin,** identical in composition and properties with that in barley.

4. A **proto-proteose,** of the same composition as leucosin, readily precipitated from its aqueous solution by adding an equal weight of alcohol.

5. A **proto-proteose,** less readily precipitated by alcohol, and differing in composition from the preceding.

6. A **deutero-proteose.**

7. A **hetero-proteose,** present in extremely small amount.

8. A protein insoluble in water, saline solutions, and dilute alcohol.

The following proportions of these constituents are found in malt:

		%
Protein, insoluble in salt solutions and in alcohol		3.80
Bynin, soluble in dilute alcohol		1.25
Bynedestin, leucosin, and proteoses soluble in water and salt solutions	coagulable	1.50
	uncoagulable	1.29
Total protein		7.84

The nitrogenous constituents of malt and the nitrogen question in brewing form the subject of very complete papers by Horace Brown,[1] to which it is impossible to make more than brief reference here.

The estimation in barley of the total amount of nitrogenous substances soluble in water[2] demands as the one essential condition the maintenance of a constant relation between the weight of the barley and that of the extraction water. This is due to the influence of the inorganic salts of the grain on the solvent action of the water on the globulins. It is therefore difficult to make a strict comparison between barleys of different origin. The same difficulty is not experienced with malt.

The standard adopted by Brown is a concentration of 20 grm. of dry barley per 100 c.c. of the mixture of grain and water, extraction being continued for 6 hours at 17°.

The interest from a practical standpoint of the nitrogenous constituents of malt centres on those substances which are soluble in water and are not thrown down on boiling.

An ordinary boiled wort contains only 0.56% of permanently soluble nitrogen compounds. Of this 51% is precipitable by phosphotungstic acid; the remainder of 49% left in the filtrate is classified as follows:

Ammonia	1.34%
Amides	3.44%
Monoamino-acids[3]	9.15%
Unclassified	35.07%
	49.00%

Extraction of malt with 70% alcohol dissolves all the organic bases,

[1] *Trans. Guinness Research Lab.*, Part ii, 1906, 169. *J. Inst. Brewing*, 1907, 13, 394; 1909, 15, 170.

[2] *Trans. Guinness Research Lab.*, Part i, 1903, 61.

[3] The ratio of half the amount of nitrogen liberated by nitrous acid to the total nitrogen multiplied by 100 gives the apparent percentage of nitrogen present as amino-acids; this is termed the amino-index and denoted by the symbol AoN. Compare the subsequent work of van Slyke, pages 86 and 486.

amides and amino-acids as well as a good deal of the unclassified substances which mainly consist of malt albumoses and malt peptones representing a series of transformation products derived from hordein.

The nitrogen in this extract is divided as follows:

Ammonia	2.0%
Malt peptones	58.0%
Malt albumoses	16.0%
Amides and amino-acids	16.0%
Organic bases (betaine and choline)	8.0%
	100.0%

The distribution of nitrogen in the cold water extract of malt is

Ammonia	3.5%
Malt albumose	20.0%
Malt peptone	35.0%
Amide and amino-acid	8.5%
Organic bases	4.0%
Unaccounted for	33.0%
	100.0%

The permanently soluble nitrogen of malt extracts, worts and beers can be differentiated into "assimilable" and "non-assimilable" nitrogen; the former alone favours yeast production. The relation of the assimilable nitrogen to the total permanently soluble nitrogen is fairly constant and there is also a close connection between the total nitrogen of a barley and the soluble nitrogen which can be extracted from the malt resulting from it. In barleys of the same class a fairly constant percentage of the initial nitrogen is rendered soluble during malting. 48% of the total soluble nitrogen is due to the germ and 52% to the endosperm, whereas in the case of the assimilable nitrogen 52 parts are derived from the germ and 48 parts from the endosperm. The soluble nitrogen extracted during mashing under standard conditions increases with the temperature at first uniformly up to 38° and then more rapidly to a maximum at 49°; when it amounts to 40% of the total nitrogen of the malt. It decreases somewhat from 49 to 60° and still more from 60 to 65.6°, the temperature of the ordinary infusion mash.

Maize.

Maize or Indian Corn, like the other cereals, contains 5 proteins but differs from wheat in giving no gluten. The amount of globulin, albumin and proteose is less, that of prolamin more than in wheat.

Zein, the alcohol soluble protein, dissolves in 90% alcohol but is insoluble in 50–60% alcohol and thus differs from gliadin. It has C 55.23, H 7.26, N 16.13, S 0.60, O 20.78. The products of hydrolysis of zein are somewhat abnormal; it contains neither glycine,

lysine nor tryptophane. The full amount of glutamic acid is isolated only with difficulty and is less than in gliadin. The proportion of leucine and phenylalanine is unusually high (Osborne and Clapp, *Amer. J. Physiol.*, 1907, **20**, 477; Willcock and Hopkins, *J. Physiol.*, 1906, **35**, 88). $[\alpha]_D^{20} = -28°$ in 90% alcohol.

The **glutelin** has been incompletely investigated; it contains considerable proportions of arginine, hystidine and lysine.

The amount of proteins is as follows:

Globulin, albumin, proteose	0.45%
Zein	5.0 %
Glutelin	3.15%
	8.6 %

The mean nitrogen content of the maize proteins is 16%.

Oats.

The study of these presents considerable difficulty and is as yet incomplete (Osborne, *Amer. Chem. J.*, 1891, **13**, 327, 385; **14**, 212). The aqueous extract is very acid and the proteins in solution are rapidly changed by proteolytic enzymes.

Very little if any *albumin* is present. 10% sodium chloride extracts about 1.5% of a *globulin* in the cold. Sodium chloride at 60° extracts a crystalline globulin, very similar to the first but containing 1% more nitrogen; it is perhaps a purer form.

The **prolamin** amounts to 1.25%. It has the following composition: C 53.01, H 6.91, N 16.43, S 2.26, O 21.39, the amount of sulphur being abnormally large. It differs in solubility from other prolamins.

Rice.

The proteins at present identified are an *albumin* coagulating at 85° present only in small quantity, a *globulin* coagulating at 70° and a *glutelin* named *oryzenin*. Prolamin is not present (Rosenheim and Kajura, *J. Physiol.*, 1908, **36**, liv). The results of analyses of 159 samples of Indian rice are given by Hooper (*Agric. Ledger*, 1908–9, 63; *J. Soc. Chem. Ind.*, 1910, **29**, 38). The amount of protein varies from 6.58 to 7.69%. (See also *J. Biol. Chem.*, 1912.)

Rye.

Rye does not form gluten and contains much gummy carbohydrate which renders the isolation of the proteins a matter of difficulty.

They are closely related to those of wheat, those soluble in water and salt solution being identical in the two cereals.

The **prolamin** in rye is very similar to gliadin and the two yield almost identical products on hydrolysis (Osborne and Clapp, *Amer. J. Physiol.*, 1908, **20**, 494). Rye gliadin contains somewhat less arginine and histidine: this in agreement with the somewhat smaller value for the basic nitrogen. Rye also contains a little glycine and more proline than gliadin. The distribution of nitrogen is: as ammonia 4.05%, basic 0.91%, non-basic 12.65%, precipitated by magnesium oxide 0.11%, total 17.72%. The chief difference between wheat and rye gliadin is in the specific rotation. In 55% alcohol, rye gliadin has $[\alpha]_D^{20} = -121°$, wheat gliadin $-95°$.

The **glutelin** of rye has not been isolated.

The distribution of the proteins is approximately

Albumin	0.4%
Globulin and proteose	1.7%
Gliadin	4.0%
Glutelin	2.5%
	8.6%

Extensive analyses of cereal grains are given in *Bulletin* 13, Division of Chemistry, U. S. Dept. of Agriculture. The mean proteins are there given as follows; the figures refer to the American grown products and there is a considerable difference between the maximum and minimum values:

Cereal	Mean	Range
Barley	11.5%	
Buckwheat	10.8%	
Maize	9.9%	8.6–11.5%
Oats	12.1%	9.1–15%
Rice (polished)	7.2%	
Rye	12.4%	8.4–19%
Wheat	12.2%	8.5–17%

Oil Seeds.

Oil seeds, in which starch is replaced by oil, contain considerable quantities of protein; it is remarkable that, though these are very similar, in no case has the same protein been found in two different species even when they belong to the same family. Many of the proteins have been obtained crystalline. They contain a relatively large proportion of arginine and in consequence have more nitrogen than other proteins. The solubility in 10% salt solution characterises them as globulins. The present knowledge of them is but scanty.

PROTEINS OF OIL SEEDS

Protein	Source	$[\alpha]_D^{20}$ in 10% sodium chloride	Chief constituents
Excelsin	Brazil nut. *Bertholletia Excelsa*	−43°	Glutamic acid, 13% Arginine, 14.3%
Globulin	Squash seed. *Cucurbita maxima*	−38.7°	Glutamic acid, 12.4% Arginine, 14.4%
Ricin	Castor bean. *Ricinus communis*	−28.9° (in water)	A toxalbumin.
Edestin	Hemp seed. *Cannabis sativa*	−41.7°	Glutamic acid, 14.5% Arginine, 14.4% Leucine, 14.5%
Linseed	Flax seed. *Linum usitatissimum*	−43.5°	Valine, 12.7%
Globulin	Cotton seed. *Gossypium herbaceum*		Glutamic acid, 17.2% Arginine, 13.5% Leucine, 15.5%
Amandin	Almonds. *Prunus amygdalus*	−56.4°	Glutamic acid, 23% Arginine, 11.9%
Corylin	Hazel nut. *Corylus avellena*	−43°	Glutamic acid, 18%
Juglansin	Walnut. *Juglans cinerea*	−45°	

Amandin is the chief protein of the almond, peach or plum kernel; it is obtained by extraction of the oil-free meal with 10% salt solution and purified by dialysis or precipitation with ammonium sulphate. The quantity amounts to some 25% of the oil-free meal (Osborne and Campbell, *J. Amer. Chem. Soc.*, 1896, **18**, 609).

It is only partially coagulated by heat. Distribution of nitrogen: as ammonia 3.05, basic 4.15, non-basic 11.55, total 19.0% (Osborne and Harris, *J. Amer. Chem. Soc.*, 1903, **25**, 323).

It yields on hydrolysis glutamic acid 23%, arginine 11.85%. (For the complete hydrolysis see Osborne and Clapp, *Amer. J. Physiol.*, 1908, **20**, 470.)

Edestin, the globulin of hemp seed (*Cannabis sativa*) is prepared by extraction of the ground seed with 10% sodium chloride solution to which preferably a little barium hydroxide has been added. When the extract is dialysed the crystalline globulin separates (Osborne, *J. Amer. Chem. Soc.*, 1894, **16**, 633, 703, 757). The oil-free meal contains about 20–24% of protein.

The solubility in inorganic salt solutions has been fully investigated by Osborne and Harris (*Amer. J. Physiol.*, 1905, **14**, 151). The chief products of hydrolysis are leucine 14.5%, glutamic acid 14.5%, arginine 14.17%.

Linseed.—The proteins have been investigated by F. W. Foreman (*J. Agric. Sci.*, 1910, **3**, 358). They were extracted with 0.2% potassium hydroxide. According to his results they are abnormal in containing much valine (12.7%) and very little tyrosine (0.65%).

The castor bean contains an albumin and a crystalline globulin of similar nature to the other seed globulins. It is separated by dialysis of the sodium chloride extract.

Ricin, the soluble albumin of the castor bean, is closely connected with the toxic principles of this plant as only those fractions which contain coagulable protein are poisonous. The nitrogen is distributed as ammonia 1.74, basic 4.29, non-basic 10.42%. It does not contain phosphorus (Osborne, Mendel, and Harris, *Amer. J. Physiol.*, 1905, **14**, 259).

It is uncertain whether *abrin* (*Abrus precatorius*), *crotin* (*Crotin eluteria*), *robin* (*Robinia pseudacacia*) which are toxic in a very similar manner are also albumins. These substances are grouped by Osborne as *toxalbumins*. (For an account of them see his monograph, page 92.)

Ricin is obtained by fractional precipitation of the aqueous solution with ammonium sulphate, the toxic substance being sharply concentrated in fractions obtained within narrow limits.

For the detection in cattle foods, W. Mooser (*Chem. Zentr.*, 1911, 638) makes use of an antiricin prepared by the intravenous injection of ricin into goats, which gives a turbidity or precipitate with ricin alone. The cattle food, 10 grm., is warmed at 37° for 24 hours with 100 c.c. of glycerol and the filtered extract added to about 10 times its volume of a mixture of equal volumes of ether and alcohol. The precipitate is collected, washed with alcohol and ether, dried at 37° and triturated with 20 c.c. of a 10% solution of sodium chloride. After standing the whole is made up to 200 c.c. by more sodium chloride solution and filtered. On a layer of 1 c.c. of the filtrate is superposed 0.1 c.c. of the antiricin serum and the whole kept in the thermostat for several hours. A turbidity or precipitate indicates ricin.

Leguminous Seeds.

The proteins of leguminous seeds are in the main globulins though the seeds also contain other types of protein in small quantities; as a class they are closely alike and quite distinct from cereal or oil-seed proteins. In many respects they resemble animal proteins more closely than do those of other seeds. They lack any particularly characteristic chemical or physical properties and have been very little investigated.

PROTEINS OF LEGUMINOUS SEEDS.

Protein	Source	Chief constituents
Phaseolin	*Phaseolus vulgaris*	Glutamic acid, 14.5% Leucine, 9.65% Arginine, 4.9% Lysine, 5%
Legumin	*Pisum sativum* *Vicia sativa* *Ervum lens* *Faba vulgaris*	Glutamic acid, 18% Leucine, 9% Arginine, 11.7% Lysine, 5%
Vicilin	*Pisum sativum* *Vicia sativa* *Ervum lens* *Faba vulgaris*	Glutamic acid, 21.3% Leucine, 9.4% Arginine, 8.9% Lysine, 5.4%
Legumelin	*Pisum sativum* *Vicia sativa* *Ervum lens* *Faba vulgaris*	Glutamic acid, 13% Leucine, 9.6% Arginine, 5.5% Lysine, 3.0%
Glycinin	*Soya hispida*	Glutamic acid, 19.5% Leucine, 8.5% Arginine, 5.1% Lysine, 2.7%
Vignin	*Vigna sinensis*	Glutamic acid, 16.9% Leucine, 7.8% Arginine, 7.2% Lysine, 4.3%
Conglutin	Lupins	Glutamic acid, 21% Arginine, 11% Lysine, 2.7% Histidine, 2.5%

Legumin, or a protein very similar to it, occurs in the seeds of the pea (*Pisum sativum*), vetch (*Vicia sativa*), lentil (*Ervum lens*), and the horse bean (*Vicia faba*). It has $[\alpha]_D^{20} = -44°$ in 10% sodium chloride solution. The products of hydrolysis of pea legumin have been determined by Osborne and Clapp (*J. Biol. Chem.*, 1907, **3**, 219) and of vetch legumin by Osborne and Heyl (*Amer. J. Physiol.*, 1908, **22**, 423).

Vicilin is found in the pea, horse bean and lentil, but not in the vetch. It differs from legumin in being more soluble in very dilute salt solutions and in coagulating when heated to 100° (Osborne, *J. Biol. Chem.*, 1907, **3**, 213; 1908, **5**, 187).

Legumelin.—An albumin apparently occurs in most of the leguminous plants. It has a mean composition of C 53.31, H 6.97, N 16.26, S 1.08%. The pea contains 20%, the vetch 1.5%, the lentil and horse bean 1.25%. It is coagulated on heating in aqueous solution at 60–65°. It remains in aqueous solution when the other globulins are precipitated by dialysis and is not precipitated on saturation with sodium chloride or magnesium sulphate. It has been further investigated by Osborne (*J. Amer. Chem. Soc.*, 1903, **25**, 323; *J. Biol. Chem.*, 1907, **3**, 213; 1908, **5**, 197).

Soy Bean (*Soya hispida*).—The soy bean is characterised by containing a large proportion of protein (33%) and fat (18%) and for this reason it is extensively used, particularly in France, for making diabetic bread and biscuits. Soy flour, however, contains a considerable proportion of starch (28.7%) and the preparations examined by the writer have been without exception unsuitable for the diabetic dietary.

Almost the entire nitrogen of the soy bean represents protein. This is to a large extent soluble in water. It consists of a specific globulin termed **glycinin,** a second more soluble globulin and a legumelin differing from that in the pea. This last gives 4.9% of lysine on hydrolysis. (See Osborne, *J. Amer. Chem. Soc.*, 1898, **20,** 419; *Amer. J. Physiol.*, 1907, **19,** 468; 1908, **23,** 180.)

Soy is largely eaten in China and Japan as cheese, sauces and pastes and supplements the deficiences of rice in protein. It has been exported recently in very large quantities to Europe. To obtain the protein the finely ground beans are extracted several times with warm water and the filtered milky extract coagulated by the addition of lactic acid. The precipitate is dried and freed from oil by extraction with a suitable solvent, the protein being left as a white powder.

The proteins of *Phaseolus vulgaris* show slight difference in composition and products of hydrolysis. **Phaseolin** (C 52.66, H 6.93, N 15.83, S 0.36%), a crystalline globulin ($[\alpha]_D^{20} = -41.46°$ in 10% sodium chloride), is the chief constituent. The products of hydrolysis have been determined by Osborne and Clapp (*Amer. J. Physiol.*, 1907, **18,** 295).

The second protein, **phaselin** (C 51.6, H 7.02, N 14.65, S 0.49) is much more soluble. It coagulates at 87° and has an abnormally low proportion of nitrogen. The total protein in the bean meal is 23.65%. Sodium chloride extracts 17% of which 15% is phaseolin. Lima beans (*Phaseolus lunatus*) probably also contain phaseolin, but the globulin of *Phaseolus radiatus* appear to be slightly different.

Lupins.

The greater part of the protein is a globulin, insoluble in water, soluble in sodium chloride, which is termed **conglutin.** A second **globulin,** present in small quantity only, coagulates about 60°. In addition there is somewhat more of an albumin present, coagulating at 85°. Osborne and Campbell have shown that yellow lupins contain two different conglutins, α and β, both of which differ slightly again from that in blue lupins (*J. Amer. Chem. Soc.*, 1897, **19,** 454).

PROTEINS OF MILK.

By L. L. VAN SLYKE.

The scope of this article limits the treatment to a selection of some of the important facts from the very extensive literature.

Nomenclature.—In examining the literature, one is impressed by the extreme confusion prevailing in nomenclature in relation to casein. It is essential that we state at the start what we mean by the terms used in this article, although, in the present incomplete state of knowledge, any nomenclature must be purely provisional. *Casein* or *free casein* is the base-free or uncombined protein; *calcium casein* or caseinate is the neutral compound that is believed to be present in fresh, normal milk, consisting of casein in combination with about 1.50% CaO; *basic calcium casein* or caseinate is the compound consisting of casein in combination with about 2.50% of CaO. *Calcium paracasein* or paracaseinate is the insoluble compound formed by the action of rennet on calcium casein; *paracasein* or *free paracasein* is the base-free or uncombined protein.

The number of proteins in milk reported by different investigators varies from one to eight. Without discussing details, the weight of chemical evidence now at hand justifies the belief that fresh, normal milk contains three distinct protein compounds, viz., *calcium casein*, *albumin* (or lactalbumin) and *globulin* (or lactoglobulin). While there may be others, their existence cannot yet be regarded as satisfactorily established. The multiplicity of milk proteins described in literature is largely due to the action of reagents or ferments on milk, forming a variety of compounds, usually in the nature of decomposition products, which do not exist in fresh, normal milk.

Of the three protein compounds in milk, calcium casein is by far the most important, since it constitutes about 80% of the milk proteins and has commercial uses of considerable interest. The literature about casein and its compounds is very extensive, while that of milk albumin and globulin is meagre. This article will, therefore, be devoted largely to a discussion of the properties and relations of casein and its compounds. For convenience, our treatment will follow this

outline: (1) Amount of proteins in milk, (2) commercial relations of casein and its compounds, (3) method of preparing casein, (4) physical properties, (5) composition, (6) action of acids, (7) action of bases, (8) action of salts, (9) action of heat, (10) action of formalin, (11) action of rennet-enzyme, (12) albumin, (13) globulin and (14) methods of estimation.

Amount of Proteins in Milk.—The writer has studied the results furnished by several thousand analyses of cow's milk made at the New York Agricultural Experiment Station, representing milk of individuals and herds of different breeds, in which total proteins and casein were carefully estimated (*J. Amer. Chem. Soc.*, 1908, **30**, 1166). In single milkings of individual cows, there have been found total proteins as low as 2.19%, and as high as 8.56%, the casein varying from 1.59 to 4.49%, and other proteins, chiefly albumin, from 0.30 to 5.30%. The highest percentages are usually found in the case of cows far along in lactation and giving only small amounts of milk; such results would be regarded as abnormal.

In the case of single herds of cows, the total proteins range from 2.31 to 3.71%; casein, from 1.79 to 3.02%; and other proteins, from 0.41 to 0.97%. In the case of milk consisting of a mixture of the product of numerous different herds of cows, the proteins vary from 2.53 to 3.76%; casein, from 1.93 to 3%; other proteins, from 0.47 to 0.88%. These results permit us to state that in the fresh, normal milk of cows the percentages of proteins will commonly be found within the following limits:

Total proteins	2.50 to 3.75%
Casein	2.00 to 3.00%
Albumin and globulin	0.45 to 0.90%

The percentages of proteins in milk, as well as the relative amounts of proteins and fat, are affected by a variety of conditions, such, for example, as the individuality of cows, breed, stage of lactation, time and manner of milking, food, season, etc. For illustration, in the milk of seven different breeds of cows studied by the writer (Holstein-Friesian, Ayrshire, American Holderness, Shorthorn, Devon, Guernsey and Jersey) the proteins vary, on an average, from 2.20% in the case of Holstein-Friesian milk to 3.93% in the case of Devon milk. As between the early and later stages of lactation, there is commonly an increase of 0.60 to 0.70% of total proteins, the percentage of proteins increasing relatively more than fat. Another illustration is furnished by seasonal conditions; the writer has found that, in times of drouth, the proteins of milk decrease in the case of cows wholly dependent upon pasturage, while the fat may increase.

Regarding the relative amounts of casein and non-casein proteins (albumin and globulin) in milk, the general statement has been prominently current in the literature to the effect that the proteins in cow's milk are present in very constant relative proportions, the amount of casein being five times that of other proteins. Taking the milk of herds of cows, we have found that the casein varies all the way from 2.6 to 5.6 parts for 1 part of other milk proteins. In single milkings of individual cows the variations are still wider. This relation varies with individuals, breeds, stage of lactation and even with the time and manner of milking (*J. Amer. Chem. Soc.*, 1908, **30**, 1180). To illustrate, casein forms an average of 77.5% of the total proteins in the milk of Holstein-Friesian cows and 82.3% in the case of milk of Jersey cows; or, stated in another way, there are, for 1 part of albumin and globulin, 3.4 parts of casein in one case and 4.7 parts in the other. When cows are about 10 months or more along in lactation, the casein tends to decrease relative to other milk proteins.

Commercial Relations of Casein.—Casein is the most important protein in milk, not only because it is the one present in largest amount, but especially because (1) it has a high food value, (2) its presence makes it possible to convert milk into cheese, and (3) it finds an increasing multiplicity of applications in the arts, covered by hundreds of patents.

Special preparations of casein, usually made from skim-milk, are used as concentrated foods, particularly by diabetic patients; they are sold under various trade-names, among which are Sanatogen, Eulactol, Plasmon, Nutrose, Lacto-somatose, Sanose, etc. These preparations are generally put up in the form of a fine, white powder and are readily soluble in water. Some are mixtures of protein with some form of carbohydrate, as cereal flours, sugar, etc.; others contain some medicinal agent, as for example, Sanatogen, which contains 5% of sodium glycerophosphate. Some of these preparations are made into bread after being mixed with flour, thus increasing the protein content. In most cases free casein is not present, but some of its soluble compounds are used, prepared by treatment with some solvent, among which can be mentioned sodium bicarbonate, sodium citrate, sodium or potassium phosphate and some ammonium compounds. The percentage of protein varies greatly in the different preparations, ranging from 20 to 95%.

Casein compounds are found very useful as a means of administering medicinal agents, such as salicylates, alkaloids, lithium, mercury, silver, iron, arsenic, etc. The combination of such substances with

casein favorably modifies their properties for medicinal purposes. For example, the silver compound, commercially known as *argenin*, is a white powder, readily soluble in warm water; it is neutral and is as strongly bactericidal as silver nitrate, but is free from caustic action and is, therefore, well adapted for the treatment of sensitive tissues.

The behavior of calcium casein to rennet-enzyme lies at the basis of the manufacture of most kinds of cheese from milk. The coagulum produced by the action of rennet-enzyme, when made into cheese, retains most of the milk-fat and a considerable amount of insoluble salts of the milk, especially phosphates, with smaller amounts of milk-sugar and albumin. Cheese, like cottage or Dutch cheese, made by coagulation of milk with acid, is, in almost every detail, unlike that made with rennet.

The applications of casein preparations in the arts may be briefly summarized as follows: (1) Painting materials; (2) adhesives, putties, etc.; (3) plastic materials as a substitute for horn, ivory, celluloid, bone, etc., in the manufacture of insulating materials, tubes, rods, handles of all kinds, buttons, picture frames, etc., etc.; (4) as a medium for fixing colors in textiles; (5) for water-proofing colored papers, art papers, transfer papers, washable wall-paper, drawing-paper and writing-paper, cardboard, boxes, cartridge-cases, paper flasks for holding oils, wood-pulp vessels, etc.; (6) mixed with asbestos paper and board to form water-proof and fire-proof materials; and (7) miscellaneous uses, such as paint-removers, shoe-polishes, photographic plates, roofing-pulp, glazing for inside of casks, preparation of artists' canvasses, solidifying mineral oils, soap-making, etc., etc.

It can thus be seen that the uses of casein are practically without limit, but the scope of this article does not permit us to go into further details. The source of casein used in these numerous ways is largely the skim-milk of creameries.

Method of Preparing Casein.—Crude commercial casein is prepared from skim-milk by treatment with sulphuric or hydrochloric acid, which precipitates the casein; this precipitate is washed and dried more or less completely. This material in granular form and containing about 10% of water is the kind generally used for technical purposes. On an average, it is found that 100 pounds of skim-milk yield about 8 1/2 pounds of damp casein or 3 1/2 pounds of dry.

Free casein in purer form is usually prepared by diluting skim-milk with water, precipitating with dilute acid, dissolving the washed precipitate in dilute alkali, repeating the operations of precipitation and solution several times, finally treating with alcohol and ether and

drying. The method described below enables one to prepare casein in a very satisfactory way for use in laboratory work (*Am. Chem. J.*, 1907, **38**, 388); it includes some details to be followed only when one wishes to use casein for work requiring conductivity measurements.

To 1,000 c.c. of fresh, separator skim-milk, are added about 6 litres of distilled water and enough dilute acetic acid (10 to 15 c.c. of strong acid diluted with water to 1,000 c.c.) to cause complete precipitation of casein, avoiding any marked excess of acid. The mixture is kept vigorously agitated while the acid is being gradually added. The precipitate is allowed to settle, the supernatant liquid is decanted or siphoned off, after which the precipitate is washed with copious amounts of distilled water, until the wash-water no longer shows an acid reaction to litmus. The precipitate is then treated with just enough dilute ammonia water (about 5 c.c. of strong ammonia diluted to 1,000 c.c.) to dissolve the casein, forming a solution neutral to litmus. The solution is diluted to about 6 litres with distilled water and reprecipitated by dilute acetic acid. Much less acid is required for the second and subsequent precipitations than for the first. The precipitate is washed free from acid as before and redissolved in dilute ammonia. Reprecipitation and redissolving should be performed five or six times. The casein should not be allowed to stand longer than necessary in contact with either acid or alkali. The final filtration and washings are completed on a Buchner funnel, the precipitate being washed until free from acid. The washed precipitate is then suspended in 1,000 c.c. of $N/1000$ HCl and agitated for 2 hours, in order to remove any remaining inorganic salts as completely as possible. Two treatments of this kind are given. The casein is finally washed until free from hydrochloric acid and, when it is to be used for work connected with determinations of electrical conductivity, it is then agitated from 2 or 3 hours with 1,000 c.c. of very pure water (showing a conductivity not greater than 1.5 to 1.8×10^{-6}), the operation being repeated two or three times until the filtrate shows an increase of not more than 1 or 2×10^{-6} in conductivity, as compared with the conductivity of the wash-water used. The precipitate is then treated with about 1,000 c.c. each of strong alcohol and ether (these reagents, in case of conductivity work, should show no conductivity when mixed with pure water), in order to remove any fat that may adhere to the casein. The precipitate is then dried at room temperature, ground fine in a mortar and finally dried at 45 to 50°, until the moisture content is reduced to 3 or 4%. Casein thus prepared may have an ash content of 0.25 to 0.30%. This method of drying is recommended because,

according to Laqueur and Sackur (*Beitr. Chem. Physiol. u. Path.*, 1903, **3**, 206) moisture can be completely removed from casein only by heating to a temperature that may alter the nature of the protein. Correction is made for moisture, so that the amounts used in work represent water-free casein.

Some Physical Properties of Casein.—Casein is most familiar as the solid, white substance called curd, which forms in milk when it sours. Calcium casein is known as a prominent constituent of fresh separator-slime (the material collecting in the bowl of a centrifugal machine used for separating cream from milk); the protein is here in the form of combination existing in the milk, being separated unchanged chemically.

The first point of interest attaches to the physical condition in which calcium casein is present in milk. For a long time all milk proteins were believed to be in complete solution in milk; later it was held by some that the chief protein of milk is in a state of semi-solution. The view which must now be regarded as representing the truth is that calcium casein exists in milk in the form of a colloid, consisting of extremely minute, gelatinous particles in suspension. The evidence sustaining the correctness of this view is three-fold:

(1) While the solid particles of calcium casein are so small that they easily pass through the pores of fine filter paper, they do not go through the finer pores of unglazed porcelain, like the Chamberland filter (*Pflüger's Archiv.*, 1869, **2**, 598, and *Ann.*, **189**, 358), nor through animal membranes. It is thus possible to strain out calcium casein from the soluble portions of milk in amounts sufficient to handle and study.

(2) Calcium casein is separated from milk by means of centrifugal force, being deposited as a gelatinous film on the surrounding walls of the bowl of the centrifuge. By whirling milk for several hours at a high rate of speed, practically all of the calcium casein can be separated from the milk (*Twelfth Ann. Rept. Wisconsin. Agr. Exp. Sta.*, 1895, page 93). It is in this manner that it is deposited on the walls of the bowl of a centrifugal cream separator as separator-slime in which the calcium casein, in a gelatinous mass, is mixed with dirt and other solid substances.

(3) The most recent method of furnishing evidence on this point is by means of actual observation of the solid particles of calcium casein in milk under ultramicroscopic examination, as reported by Kreidl and Neumann (*Pflüger's Archiv.*, 1908, **123**, 523). These investigators were able to see the actual particles of calcium casein swimming

in milk, to treat them with reagents and to observe their various transformations. Not only was the milk of cows studied in this way but also that of other animals, including human milk. In the case of some animals the ultramicroscopic particles of calcium casein are present in colostrum, while in others they do not appear until later. In the case of fresh human milk, no particles could be seen except after addition of a small amount of acid. The absence of ultramicroscopic particles of calcium casein in human milk is attributed to its greater alkalinity.

The appearance of calcium casein, prepared either by means of centrifugal separation or by filtration with porous earthenware is, when wet, a white or cream-white gelatinous substance. When dried, it has much the appearance of gelatin or dried egg-albumin.

Free casein, as usually prepared, is, when dry, a fine white powder with a sp. gr. about 1.259.

Composition of Casein.—Casein is a very complex chemical compound, belonging to the general class of proteins and to a special subdivision called *phosphoproteins*. We can conveniently consider the composition of casein under three heads, as follows: (1) ultimate composition, (2) the relation of the protein molecule to calcium, and (3) the structure of the protein molecule.

The following statement gives approximately the average percentages of constituents found in the uncombined protein:

Carbon	53.00%
Oxygen	22.70%
Nitrogen	15.70%
Hydrogen	7.00%
Phosphorus	0.85%
Sulphur	0.75%

The results of the work of several investigators lead to the conclusion that the protein, casein, as it exists in milk, is in some form of combination with calcium and not present as uncombined or base-free protein. The various views that have been held in relation to this point may be summarized under the three following statements: (1) Casein is in milk as the compound calcium casein, containing in combination about 1.50% of calcium oxide (Söldner, *Landw. Versuchs-Stat.*, 1888, **35**, 351). (2) The protein is combined with tricalcium phosphate, the casein acting as a solvent for the calcium phosphate (Hammarsten, Maly, *Jahresber. d. Thierchem.*, 1874, **4**, 146). (3) The compound consists of calcium casein in combination with tri-calcium phosphate (Eugling. *Landw. Versuchs-Stat.*, 1885, **81**, 392; and Schaffer, *Landw. Jahrb. d. Schweiz*, 1887, 1, 33). Taking into consideration all the evidence at hand, the first view appears to accord most completely

with all the facts known, but the question cannot yet be regarded settled beyond doubt. We consider later the relation of bases to casein in more detail.

Our present knowledge of the structure of any protein is stated by giving the percentages of the different amino-acids formed by hydrolysis of the protein. The products of the hydrolysis of casein have been extensively studied, and the following summary may be regarded as the most reliable up to the present time (Osborne and Guest, *J. Biol. Chem.*, 1911, **9**, 333): (Compare page 20.)

	%
Glycocoll or glycine (amino-acetic acid, $CH_2.NH_2.COOH$)	0.00
Alanine (α-amino-propionic acid, $CH_3CH(NH_2).COOH$)	1.50
Valine (α-amino-isovaleric acid, $(CH_3)_2CH.CH(NH_2).COOH$)	7.20
Leucine (α-amino-caproic acid, $(CH_3)_2CH.CH_2.CH(NH_2).COOH$)	9.35
Proline (pyrrolidine-carboxylic acid, )	6.70
Phenylalanine (phenyl-α-amino propionic acid, $C_6H_5CH_2.CH(NH_2).COOH$)	3.20
Glutaminic acid (amino-glutaric acid, $COOH.CH_2.CH_2.CH(NH_2).COOH$)	15.55
Aspartic acid (amino-succinic acid, $COOH.CH_2.CH(NH_2).COOH$)	1.39
Cystine (amino-thiolactic acid, )	
Serine (α-amino-β-hydroxy-propionic acid, $CH_2OH.CH(NH_2).COOH$)	0.50
Tyrosine (oxyphenyl-α-amino-propionic acid, $C_6H_4(OH).CH_2.CH(NH_2).COOH$)	4.50
Oxyproline ($OH.C_4H_7N.COOH$)	0.23
Histidine (α-amino-β-imidazol-propionic acid, )	2.50
Arginine (δ-guanidino-α-amino-valeric acid, $(H_2N)C.NH.CH_2.CH_2.CH_2.CH(NH_2).COOH$)	3.81
Lysine (α, ϵ, diamino-n-caproic acid, $CH_2(NH_2).CH_2.CH_2.CH_2.CH(NH_2).COOH$)	5.95
Tryptophane (indol-amino-propionic acid, )	1.50
Diamino-trioxy-dodecanic acid ($C_{11}H_{18}(OH)_3(NH_2)_2.COOH$, structure unknown)	0.75
Ammonia	1.61
Sulphur	0.76
Phosphorus	0.85

In what form of combination sulphur is present in casein we have little information as yet; and the same is true of phosphorus, though it is probable that it is a constituent of some complex organic compound rather than in union as a salt-like combination of a phosphorus-containing acid with a protein base.

In connection with the composition of casein, the question has been raised as to whether the casein in the milk of different animals is the same compound. Without going into details, we can say that the evidence is conflicting and the question cannot be regarded as settled. (The following references bear on this subject: Tangl, *Pflüger's*

Archiv., 1908, **121**, 534; Ellenberger, *Archiv. f. Physiol.*, 1902, *Supplement*, 313; Burow, *Beiträge zur Entscheidung der Frage, ob die Caseine verschiedener Tierarten identisch sind. Diss.* Basel, 1905; Wroblewski, *Beiträge zur Kenntniss des Frauencaseins, Diss.* Bern., 1894; *Bergellund Langstein, Jahrb. f. Kinderheilkunde*, 1908, **68**, 568; Abderhalden und Schittenhelm, *Zeitschr. f. Physiol. Chem.*, 1906, **47**, 458.)

Action of Acids on Casein.—Casein is basic in character and, under certain conditions, unites with acids. In this connection we have to consider (1) the action of acids on calcium casein, (2) the action of dilute acids upon the free protein, and (3) the action of acids of greater concentration upon free casein.

When milk is treated with acid or acid salts, the casein is precipitated as a heavy, white solid in more or less flocculent form, depending on conditions of treatment. When milk sours in the ordinary way, the lactic acid that is formed produces the same effect as the addition of any dilute acid; the precipitation or curdling occurs at ordinary temperatures, when the acidity reaches 0.6 to 0.7%, expressed as lactic acid. When dilute artificial lactic acid is added directly to fresh milk, precipitation takes place at ordinary room temperature when the acidity reaches 0.57%. Increase of temperature enables a smaller amount of acid to precipitate casein; for example, milk curdling at room temperature with 0.55% of added lactic acid, precipitates with 0.35% at boiling temperature, according to work done in the laboratory of the writer. The precipitating effect of acids is made the basis of methods of separating and estimating casein.

When the casein of milk is precipitated by acids, the first action of the acid is combination with the calcium of calcium casein, forming calcium lactate and free casein. The accompanying physical change that takes place is the formation of the white curd produced by aggregation of the very minute particles of protein. The exact cause of this change has not been satisfactorily explained, but the effect is probably to be ascribed to the formation of soluble calcium salts.

When more acid is present than is sufficient to combine with the calcium of the calcium casein, the insoluble phosphates of the milk are changed to mono-calcium phosphate, and further amounts of acid are more or less extensively adsorbed by the base-free protein. It was formerly held that insoluble, precipitated casein under these circumstances combines directly with a definite amount of acid, forming an insoluble compound of definite composition and that, under this supposition, the curd of ordinary sour milk is the compound, casein lactate

(Laxa, *Milchwirtschaftl. Zentralbl.*, 1905, **1**, 538; Van Slyke and Hart, *Am. Chem. J.*, 1905, **33**, 461). But more recent and accurate work (L.L. Van Slyke and D.D. Van Slyke, *Am. Chem. J.*, 1907, **38**, 383) furnishes evidence that the action is one, not of chemical combination, but of adsorption, by which is meant the concentration of a dissolved substance upon the surface or within the meshes of a solid substance in contact with the solution, whereby a portion of the dissolved substance is removed from solution, without forming a definite chemical compound with it. The amount of acid thus adsorbed varies (a) with the concentration of the acid, (b) with the duration of contact and degree of agitation until equilibrium is reached, (c) with the temperature and (d) with the kind of acid. For example, the amount of hydrochloric acid adsorbed by casein varies from 8 to 50% of the acid originally in solution, according to the concentration of the acid; in the case of sulphuric acid, about 75% of the acid in solution is quickly adsorbed at ordinary room temperature. Lactic and acetic acids are taken up less completely. The acid thus removed from solution can be washed out of the casein by agitating with water.

Free casein dissolves easily in moderately dilute acids, more easily at higher temperatures, forming soluble compounds, which are either combinations of acid with the protein or decomposition products, depending on the concentration of the acid, the temperature, length of contact, etc. For example, solutions of acids of a concentration of $N/125$ dissolve casein quite readily at 25°. Little or no solution of casein occurs with solutions of acids not stronger than $N/1{,}000$ at 25°. The solvent action of the following acids is in this order, from strongest to weakest: Hydrochloric, lactic, sulphuric, acetic. The dissolved protein, at least in case of hydrochloric acid, contains more acid than the adsorption product. According to Long (*J. Amer. Chem. Soc.*, 1907, **29**, 1334), 1 grm. of free casein combines with about 7 c.c. of $N/10$ acid in case of hydrochloric, hydrobromic, hydriodic, sulphuric and acetic to form soluble, salt-like compounds. At higher temperatures, much more acid appears to combine, probably due to hydrolysis.

The casein-acid compounds formed by dissolving casein in an acid are unstable; they decompose on dialysis, with separation of free casein; they are denaturised on drying. They are precipitated from solution by addition of just enough alkali to neutralise the combined acid, also by treatment with such salts as sodium or calcium chloride, sodium sulphate, etc.

When casein is treated, for example, with $N/125$ HCl at 25° for 3 hours, the undissolved residue undergoes marked changes, presenting

a swollen, gelatinous appearance; it melts at 100° and, when dried, looks like dried albumin. The exact chemical nature of the substance has not been studied yet.

Action of Bases on Casein.—Casein is also acid in character, in that it unites with fixed alkalis, ammonia and alkaline carbonates, forming salts easily soluble in water. Thus, the curd of sour milk, as well as of fresh cheese, can be dissolved by treatment with dilute sodium carbonate or ammonia. The acid character of casein is shown also by the fact that free casein rubbed in a mortar with moist calcium carbonate displaces carbon dioxide, forming a calcium-casein compound. Bases of the other alkaline earths also can form compounds with casein. These compounds are often called *caseinates*. Their solutions in water seem to dissociate with formation of free casein, this change being more marked in the case of weaker bases, as shown by the appearance of the solutions. While the compounds formed with the alkali bases make solutions nearly transparent or slightly opalescent, which become turbid only after heating above 50°, the compounds with the alkali earth bases produce milk-white, opaque solutions, especially in presence of soluble salts of such bases. Solutions becoming turbid on heating do not become perfectly clear on cooling.

Compounds of casein and bases are precipitated from their solutions very easily by addition of dilute acids, the precipitate redissolving in excess of acid. They are thrown out of solution also by common salt, sulphates of magnesium, sodium, ammonium, zinc, copper, aluminum and other salts. The alkali salts of casein, but not those of the alkaline earths, diffuse through porous earthenware.

Of the compounds formed by casein with bases, those of calcium are of special interest. The existence of two calcium-casein compounds is fairly well established, which contain, respectively, about 1.50% and 2.50% calcium oxide (Söldner, *Landw.-Versuchs-Stat.*, 1888, **35**, 351; Van Slyke and Hart, *Am. Chem. J.*, 1905, **33**, 461). When a solution of free casein in lime-water is made neutral to phenolphthaleïn, the solution becomes somewhat milky; basic calcium casein is formed, containing about 2.5% CaO. When the addition of acid is carried farther, a point is reached where the solution becomes neutral to litmus and much more milky in appearance than the solution neutral to phenolphthaleïn; neutral calcium casein is formed, containing about 1.5% CaO.

A solution of basic calcium casein is not coagulated by rennet-enzyme either in the presence or absence of soluble calcium salts; it is, however, precipitated by soluble calcium salts on warming to 35 to 45°. Neutral

calcium casein is precipitated by rennet-enzyme in the presence of soluble calcium salts and also by a soluble calcium salt alone, on warming. Neutral calcium casein appears to possess the properties that are characteristic of the casein compound that exists in milk.

Courant reported that casein forms a third compound with calcium, containing 0.87% calcium oxide (*Pflüger's Archiv.*, 1891, **50,** 109), but many efforts to prepare such a compound in the writer's laboratory have so far failed, while Robertson (*J. Biol. Chem.*, 1907, **2,** 317) furnishes evidence against the existence of such a compound. However, recent work (not yet published), in the writer's laboratory, indicates a compound containing 0.31% CaO; while Robertson (*J. Physical Chem.*, **13,** 469) has prepared a corresponding sodium compound.

According to the work of Laqueur and Sackur (*Hofmeister's Beiträge*, 1902, **3,** 193) casein obtained from cow's milk is 4 to 6 basic, and its equivalent weight about 1,135. According to Matthaipoulos (*Zeitsch. anal. Chem.*, 1908, **47,** 495), 1 grm. of free casein neutralises 8.8378 c.c. of $N/10$ sodium hydroxide, or 1 c.c. of $N/10$ sodium hydroxide combines with 0.11315 grm. of casein, making the equivalent weight 1131.5. According to Long (*J. Amer. Chem. Soc.*, 1906, **28,** 372) the equivalent weight of casein in cow's milk is 1,124; in goat's milk, 1,190. If the equivalent weight of casein is 1131.5, the molecular weight would be 4,526 if casein is tetrabasic, or 6,789 if casein is hexabasic.

Action of Salts.—Casein is insoluble in sodium chloride, sulphate, nitrate, potassium chloride, lithium nitrate, but soluble in ammonium nitrate or acetate, sodium propionate, butyrate, valerianate, oxalate, rhodonate and potassium cyanide (Robertson, *J. Biol. Chem.*, 1907, **2,** 317). It is soluble also in potassium oxalate and sodium fluoride (Arthus, *Compt. rend. soc. biol.*, 1893, **45,** 327). Casein is also easily soluble in di- and tri-phosphates (Hammarsten, *Maly's Jahresb*, 1875, **5,** 119).

Calcium casein is precipitated, apparently unchanged chemically, by saturating milk with common salt, ammonium sulphate, magnesium sulphate, etc., at ordinary temperatures. It is also precipitated by small amounts of alum, zinc sulphate and many other metallic salts. Calcium chloride and some other salts precipitate calcium casein in milk heated to 35 to 45°.

Action of Heat.—Heat alone under ordinary conditions, even at the boiling-point of water, does not coagulate the calcium casein in milk. However, heated under pressure to 130–140°, casein salts are changed in their properties and casein itself is coagulated. The

browning of milk heated under pressure is more or less due to changes in casein. The formation of a peculiar skin (haptogen membrane) on milk heated above 60° is largely due to the calcium casein of the milk and not, as was formerly supposed, to albumin. The skin itself contains practically all of the constituents of the milk and may be regarded as a kind of evaporated milk. On removing the membrane, a new layer is formed and, by removing these one after another, practically all of the milk can be transformed into the membrane condition. It appears to be due to surface evaporation. When milk is diluted with water and the surface covered with oil, no formation of skin takes place at boiling temperature (Sembritzky, *Pflüger's Archiv.*, 1885, **37**, 460; Jensen and Plattner, *Landw. Jahrb. Schweiz.*, 1905).

Action of Formalin.—Formalin has been a favorite milk preservative. The casein is profoundly changed in some of its characteristics, so that the precipitate formed by acids is not redissolved by excess of acid. This is noticeable in the application of the Babcock method in estimating fat in milk; the strong sulphuric acid used (1.825 sp. gr.) precipitates the casein but does not redissolve it promptly as in case of normal milk. The action of formalin has the effect of decreasing the basicity and increasing the acidity of casein. For example, when 100 c.c. of pure milk requires for neutralisation 15.65 c.c. $N/10$ sodium hydroxide, the addition of 1 c.c. of formalin increases the amount of alkali required for neutrality to 27.8 c.c. (Seigfeld, *Molkereizeitung*, Hildesheim, 1906, No. **47**; Steinegger, *Landw. Jahrb. Schweiz*, 1903 and 1905).

Action of Rennet-enzyme.—One of the most characteristic properties of calcium casein is its coagulation by the enzyme or chemical ferment contained in rennet (the name applied to the fourth stomach of a suckling calf). The rennet extracts of commerce, largely used in cheese-making, are prepared by extracting the rennet with salt brine. Such extracts contain rennet-enzyme in a dilute and impure form. Rennet-enzyme is also prepared in more concentrated state in the form of powders and tablets. Enzymes having similar action are found also in plants and in many animals. Some bacteria produce a coagulating enzyme like that in rennet.

The coagulating action of rennet extract makes possible the manufacture of many kinds of cheese from milk. The results of the action of rennet in precipitating casein are wholly unlike the effects produced by acids, considered from a chemical point of view. The chief protein of the curd formed by the action of rennet-enzyme is usually called

paracasein or, more properly, *calcium paracasein* (*Amer. Chem. J.*, 1905, **33**, 461). Calcium paracasein behaves, in general, much like calcium casein toward acids and alkalis.

A large amount of study has been devoted to the action of rennet-enzyme. There has been a difference of belief as to whether rennet extract contains one enzyme acting in two different ways on casein, or two different enzymes, each with its own characteristic action. So far as the essential facts are concerned, rennet extracts possess the power of effecting two distinct kinds of change: (1) Coagulation of calcium casein in milk, and (2) solution or digestion of the coagulated protein. Those who regard these two actions as due to two different enzymes contained in rennet call the coagulating enzmye *rennin* or *chymosin*, and the proteolytic enzyme, *pepsin*. The weight of evidence at hand at present rather favours the existence of two enzymes (Hammarsten, *Zeits. physiol. Chem.*, 1908, **56**, 53; Bang, *Pflüger's Archiv.*, 1900, **79**, 425; Pawlow and Parastschuk, *Zeits. physiol. Chem.*, 1905, **42**, 415; Sawjalow, *Zeits. physiol. Chem.*, 1906, **46**, 307; Schmidt-Nielsen, *Hofmeister's Beiträge*, 1907, **9**, 311; Sawitsch, *Zeits. physiol. Chem.*, 1907, **55**, 84; Gewin, *Zeits., physiol. Chem.*, 1907, **54**, 32; Meunier, *Bull. Soc. Pharm.*, **7**, 125; Taylor, *J. biol. Chem.*, 1909, **5**, 399; Grützner, *Pflüger's Archiv.*, 1878, **16**, 118; Lörcher, *Pflüger's Archiv.*, 1898, **69**, 71; Glässner, *Hofmeister's Beiträge*, 1901, **1**, 1).

Much study has been devoted to the coagulating effect of rennet-enzyme in order to ascertain just what change, if any, the calcium casein undergoes. Many different explanations have been offered, but in the present incomplete state of our knowledge it is impossible to give an explanation of the process that can be regarded as satisfactory and conclusive. The most we can do here to advantage is to present the details of the process so far as they appear to be worked out. The action of rennet in the coagulation of calcium casein is believed to take place in three quite distinct stages as follows: (1) Change of calcium casein into calcium paracasein; (2) change of the calcium salts of the milk into soluble form; and (3) precipitation of uncoagulated calcium paracasein by the soluble calcium salts.

(1) *First Stage of Rennet Reaction; Change of Calcium Casein into Paracasein.*—The change of calcium casein into calcium paracasein is wholly dependent on the action of rennet-enzyme. There is no change visible to the eye, nor any apparent coagulation. Gutzeit states that there is gradual increase of viscosity (*Milchzeitung*, 1895, **24**, 745). In the absence of soluble calcium salts, the calcium paracasein that has been formed remains in this uncoagulated condition.

The action in this stage of the process takes place as well in the cold as at higher temperatures (Morgenroth, *Zentralbl. f. Bakteriol. u. Parasitenk.*, 1899, **26**, 349; Fuld, *Biochem. Zeits.*, 1907, **4**, 54). What evidence have we that calcium casein is changed into calcium paracasein before coagulation takes place? This is shown experimentally as follows: To a solution containing some salt of casein, free from soluble calcium salts, we add rennet extract. No coagulation takes place. This solution is heated high enough to destroy the power of the rennet to act and then cooled, after which calcium chloride or some other soluble calcium salt is added, when coagulation appears at once. It may be stated here that one of the most characteristic differences between calcium casein and calcium paracasein is that soluble calcium salts do not coagulate calcium casein at ordinary temperatures, but they do cause coagulation of calcium paracasein. In the foregoing experiment, rennet does something to the casein compound which causes the casein to do what it could not do before; that is, coagulate at ordinary temperatures by addition of soluble calcium salts, even when the rennet-enzyme itself had been removed from the field of action.

(2) *Second Stage of Rennet Action; Change in the Calcium Salts of Milk.*—In the second stage of rennet action it is believed that the rennet-enzyme acts upon the insoluble calcium salts of the milk, converting them into a form sufficiently soluble to enable them to coagulate the calcium paracasein. This action appears to take place more slowly than does the conversion of calcium casein into calcium paracasein. This accounts for the period of time that elapses between addition of rennet and coagulation; this time can be shortened by addition of soluble calcium salts (Loevenhart, *Zeits. physiol. Chem.*, 1904, **41**, 177).

(3) *Third Stage of Rennet Action; Precipitation of Uncoagulated Calcium Paracasein.*—During this period, increased viscosity (thickening) and visible coagulation take place. This change, it is generally agreed, is caused by the action, either physical or chemical, of soluble calcium salts upon the uncoagulated calcium paracasein formed during the first stage of the process. After the second stage is completed or nearly so, coagulation commences and proceeds rapidly. The paracasein coagulum (curd) formed in milk always contains insoluble calcium phosphate, which is probably held in a purely mechanical way, although some believe that it is in combination with paracasein.

What is the evidence leading us to believe that a soluble calcium

salt is necessary for the coagulation of calcium casein? Two lines of experimental evidence have been furnished. (1) If we prepare a pure solution of neutral calcium casein or sodium casein, containing no soluble calcium salts, rennet extract will not coagulate such a solution, but, after the addition of some soluble calcium salt, as calcium chloride, coagulation takes place promptly. (2) Milk from which the soluble calcium salts have been removed by precipitation with ammonium oxalate or by dialysis is not coagulated by rennet-enzyme until a soluble calcium salt is added.

We may, therefore, summarise as follows what appears to be fairly well established in explanation of the coagulating action of rennet: (1) That calcium casein is the only substance in milk involved in the rennet coagulation, excepting phosphates of calcium and other soluble salts of calcium. (2) That in rennet coagulation, no change of reaction or acidity occurs; the milk becomes neither acid nor alkaline through rennet action. (3) That the two active agents in the rennet coagulation of milk are rennet-enzyme and soluble calcium salts.

In the foregoing discussion of the process of rennet coagulation, there is nothing to indicate just what happens to calcium casein in being changed into calcium paracasein, or, in other words, just how one really differs from the other. It must be confessed that we do not know at all clearly, although there are many suggestions. We know only this with certainty, that calcium casein does not readily coagulate in the presence of dilute calcium salts at ordinary temperatures, but calcium paracasein does. Otherwise the general properties of casein and paracasein compounds are very similar. Some hold that the difference is purely physical, the paracasein consisting of larger particles than the casein. While the ultramicroscopic study of rennet coagulation enabled the observers to see the minute particles of casein come together and form larger aggregations under the action of rennet, this does not show whether this physical change is accompanied by any chemical change in the protein or its calcium compound.

Rennet-enzyme has the power of forming soluble protein from calcium casein, this peptic action continuing for a long time, especially as shown by a study of its action in cheese (Van Slyke, Harding and Hart, *Bulletin* 233, 1903, or *22d Ann. Rept. New York Agr. Exp. Sta.*, "Rennet-enzyme as a factor in cheese-ripening"). The first conception, that of Hammersten (*Maly's Berichte*, 1872, **2**, 118 and 1874, **4**, 135), was to the effect that rennet-enzyme splits calcium casein into two compounds—one the insoluble curd, called paracasein, always present in large proportions; the other, soluble, albumose-like, called

whey-protein, found in small amounts. This view has been more recently supported by Lindet and Amman (*Revue général du Lait*, 1906, 361) and also Schmidt-Nielsen (*Hofmeister's Beiträge*, 1907, **9**, 422). Several recent investigations support the view that the coagulation by rennet-enzyme is simply the beginning of peptic digestion (Pawlow and Parastschuck, *Zeits. physiol. Chem.*, 1905, **42**, 415; Sawjalow, *Zeits. physiol. Chem.*, 1906, **46**, 307; Gewin, *Zeitschr.*, 1907, **54**, 42; Sawitsch, *Zeitschr. physiol. Chem.*, 1907, **55**, 84). Various other views have been expressed about the character of the soluble protein (Arthur and Pagès, *Arch. physiol.*, 1890, 540; Van Hernerden, *Zeits. physiol. Chem.*, 1907, **52**, 184; Basch, *Jahrb. f. Kinderh.*, 1898, **47**, 90).

The conditions under which rennet-enzyme coagulates calcium casein have been extensively studied; the more important may be summarised as follows:

(1) The action in respect to rapidity or completeness of coagulation or both is favored by the presence of soluble salts of the alkaline earths, especially calcium, by acids or acid salts, by finely divided inert matter in suspension like starch or sawdust, by temperature up to about 42°.

The relation of soluble calcium salts to rennet action has been discussed already. All dilute acids, organic or inorganic, show very marked effect, though it varies with different acids. Acids of the same concentration promote coagulation by rennet-enzyme in the following order: Hydrochloric, nitric, lactic, acetic, sulphuric, and phosphoric (Pfleiderer, *Pflüger's Archiv.*, 1897, **66**, 605). Milk sour enough to curdle is not coagulated by rennet-enzyme, according to the writer's observation; thus sour buttermilk is not coagulated. This effect of acids upon rennet action is commonly explained by saying that the added acid dissolves the insoluble calcium phosphates in milk and thereby increases the amount of soluble calcium salts. When acids are added beyond certain concentrations, action is delayed. Temperature affects the time of coagulation and the character of the coagulum. Coagulation in a given time is more complete at 41 to 42° and less complete at lower temperatures. At 15° the curd is flocculent, spongy and soft and increases in solidity up to 45°; at 50° and above it is very soft, loose and with a tendency to be gelatinous. Milk heated above 65° for a considerable length of time coagulates less rapidly than normal milk and the coagulum is highly flocculent, never a firm, solid mass, in the absence of soluble calcium salts or acids. Boiled milk fails to coagulate normally, if at all, by rennet-enzyme, unless treated with some soluble calcium salt or some acid.

(2) The action is retarded or prevented by dilution of milk with

water, by alkalis and alkali salts, by numerous neutral salts, by agitation and by high temperature as already shown.

Dilution of milk with more than 10% of water shows marked effect in decreasing time of coagulation. Among the compounds whose presence, even in small amounts, retards the coagulating action of rennet-enzyme are the following: Sodium chloride, acetate, borate, boracic acid, ammonium chloride, chloroform, formalin, and many others (Hammarsten, *Zeits. physiol. Chem.*, 1908, **56**, 18; Lörcher, *Pflüger's Archiv.*, 1898, **69**, 141; Peters, *Untersuch-über das Lab., etc., Rostocker Preisschrift*, 1894; Weitzel, *Arb. d. kais. Gesundheitsamts*, 1902, **19**, 126; Gerber, *Compt. rend. Soc. Biol.*, 1907, **63**, 783; Reichel und Spiro, *Hofmann's Beiträge*, 1904, **6**, 68, and 1906, **8**, 15; Amberg and Loevenhart, *J. Biol. Chem.*, 1908, **4**, 149; Vandevelde, *Hofmann's Beiträge*, 1904, **5**, 558; Sigmund, *Zentralbl. f. Bakt.*, 1905, **14**, No. 12). Vigorous agitation of rennet extract renders the enzyme less active (Schmidt-Nielsen, *Zeitschr. physiol. Chem.*, 1909, **60**, 426). The action of rennet-enzyme is made imperfect when milk is agitated, even slightly, after the milk has begun to thicken and before it becomes fairly solid.

(3) The coagulating power of rennet-enzyme is weakened by exposure to sunlight, by treatment with many different reagents, and by increase of temperature. Heated above 60° for some time rennet extract becomes permanently weaker or inactive; it begins to suffer injury at about 49°. High temperatures destroy the activity gradually, not instantaneously.

Testing of Rennet.—For general purposes a simple test and some arbitrary standard is adopted. The Mourad test is as good as any. For this purpose 5 c.c. of the rennet extract are measured into a 50 c.c. flask and diluted to the mark, washing out the pipette into the flask. 160 c.c. of milk are placed in a beaker and heated to 84°–86° F. and a few small particles of straw dropped in. The rennet extract dilution is brought to the same temperature and 5 c.c. are then quickly run into the milk, stirring at the same time. When the contents of the pipette are half out, a stop watch is started, and the milk having been given a strong rotary motion with the stirrer or thermometer, this last is withdrawn, and the watch stopped the moment the straw floats cease to move, indicating coagulation. In this manner a certain dilution being taken as a standard, other extracts can be standardised by making the same dilution and comparing the times of coagulation. The relative strengths of the extracts are then given in terms of the coagulation time. Other temperatures may be employed, but at

37° rennet is seriously influenced both by hydrion and hydroxyl ion, and as for cheese making, acidity is an all important factor, the lower temperature given above is better.

The time of rennet coagulation is influenced by many factors, especially acidity, and the conditions in comparative experiments must therefore be identical; see in this connection, Zimmermann, (*J. Ind. and Eng. Chem.*, 1912, **4**, 506.)

For a very full and elaborately scientific method of testing rennet, see Van Dam. (*Landed Versuchs Stat.*, 1912, **78**, 133; also *Analyst*, 1912, **37**, 564.) The method is made of a standard character by the use of purified casein in a fixed low hydrogen ion concentration.

Milk-albumin, also known as *lactalbumin*, differs from casein in composition and properties. It contains no phosphorus but about twice as much sulphur as casein. Milk-albumin (1) is not acted on by rennet, (2) is not coagulated by acids at ordinary temperatures, (3) is coagulated by heat, (4) is in solution in milk, and (5) is precipitated with magnesium sulphate in presence of dilute acid, also by ammonium sulphate added nearly to saturation. Wichmann obtained it in crystalline form. (*Zeits. physiol. Chem.*, 1899, **27**, 575.) It was first carefully studied by Sebelien (*Zeits. physiol. Chem.*, 1885, **9**, 445).

Milk-globulin or *lactoglobulin* is obtained from milk by removal of casein with acid or saturation with common salt and then saturating the filtrate with magnesium sulphate. This has been studied especially by Sebelien (*Zeits. physiol. Chem.*, 1885, **9**, 445), Emmerling (*Zentralbl. Agr. Chem.*, 1888, 861) and Tiemann (*Zeits. physiol. Chem.*, 1898, **25**, 363). It is present in the normal milk of cows in only minute amounts, but is present in colostrum in large amounts, 8% or more, dropping rapidly to normal in a few days.

Methods of Estimation of Casein.

The gravimetric method of estimating casein by precipitation with acid, washing with ether and alcohol to remove fat, drying and weighing, has been wholly superseded by the method of determining nitrogen in the washed precipitate, the precipitation being made with some acid. Salting-out with magnesium sulphate is extremely tedious and is rarely, if ever, used.

Of the acids used as precipitants for casein, acetic acid possesses the advantage over the other ordinary acids of causing less solution of precipitated casein when the acid is used in slight excess (L. L. Van

Slyke and D. D. Van Slyke, *Am. Chem. J.*, 1907, **38**, 408). Of the salts used, alum appears to be most convenient.

Analytical methods will be considered for (A) total proteins, (B) casein, (C) albumin, (D) albumin and globulin.

A. **Total Proteins.**—In 2 to 5 grm. of milk, determine nitrogen by the Kjeldahl method. Multiply the percentage of nitrogen by 6.38 to obtain proteins.

B. **Casein.**—Of the different methods employed in estimating casein in milk, the following may be selected as being the most desirable: (1) Precipitation with acetic acid and determination of nitrogen in precipitate; (2) precipitation with alum; (3) direct volumetric estimation; neutralisation with alkali, followed by precipitation with standard acetic acid and subsequent titration of filtrate with standard alkali; and (4) determination of refractive index of casein in form of a soluble salt.

(1) **Precipitation with Acetic Acid.**—Dilute 10 grm. of milk with about 90 c.c. of water at 40 to 42° and add at once 1.5 c.c. of a 10% acetic acid solution. Stir vigorously and let stand a few minutes. Decant on filter, wash two or three times with cold water by decantation and transfer completely to filter. Wash once or twice on filter. The filtrate should be clear or nearly so. Sometimes two or three repeated filtrations may be required before obtaining a clear filtrate. Determine nitrogen in washed precipitate and filter paper by the Kjeldahl method. Use nitrogen-free filter paper or deduct amount present as determined by blank. Multiply result by 6.38 to obtain equivalent amount of casein.

Instead of precipitating at 40 to 42°, one can precipitate at room temperature, but more acid is usually required. The best plan then is to add about 1 to 1.5 c.c. of 10% acetic acid, stir and then add more acid, 0.1 c.c. at a time, stirring after each addition and continuing successive additions of acid until the casein separates, leaving a clear solution.

One can satisfactorily use an approximate standard $N/10$ solution of acetic acid, adding 25 c.c. directly to the diluted milk at room temperature and then, if the casein does not separate, continuing the addition of acid 1 c.c. at a time, and stirring after each addition, until casein separates completely. At room temperature with $N/10$ acid, there is less liability of redissolving casein than at higher temperatures (40 to 42°) with warm concentrated acid.

The foregoing method is applicable to cow's milk and to that of goats, sheep, etc., but not to human milk and that of asses.

For the precipitation of casein in human milk, Engel (*Biochem. Zeits.*, 1908, **13**, 89, and 1908, **14**, 234) recommends the following process as satisfactory in every way: Dilute 10 grm. of milk to 50 c.c. with water and add 6 to 8 c.c. $N/10$ acetic acid. Cool the mixture to 0°, hold at this temperature 2 to 3 hours and then warm on water-bath to 40° with frequent agitation. The casein separates well and permits easy filtration.

For the precipitation of casein in the milk of asses, Friedheim proposes the following method (*Biochem. Zeits.*, 1909, **19**, 32): Dilute 10 grm. of milk to 40 c.c. with water, and add 15 to 18 c.c. of $N/10$ acetic acid. Then proceed as directed above for human milk.

(2) **Precipitation with Alum.**—To 10 grm. of milk add 40 c.c. water and then add 2 c.c. of alum solution saturated at 40°, agitating the mixture well. Let the precipitate settle, transfer to a filter and wash. Determine the nitrogen in the precipitate. The method applies to all kinds of milk except human milk and that of asses (Schlossmann, *Zeits. physiol. Chem.*, 1903, **37**, 337).

(3) **Direct Volumetric Method.**—The following method has been worked out by Van Slyke and Bosworth (*Technical Bulletin* No. 10, 1909, New York Agr. Exp. Sta., and *J. Ind. and Eng. Chem.*, 1909, **1**, 768):

The method is, in brief, as follows: A given amount of milk, diluted with water, is made neutral to phenolphthaleïn by addition of a solution of sodium hydroxide. The casein is then completely precipitated by addition of standardised acetic acid; the volume of the mixture is made up to 200 c.c. by addition of water and then filtered. Into 100 c.c. of the filtrate a standardised solution of sodium hydroxide is run until neutral to phenolphthaleïn. These solutions are so standardised that 1 c.c. is equivalent to 1% of casein when a definite amount of milk is used. Therefore, the number of cubic centimetres of standard acid used, divided by 2, less the amount of standard alkali used in the last titration gives the percentage of casein in the milk examined. The operation usually requires 12 to 15 minutes when apparatus and solutions are at hand convenient for ready use; several determinations can be carried on simultaneously with much relative economy of time.

This method is based upon the following well-known facts: (1) Uncombined casein is insoluble in milk-serum, or water, or very dilute acids. (2) It is acid in properties and combines with alkalis to form definite chemical compounds, which are neutral to phenolphthaleïn.

Of the total amount of acid used in the process of precipitating

casein, a portion is taken to set casein free from combination, thus forming a soluble neutral salt and an insoluble compound (free casein) possessing the properties of an acid; and, on filtration, this amount of acid, as free casein, is removed from the mixture. The balance of the acid used in the process is accounted for in the filtrate on titration with alkali. Therefore, the difference between the total amount of acid used and that accounted for in the filtrate by titration with alkali represents the amount of acid corresponding to the casein present in the milk examined.

Since 1 grm. of free casein neutralises 8.8378 c.c. of $N/10$ sodium hydroxide (or 1 c.c. of $N/10$ sodium hydroxide equals 0.11315 grm. of casein), we have a definite basis for estimating the amount of casein in any given case, when we know the amount of alkali it neutralises.

The details of the method are carried out in the following manner:

(a) *Measuring and Diluting Sample.*—Mix well the sample of milk and run 20 c.c. into a 200 c.c. flask.

(b) *Neutralising the Milk.*—Add 1 c.c. of phenolphthaleïn solution to the diluted milk and then run into it solution of $N/10$ sodium hydroxide, in small portions, shaking vigourously after each addition of alkali, until a faintly, but distinctly, pinkish shade of colour remains even after considerable agitation. Any marked excess of alkali must be avoided.[1]

(c) *Precipitation of Casein.*—Into the neutralised sample of diluted milk, which should be at a temperature[2] of 18 to 24°, run from a

[1] *Preparation of a Colour Standard.*—More uniform and satisfactory results can be obtained in this step of the process by preparing a colour standard for comparison. One method of accomplishing this is as follows: About 20 c.c. of fresh skim-milk and 80 c.c. of water are put into a 200 c.c. flask and a small amount of mercuric chloride added to keep the milk from curdling. A few drops of ordinary carmine ink are considerably diluted with water and this is carefully added, a few drops at a time, to the diluted skim-milk until a faint but distinct pinkish colouration appears. This can be more readily and accurately perceived by placing beside the flask another flask half full of uncoloured, diluted milk. The colouration must be as slight as possible and yet be appreciably distinct when compared with uncoloured milk. After the colour standard has been prepared, the flask is stoppered and kept in a dark place when not in use. In the case of some carmine colours, the pinkish shade in the milk may deepen on standing, especially when exposed to light. If at any time this is observed, the proper shade can be reproduced by slight dilution with skim-milk. The object of using skim-milk in preparing a colour standard is to avoid the presence of fat, which in case of whole-milk separates on standing, adheres to the sides of the flask, and obscures the colour.

Use of Colour Standard.—In neutralising a sample of milk, the colour standard is placed beside the sample under examination for constant comparison after each addition of alkali. The flasks should be placed on a white surface and in a good light in order to render more sharp the observation of the colouration. In fresh milk it is usually found that 3 or 4 c.c. of $N/10$ alkali is sufficient to neutralise the milk. In cases where milk is not strictly fresh or where it has been kept for some time with mercuric chloride, usually from 5 to 10 c.c. may be required. One can usually add 2 or 3 c.c. of alkali at the start and then add it in smaller portions, until the milk begins to show signs of neutrality. After that the alkali is added a drop at a time, the flask being shaken after each addition and the colour being observed. A little experience enables one to perform the operation with rapidity and accuracy.

[2] *Influence of Temperature.*—For convenience and uniformity of results, the temperature of the mixture at the time of addition of acid may be between 18 and 24°. Under these conditions, in most milks, 30 c.c. of $N/10$ acetic acid give satisfactory results. In some cases, especially with the milk of cows far along in lactation and high in casein (3.5 to 4%), one may have to use as high as 35 to 45 c.c. of acid. Rarely will 25 c.c. of acid be excessive. The amount of acid may be 2 or 3 c.c. in excess of that required to effect complete precipitation without seriously affecting the accuracy of the results, provided the temperature

burette some of the $N/10$ acetic acid, adding the acid approximately in 5 c.c. portions and agitating vigourously for a few seconds after each addition. It is usually safe to add about 25 c.c. of acid before examining the milk to see if the casein separates in the form of white flakes. After adding 25 c.c. and shaking, the mixture is allowed to come to rest. If enough acid has been added, the casein separates promptly in large, white flakes, and, on standing a short time, the liquid above the settled casein appears clear and not at all milky. If the addition of 25 c.c. of acid is insufficient to separate the casein properly, add 1 c.c. more of acid and shake; continue the addition of acid, 1 c.c. at a time, until the casein is observed to separate promptly and completely on standing at rest a short time. The number of cubic centimetres of acid used to effect precipitation is noted and this result is recorded as A.

(d) *Filtration of Casein.*—After the casein is completely precipitated, add water to the mixture up to the 200 c.c. mark and shake the contents vigourously for 10 seconds, in order to make the distribution of acid through the mixture as uniform as possible. Then pour the contents of the flask upon a dry filter. It is generally well to allow the filtration to continue until practically all of the liquid has been filtered.[1]

(e) *Titration with Alkali.*—After filtration is completed take 100 c.c. of the filtrate and run into it $N/10$ solution of sodium hydroxide until the reaction is neutral to phenolphthaleïn. The number of cubic centimetres of alkali used is noted and this result is recorded as B.

The exact neutral point is not perfectly sharp on account of the presence of phosphates, and the appearance of the end reaction is not as pronounced as might be desired. However, with experience one should have no difficulty in getting within 1 drop of the correct amount of alkali. One should work to obtain the same shade and duration of colour every time. In general, the appearance of a

of the mixture is below 24°. At higher temperatures, good results are attainable but care must be exercised not to use much excess of acid; and, of course, the higher the temperature the less will be the amount of acid required for precipitation. In working at temperatures below 18°, the casein separates more slowly or requires more acid to separate promptly. In case of milk that is much below 18°, it is well to use for dilution water that is at a temperature of about 27°.

[1] *Rapidity of Filtration.*—The usual time of filtration should not exceed 3 to 5 minutes. The rapidity depends upon the temperature of precipitation and the completeness of the separation of casein. In general, the higher the temperature of the mixture when precipitated with acid, the more rapid should be the filtration, other conditions being uniform. In case of insufficient acid, the filtration is slower.

Appearance of Filtrate.—The filtrate should be quite clear, though this is not always a sure indication that the right amount of acid has been added to effect complete precipitation and release casein entirely from its combination. Sometimes the filtrate may be clear when not quite enough acid has been added, in which case the percentage of casein found is apt to be low; under such circumstances, filtration is usually slow. In case of milks rich in fat, a slight turbidity may appear, due to fat globules in the filtrate. The filtrate should be free from all signs of marked turbidity or anything like milkiness. If such a filtrate appears, a new sample of milk should be taken and the operation repeated from the beginning, more acid being used than before.

faint but distinct pink colouration is taken for the end reaction, which remains clearly marked through the solution for half a minute or longer before beginning to fade. In case of milks rich in phosphates, the solution usually grows quite turbid as the neutral point is approached, making it necessary to use special care in observing the colour of the end-point of the reaction.

If a second titration of the same filtrate is desired, one can use 50 c.c. of the remaining portion, multiplying the result by 2 and recording this as B.

(f) *Calculation of Results.*—The calculation of the percentage of casein from (1) the amount of acid used (A) in precipitating the casein and (2) the amount of alkali used (B) in neutralising 100 c.c. of filtrate is very simple. Divide A by 2, from the result subtract B and multiply the result by 1.0964; or, expressed as a formula,

$$\left(\frac{A}{2}-\mathrm{B}\right)\times 1.0964 = \% \text{ of casein.}$$

By using 22 c.c. of milk instead of 20 c.c., the formula becomes simply $\frac{A}{2}-\mathrm{B}=\%$ of casein; in this case each cubic centimetre of $N/10$ solution being equivalent to 1% of casein.

(g) *Use of Preservatives.*—In making casein determinations by this method, it is desirable when possible to use milk comparatively fresh. Milk that is sufficiently acid to coagulate on boiling or that is well soured cannot be used with satisfactory results. However, by adding to fresh milk powdered mercuric chloride in the approximate proportion of 1:1,000 or 1,500, and keeping the mixture in a cool place, satisfactory results can be obtained with milk that has been kept for 2 or 3 weeks. Milk thus treated should be shaken often enough to keep the fat well incorporated in the body of the milk.

(4) **Determination of Refractive Index of Casein in Form of Soluble Salts.**—The method described below has been worked out by Robertson (*J. Ind. and Eng. Chem.*, 1909, **1**, 723). The method is based upon the fact that the difference between the refractive indices of two solutions of a caseinate, which differ only in their casein content, is proportional to the difference between the percentages of casein which they contain, or, expressed in another way, that

$$n-n_1 = a\times c,$$

in which n is the observed refractive index of the solution, c is the percentage of casein which it contains, n_1 is a constant whose value

depends upon the concentration and nature of the alkaline (or acid) solution employed as solvent, and a is a constant numerically equal to the change in the refractive index of the solvent which is brought about by the addition to 100 c.c. of 1 grm. of casein. By means of the above formula, the concentration of casein in a solution can be very accurately determined, the deviations from accuracy rarely exceeding 2% of the quantity of casein contained in 100 c.c. of solution (provided that quantity exceeds 0.5 grm.) and that the change in the refractive index of a given volume of solution of a base which is brought about by the introduction of a given weight of casein is independent of the concentration of the base and of the nature of the base; if the volume be 100 c.c. and the weight of casein 1 grm., the change in the refractive index is 0.00152.

The method in detail is as follows: Dilute 50 c.c. of milk to 250 c.c. and slowly add 75 c.c. of $N/10$ acetic acid, continuously and rapidly stirring the mixture during the addition. Let the precipitate settle and pour the supernatant liquid through a 15 cm. C. S. & S. No. 589 "white band" paper. Wash the precipitate by decantation with distilled water several times, transferring the washings and subsequently the precipitate to the same filter. Let the filter and precipitate drain 1 hour and then transfer them to a dry beaker, to which add exactly 100 c.c. of $N/10$ sodium hydroxide. Macerate the filter and precipitate (using conveniently a stirring rod protected by a rubber tip) until the paper is transformed into a fine pulp and the casein completely dissolved. Complete solution of the amounts treated is usually attained within 10 minutes; the point of complete solution can easily be determined even in the presence of the paper pulp, since the casein particles are of a different color. When solution is complete, filter the mixture and determine the refractive index of the filtrate by means of a Pulfrich refractometer reading accurately to within 1′ of the angle of total reflection, if possible at temperature of 20°. Since n for $N/10$ sodium hydroxide is 1.33444 (at 20°), the results are calculated as follows:

$$\text{Grams casein in 50 c.c. milk} = \frac{n - 1.33444}{0.00152},$$

where n is the refractive index of the final solution obtained in the manner described above.

If the refractive index of the final solution be determined at temperatures above 20° and below 30°, 0.0001 must be subtracted from the value of the constant n_1 (= 1.33444 at 20° when the solvent is $N/10$

NaOH) for every degree by which the temperature exceeds 20°. If it be determined at temperatures below 20° and above 10°, 0.0007 must be added to the value of the constant n_1 for every degree by which the temperature is less than 20°.

Estimation of Casein in Compounds.[1]—Casein generally occurs commercially as dried curd of skimmed milk containing a little natural fat and natural phosphates, etc., or as a sodium compound. When it occurs in complex admixture such as massage cream, it is probably best to determine its amount by its nitrogen content (see page 132) after having removed fats and soaps, etc., by well known means and estimated ash. (See pages 121 and 123 for action of acids and bases on casein.)

Ash is estimated best by ignition in a muffle at a dull red heat.

Fat is estimated by extraction with freshly rectified light benzin or anhydrous ether, or by the Gottlieb process (p. 239) using 0.5 grm.

Free Acid.—Free mineral acid may be tested for and estimated quantitatively by the use of methyl orange as an indicator and an $N/10$ or $N/50$ potassium hydroxide solution allowing intervals of rest during the titration so that absorbed acid may have a chance to react with the alkali.

Formaldehyde may be tested for in coatings by means of methylene blue which is fastened by formaldehyde casein, but not by casein alone.

C. **Milk-albumin (Lactalbumin).**—To the filtrate obtained by precipitating casein with acetic acid and filtering add sodium hydroxide solution until the mixture is neutral to phenolphthaleïn; then add 0.3 c.c. of 10% solution of acetic acid and heat the liquid to the temperature of boiling water until the albumin is completely coagulated. In case of use of alum in precipitating casein, add to the filtrate 0.3 c.c. of 10% solution of acetic acid and boil as above. Collect the precipitate on a filter, wash and determine the nitrogen therein. Nitrogen multiplied by 6.38 equals albumin.

D. **Milk-albumin and Globulin (Lactalbumin and Lactoglobulin).**—To the neutralised filtrate obtained by the acetic acid precipitation of casein, or to the filtrate from the alum precipitation, add 10 c.c. of a solution consisting of 4 grm. of tannic acid dissolved in 8 c.c. of 25% acetic acid and made up to 200 c.c. with 40–50% alcohol. Filter the precipitate after it settles. Wash it thoroughly, determine nitrogen and multiply by 6.38.

[1] This addenda was supplied by the American editor as the volume went to press.

MILK.

By HENRY LEFFMANN.

Milk is the nutrient secretion of nursing mammalia, and in this article, unless otherwise stated, cows' milk is to be understood.

Normal milk is a white or pale yellow fluid, opaque, except in very thin layers, and with a sp. gr. slightly higher than that of water. It is often amphoteric to litmus. When first drawn it is practically sterile, but unless great precaution is taken, becomes infected with ordinary microbes and begins to decompose.

The principal types of ingredients in milk, exclusive of water, are: fat, carbohydrate, proteins and mineral matters. Several active enzymes are usually present but these are known by their effects rather than as distinct substances. In the early stages of the secretion milk is somewhat different from that produced when the function is well established. This form, known as *colostrum*, is especially characterised by the amount and condition of the proteins. Colostrum is, however, almost entirely of physiologic interest and does not need specific description here.

The fat of milk is of the general type of fats, but contains a larger proportion of esters of acids of low carbon content than other natural fats. For details concerning this point the article on "Butter" in Vol. 2 should be consulted.

Milk-fat exists in suspension in the form of globules, mostly ranging within the limits 0.0024 mm.–0.0046 mm., average about 0.0037 mm., but globules above and below these limits are sometimes found. These globules exist under conditions that interfere with spontaneous coalescence, hence masses of fat are not readily formed and even high centrifugation will not develop a clear fatty layer. The globules being specifically lighter than the liquid in which they are suspended, tend to rise when the milk is allowed to stand and form a layer much richer in fat but still showing no marked coalescence. This layer is termed *cream*. It may be removed by direct skimming or better by centrifugation. The remaining liquid is termed "skim milk." By modern centrifuges (separators) almost the whole of the fat may be

removed. The product (often termed "separator skim" or "separated milk") is still moderately opaque although containing only traces of fat. The fat of milk may be made to coalesce by mechanical agitation; this method (churning) yields it as a soft mass (butter) and leaves an opaque liquid (buttermilk). As churning is often performed on cream that has stood for a time, decomposition has set in, the buttermilk is slightly acid and the proteins slightly hydrolysed.

The carbohydrate of milk is in complete solution and as far as known is always lactose, but even this may exist in several forms. It is not unlikely that a careful study of less known milks will yield carbohydrates not identical with that of ordinary milks. Lactose is fully described in Vol. 1. Compare this Vol. p. 197.

The proteins of milk have been extensively studied. Three forms have been long distinguished: casein, lactalbumin and globulin. "Opalescein" and "mucoid" are other forms that have been lately described. Olson (*J. Biol. Chem.*, 1909, **5**, 261) has described another protein obtained from separator slime, that is, the deposit that forms in the centrifuge bowl in the commercial production of cream. This protein has proteolytic power.

Casein is by far the most abundant protein of milk and is the substance that produces the bulk of the curd. In most methods of curdling, especially those with acids or enzymes, this protein is hydrolysed, a part being precipitated and a part remaining in solution. It has been proposed to call the original predominating protein "caseinogen," but Hammarsten regards this term as objectionable. Casein is not coagulated by heat alone, but is more or less precipitated by many acids and some enzymes, the precipitates often carry with them calcium phosphate (possibly by adsorption) and entangle most of the fat, the curd so formed constituting "cheese." By special manipulation, casein may be obtained free from adherent phosphate and is found to contain a notable amount of phosphorus as part of its molecule. When milk is boiled in the open air a scum of casein and calcium phosphate is formed but this is due to surface evaporation, not to coagulation.

The liquid filtered from the curd is termed "whey." It contains under most conditions of precipitation a notable amount of proteins.

Lactalbumin is coagulated by boiling and is therefore found in an insoluble form in milk that has been heated to or near 100°.

Lactoglobulin is present in very small amount in normal milk and does not need special description. The other proteins are at present of no interest in the general analytic problems concerning milk. The

standard methods for separating and estimating the more important proteins are given below. (See page 155.)

The *mineral matters* of milk are principally calcium phosphates. Small amounts of sulphates and chlorides are also found. The total mineral matter is less than 1.0% and is usually estimated as *ash*, which, of course, does not represent the ingredients in the exact form in which they exist in the milk. "Ash" is a datum of some importance in milk inspection and for control of market-milk and the methods for ascertaining it will be given in detail.

Quantitative Composition of Milk.—Many thousands of analyses of milks, especially of cows' milk, have been published, but unfortunately many of them have been made by untrustworthy methods. In the case of undomesticated animals the analyses are too few to eliminate errors due to accidental abnormal conditions. The following table shows some of the published analyses, but the figures must be regarded as approximate and in some cases doubtful; at least they may not represent average composition.

Human milk is notable for a low protein content hence the curd is less bulky and more friable than that from cows' milk. The milk of all animals is subject to modification by breed, climate, season, feed, housing, exercise, time of lactation, and in human beings (and possibly in some other animals) by psychic influences. It will be

COMPOSITION OF MILKS.

	Human	Cow	Mare	Goat	Ass	Gamoose	Whale	Elephant
Fat	3.5	4.0	1.1	4.3	1.6	5.6	19.4	19.6
Protein	1.5	3.5	1.9	4.6	2.2	3.8	9.5	3.1
Lactose	6.8	4.8	6.6	4.0	6.1	5.4	None	8.8
Ash	0.2	0.7	0.3	0.6	0.5	1.0	1.0	0.5
	12.0	13.0	9.9	13.5	10.4	15.8	29.9	32.0

noted that as regards the proportion of proteins and lactose the milks of the mare and ass agree closely with human milk.

Citrates are normal constituents of several kinds of milk, and do not seem to be dependent on citrates in the food. The proportion in cows' milk is about 0.5%; in human milk less.

Lecithin is also a usual ingredient of milk. Nerking and Haensel by a process given on page 162 found a range in cows' milk from 0.03 to 0.11%.

Composition of Cows' Milk.—For the general purposes of analysis it is sufficient to distinguish two groups of solids, the fat and solids not fat, the latter being, of course, obtained by subtracting the fat from the total solids.

The most extensive series of data on these points are those of Vieth,

who tabulated the results of 120,540 analyses by trustworthy methods. The average is

Fats	4.1
Solids not fat	8.8
Total solids	12.9

Richmond reported (*Anal.*, 1909, **35**, 231) the average composition of 18,519 samples as: fat, 3.74; solids not fat, 8.92; total solids, 12.66, sp. gr., 1032.1.

Seasonal Variations.—The poorest quality usually occurs in the early part of the year, especially in April. Low figures are also found in July. In autumn the quality rises.

Deficient Solids.—The great majority of normal milks will be found to comply with the standards indicated by the above averages, but many analyses of pure milks below the standards have been reported. The following are some data of this character:

Sp. gr.	Fat	S. N. F.	Total solids	Analyst
1029.6	3.38	7.95	11.33	Cochran.
1030.0	3.62	8.31	11.93	Cochran.
1029.3	3.63	8.02	11.65	Cochran.
.....	3.99	8.36	12.35	Leffmann and Beam.
.....	3.11	8.33	11.44	Monthly averages N. J. State Agricultural Exp. Station.
.....	3.05	8.33	11.38	
.....	3.23	8.44	11.67	
.....	.....	8.1	.....	Richmond (*Anal.*, 1909, 35, 231).

The following analyses of milk from individual cows were made by Cochran. The samples were taken under precautions which insured their genuineness. The data are all direct estimations. The total solids were obtained by drying in the usual manner, and the fat by the L-B. method. (See page 150.) Low milks have been often noted in the vicinity of Philadelphia.

Sp. gr.	Fat	S. N. F.	Total solids
1026.6	2.35	6.78	9.13
1028.8	2.95	7.56	10.51
1028.8	2.40	7.56	9.96
1033.5	2.90	8.68	11.58

The mixed milk from a herd of any considerable number will rarely, if ever, show a proportion of non-fatty solids less than 8.5% nor less than 3.5% of fat.[1] Cochran examined the milk from each cow of a herd of 59, with the following results:

[1] According to Revis this is not true of English experience.

Fat	2.60 to 5.40
Total solids	9.86 to 13.78

The average milk of the entire herd was:

Fat	3.76%
Solids not fat	8.57%

The average of nearly 100 analyses at the University of Wisconsin creamery during a protracted drought in 1895 gave but a trifle over 8.5% solids not fat. The casein was low in this milk, while the sugar was about normal in amount. Similar conditions have been observed by Van Slyke at the New York station.

It has been thought that when the solids not fat are deficient in genuine milk the lactose is principally lacking, but recent investigations have thrown doubt on this.[1]

Lythgoe (41st *Annual Rep., S. B. of Health of Mass.*, 1909) gives a table of averages of composition of 51 samples of genuine milk, each set of averages being deduced by analysis of 10 samples. The following data are selected from this table. For explanation of the figures in the last column, see page 164.

Total solids	Fat	Proteins	Lactose	Ash	Solids not fat	Refraction of copper serum, 20°
15.70	6.01	4.13	4.79	0.77	9.69	38.1
15.00	5.62	3.75	4.87	0.76	9.38	38.3
14.50	5.30	3.61	4.82	0.77	9.20	38.3
14.00	4.78	3.51	4.98	0.73	9.22	38.5
13.50	4.61	3.37	4.77	0.75	8.89	38.1
13.00	4.24	3.17	4.86	0.73	8.76	37.9
12.50	3.99	2.84	4.94	0.73	8.51	38.0
12.00	3.45	2.88	4.96	0.74	8.55	37.7
11.50	3.33	2.67	4.80	0.70	8.17	37.3
11.00	3.02	2.64	4.63	0.71	7.98	37.0
10.70	2.90	2.60	4.49	0.71	7.80	36.4

From these figures Lythgoe derives the rule that differences in proportion of solids not fat in unadulterated milks are principally due to differences in the amount of proteins. Lactose and ash are fairly constant. On these facts depend recently introduced methods of detecting watering of milk, as will be pointed out later.

Derivatives from Milk.—Among these are: cream, buttermilk, whey, skim-milk, condensed milk, and fermented and otherwise specially modified milk. All these are considered fully in the section on "Milk Products."

Sterilisation and Pasteurisation.—Boiling produces coagulation of the albumin, some caramelisation of the sugar, and develops a greater facility of coalescence on the part of the fat globules. Enzymes are rendered inert and most microbes are killed.

[1] In England, the experience of Richmond and of Revis supports the view that low solids, not-fat, are due to a deficiency of lactose.—W. A. D.

Pasteurisation is usually carried out by heating milk to about 75° (167° F.) for a limited time. Little effect is produced on the important ingredients, but only certain bacteria are killed such as those that cause lactic acid fermentation, so that pasteurised milk keeps better than ordinary milk but may not be free from dangerous organisms.[1]

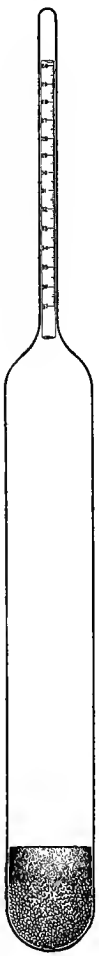


FIG. 4.—Shaw and Eckel's lactometer. *Bull. Bur. An. Ind.*, 134, U. S. Dept. Agric.

Very low temperatures produce also marked changes. Ravenel, Hastings and Hammer (*J. Inf. Dis.*, 1910, **7**, 38) have found that in milk held for some time at −9° the casein and fat become clumpy, and an increase in soluble nitrogen occurs. Bacteria capable of growing on ordinary gelatin and agar media do not sensibly increase. In milk held at 0° a marked increase of bacterial content was noted, together with increase of acidity and of soluble nitrogen, which may amount in time to 70% of the total originally present. There is also some loss due possibly to the liberation of nitrogen in the free state. Cold storage and pasteurisation kill organisms producing lactic acid, but not all pathogenetic organisms.

Analytical Processes.—The great importance of milk and milk products as foods, and the great liability of them to adulteration have led to extensive laboratory studies and to the development of many analytical processes. These are of two types—those especially adapted to ordinary milk inspection and control, and those adapted to research or to the verification of the ordinary methods.

The methods for ordinary inspection and sanitary control are principally the estimation of fat and total solids. Sp. gr. and ash are also often ascertained, and special points such as detection of thickeners, colours, and preservatives are matters of great moment in sanitary administration. Methods for the detection of contamination by dangerous animal products, such as pus cells, though of great practical importance, do not come within the scope of this article.

Specific Gravity.—Air-bubbles are held rather tenaciously by milk, and care must be taken in mixing to avoid as far as possible

[1] The U. S. Dept. Agric. (Cir. of Inform. released Aug. 1, 1913) advises as a result of careful experiments, pasteurization for 30 minutes at 63° (145° F) in which the bacteria that survive are principally those that produce lactic acid. The new process is also more economical.

the inclosure of the air, and to allow sufficient time for the escape of any bubbles that may be present. The sp. gr. of milk is understood to be taken at 15.5°; samples should be brought near to this. If at a few degrees above or below, it will suffice to make the estimation at once and obtain the correct figure by reference to the annexed table. The sp. gr. of normal milk ranges between 1.028 and 1.035. The figure alone does not indicate the character of the sample, but taken in conjunction with the figure for fat or for total solids, it is of value as a check on the results furnished by other determinations.

The sp. gr. of milk rises gradually for some time after it has been drawn, and the observation is to be made only after this action has ceased. This will require about 5 hours after the milk is drawn, if it has been kept below 15°, but at a higher temperature it will be necessary to allow at least 12 hours. For all other data, the analysis should be made as soon as possible.

The sp. gr. of milk is usually taken with the *lactometer*, a delicate and accurately graduated hydrometer. The instrument must be immersed carefully so as not to wet the stem above the point at which it will rest. Its accuracy should be verified by immersion in distilled water at 15.5° and milks of known sp. gr.

Shaw and Eckels (*Bull.* 134, *Bur. Animal Ind.*, U. S. Dept. Agric.) have devised a simple form of lactometer shown in Fig. 4.

More accurate determinations may be made with a balance. The Westphal balance is adapted to the determination of sp. gr. only, the weights being so arranged that a simple enumeration of them gives the gravity directly. The cheap forms of this instrument are not satisfactory, but the best forms are accurate.

Sp. gr. may be accurately taken with a plummet on the ordinary analytic balance. The Westphal plummet is convenient, but a glass tube of a bulk of about 10 c.c., weighted with shot or mercury and sealed, may be used. It is suspended from the hook of the balance by means of fine platinum wire and the weight ascertained. It is then submerged in distilled water and the weight also noted. The water is contained in a narrow upright cylinder resting on a bench or support above the scale pan. The loss of weight of the plummet is, of course, the weight of the bulk of water that it displaces. The sp. gr. of any sample can be determined by weighing the plummet immersed in the sample and dividing the loss in weight by the loss in water. The quotient is the sp. gr.

The sp. gr. bottle is not convenient for milk on account of the

Find the temperature of the milk in one of the horizontal lines and the specific gravity in the first vertical column. In the same line with this and the temperature the corrected specific gravity is given.

° F.	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73
Sp. gr.																						
21	20.3	20.4	20.5	20.6	20.7	20.8	20.9	20.9	21.0	21.1	21.2	21.3	21.4	21.5	21.6	21.7	21.8	22.0	22.1	22.2	22.3	22.4
22	21.3	21.4	21.5	21.6	21.7	21.8	21.9	21.9	22.0	22.1	22.2	22.3	22.4	22.5	22.6	22.7	22.8	23.0	23.1	23.2	23.3	23.4
23	22.3	22.4	22.5	22.6	22.7	22.8	22.8	22.9	23.0	23.1	23.2	23.3	23.4	23.5	23.6	23.7	23.8	24.0	24.1	24.2	24.3	24.4
24	23.3	23.4	23.5	23.6	23.6	23.7	23.8	23.9	24.0	24.1	24.2	24.3	24.4	24.5	24.6	24.7	24.9	25.0	25.1	25.2	25.3	25.5
25	24.3	24.4	24.5	24.6	24.6	24.7	24.8	24.9	25.0	25.1	25.2	25.3	25.4	25.5	25.6	25.7	25.9	26.0	26.1	26.2	26.4	26.5
26	25.2	25.3	25.4	25.5	25.6	25.7	25.8	25.9	26.0	26.1	26.2	26.3	26.5	26.6	26.7	26.8	27.0	27.1	27.2	27.3	27.4	27.5
27	26.2	26.3	26.4	26.5	26.6	26.7	26.8	26.9	27.0	27.1	27.3	27.4	27.5	27.6	27.7	27.8	28.0	28.1	28.2	28.3	28.4	28.6
28	27.2	27.3	27.4	27.5	27.6	27.7	27.8	27.9	28.0	28.1	28.3	28.4	28.5	28.6	28.7	28.8	29.0	29.1	29.2	29.4	29.5	29.7
29	28.2	28.3	28.4	28.5	28.6	28.7	28.8	28.9	29.0	29.1	29.3	29.4	29.5	29.6	29.8	29.9	30.1	30.2	30.3	30.4	30.5	30.7
30	29.1	29.2	29.3	29.4	29.6	29.7	29.8	29.9	30.0	30.1	30.3	30.4	30.5	30.7	30.8	30.9	31.1	31.2	31.3	31.5	31.6	31.8
31	30.1	30.2	30.3	30.4	30.5	30.6	30.8	30.9	31.0	31.2	31.3	31.4	31.5	31.7	31.8	32.0	32.2	32.2	32.4	32.5	32.6	32.8
32	31.1	31.2	31.3	31.4	31.5	31.6	31.7	31.9	32.0	32.2	32.3	32.5	32.6	32.7	32.9	33.0	33.2	33.3	33.4	33.6	33.7	33.9
33	32.0	32.1	32.3	32.4	32.5	32.6	32.7	32.9	33.0	33.2	33.3	33.5	33.6	33.8	33.9	34.0	34.2	34.3	34.5	34.6	34.7	34.9
34	33.0	33.1	33.2	33.3	33.5	33.6	33.7	33.9	34.0	34.2	34.3	34.5	34.6	34.8	34.9	35.0	35.2	35.3	35.5	35.6	35.8	36.0
35	33.9	34.0	34.2	34.3	34.5	34.6	34.7	34.9	35.0	35.2	35.3	35.5	35.6	35.8	35.9	36.1	36.2	36.4	36.5	36.7	36.8	37.0
°C.	11.1	11.6	12.2	12.7	13.3	13.8	14.4	15.0	15.5	16.1	16.6	17.2	17.7	18.3	18.8	19.4	20	20.5	21.1	21.6	22.2	22.7

liability of the upper layer of the liquid to be richer in fat than the lower; the overflow, therefore, does not represent the mixture.

For information as to the scientific principles involved in the construction and use of apparatus for ascertaining sp. gr., see Introduction, Vol. 1.

Total Solids.—This estimation may often be made with sufficient accuracy for practical purposes by evaporating a measured volume (*e.g.*, 3 or 5 c.c.) in a shallow nickel dish from 5 to 8 cm. in diameter. Nickel crucible-covers are suitable. The thin glass (Petri) dishes used for bacterial cultures are convenient. When greater accuracy is required, and especially when the ash is to be ascertained, platinum dishes must be used.

Bevan found that long keeping of samples had an appreciable effect on the amount of solids, as the following figures obtained by him show:

	Total solids	Loss
Evaporated immediately	11.73	.....
Evaporated after 24 hours	10.79	0.94
Evaporated after 48 hours	10.38	1.35
Evaporated after 120 hours	9.42	2.31

The decomposition is irregular. See page 176.

The pipette used for taking portions for analysis should have a rather wide opening that no cream may be retained.

When rigid accuracy is not essential, it will suffice to measure the portions of milk with a pipette. Vieth uses one graduated to deliver 5 grm. and finds that, working with whole and skimmed milk, under the ordinary differences of temperature, the error will not exceed 0.1 on the total solids and is less on the fat.

Satisfactory results may be secured by the following simple method: A flat platinum dish, 3.5 cm. in diameter, with sides 0.5 cm. high, is provided with a thin flat watch-glass cover that fits rather closely. The total weight of the cover and dish is noted. 2 or 3 c.c. of the sample are run into the dish, the watch-glass placed on, and the weight taken as rapidly as possible. The glass prevents appreciable loss from evaporation during an ordinary weighing. The cover is removed, the dish heated on the water-bath or in the water-oven, and weighed from time to time (with cover on it) until the weight is sensibly constant. The percentage of residue can be easily calculated. About 3 hours may be required to secure constant weight.

A. O. A. C. Method.—Heat at 100° to constant weight, about 3 grm.

in a tared platinum, aluminum or tin dish of 5 cm. diameter, with or without the addition of 15 to 30 grm. of sand. Cool and weigh.

The use of aluminum or tin as substitutes for platinum is inadvisable; much better results will be obtained with nickel, porcelain, glass, or fused quartz. Dishes made of fused, transparent quartz will probably be useful.

Acetone Method.—2.5 grm. of milk are weighed into a flat porcelain capsule and mixed with 1 c.c. of acetone. The capsule is then placed on a *rapidly* boiling water bath and left there for 30 minutes, after which it is removed, the outside wiped dry, and it is then placed in the water oven for 1 hour, and weighed. The results are practically identical with those given by official processes, as skin formation is prevented and no combination with the protein takes place, as is the case if acetic acid be used.

For a special method with asbestos as recommended by the A. O. A. C., see page 149.

Ash.—The residue from the estimation of total solids is heated cautiously over the Bunsen burner, until a white ash is left. The result obtained in this manner is apt to be slightly low from loss of sodium chloride. This may be avoided by heating the residue sufficiently to char it, extracting the soluble matter with a few cubic centimetres of water, and filtering (using paper extracted with hydrofluoric acid). The filter is added to the residue, the whole ashed, the filtrate then added, and the liquid evaporated carefully to dryness. The ash of normal milk is about 0.7% and faintly alkaline. A marked degree of alkalinity and effervescence with hydrochloric acid will suggest the addition of a carbonate. Probably the safest and best method to follow is to heat to dull redness in a muffle.

A. O. A. C. Method.—In a weighed dish put 20 c.c. of milk from a weighing bottle; add 6 c.c. of nitric acid, evaporate to dryness, and burn at a low red heat till the ash is free from carbon.

Fat.—Many methods for fat estimation have been devised.

Wanklyn's Method—extraction of the solid residue with several portions of ether, evaporating these and weighing the fat—is now known to be inaccurate.

Adams' Method (*Anal.*, 1885, **10**, 46, 85).—This consists essentially in spreading the milk over absorbent paper, drying, and extracting the fat in an extraction apparatus; the milk is distributed in an extremely thin layer, and by a selective action of the paper the larger portion of the fat is left on the surface. A paper, manufactured especially for this purpose by Schleicher & Schüll, is obtainable in

strips of suitable size. Each of these yields to ether only from 0.001 to 0.002 grm. of extract.

Coils made of thick filter paper, cut into strips 6 by 62 cm., are thoroughly extracted with ether and alcohol, or the weight of the extract corrected by a constant obtained for the paper. From a weighing bottle about 5 grm. of the milk are transferred to the coil by means of a pipette, care being taken to keep dry the end of the coil held in the fingers. The coil is placed, dry end down, on a piece of glass and dried for 1 hour, preferably in an atmosphere of hydrogen; it is then transferred to an extraction apparatus and extracted with absolute ether, petroleum spirit of b. p. about 45° or carbon tetrachloride. The extracted fat is dried and weighed.

The above procedure is very satisfactory, but the drying in hydrogen may usually be omitted. After the coil has received at least 20 washings, the flask is detached, the ether removed by distillation, and the fat dried by heating in an air-oven at about 105°, and occasionally blowing air through the flask. After cooling, the flask is wiped with a piece of silk, allowed to stand 10 minutes, and weighed.

Richmond states that to perform a rigidly accurate estimation attention to the following points is necessary: The ether must be anhydrous (drying over calcium chloride and distilling is sufficient). Schleicher & Schüll's fat-free papers should be used, and one should be extracted without any milk on it, as a tare for the others. 4 or 5 hours' extraction is necessary, and the coils should be well dried before extraction is begun.

Thimble-shaped cases made of fat-free paper are now obtainable and are convenient for holding the absorbent material on which the milk is spread. The fine texture prevents undissolved matter escaping. A case may be used repeatedly. Sour milk may be thinned with ammonium hydroxide before taking the portion for analysis.

Babcock Asbestos Method (A. O. A. C).—Provide a hollow cylinder of perforated sheet metal 60 mm. long and 20 mm. in diameter, closed 5 mm. from one end by a disc of the same material. The perforations should be about 0.7 mm. in diameter and 0.7 mm. apart. Fill the cylinder loosely with from 1.5 to 2.5 grm. of freshly ignited woolly asbestos free from fine or brittle material. Cool in a desiccator and weigh. Introduce a weighed quantity of milk (about 4 grm.) and dry at 100°. The cylinder is placed in the extraction tube and extracted with ether in the usual way. The ether is evaporated and the fat weighed. The extracted cylinder may be dried at 100° and the fat checked by the loss in weight. A higher degree of accuracy is

secured by performing the drying operation in hydrogen. For details as to arrangement and operation of continuous extraction apparatus, see Introduction, Vol. 1.

Röse-Gottlieb Method.—This process is now widely adopted, being convenient and accurate. It is applicable to all forms of milk products as well as to whole milk. 5 grm. of milk should be employed. It is fully described in the section on Milk Products (page 188).

Centrifugal Methods.—Although almost all the fat of milk may be separated by the centrifuge, the emulsion is not destroyed and the volume of cream is merely suggestive as to the fat-content of the milk. To obtain a clear fatty layer in condition for close measurement it is necessary to use chemicals. The methods at present most employed depend essentially on one devised by Gustaf DeLaval, who, on July 1, 1885, took out a patent in Sweden for the use of a mixture of 20 volumes of strong acetic acid and 1 volume of strong sulphuric acid. This mixture coagulates and then dissolves the proteins, destroys the emulsion, but does not otherwise affect the fat and does not act on the lactose. By brief whirling in a centrifuge the fat collects in a clear sharply defined layer. DeLaval took out patents in several countries subsequent to the above date.

In 1889, Leffmann and Beam devised a method in which a small amount of amyl alcohol with an equal volume of hydrochloric acid was added to the milk, and the proteins thus coagulated dissolved by strong sulphuric acid. About the same time Babcock devised a process in which sulphuric acid was used alone. Subsequently Gerber published a process in which the essential feature of the Leffmann-Beam method, namely, the use of amyl alcohol, was advised.[1]

Leffmann-Beam Method.—The test-bottles have a capacity of about 30 c.c. and are provided with a graduated neck, each division of which represents 0.1% by weight of butter fat.

15 c.c. of the milk are measured into the bottle, 3 c.c. of a mixture of equal parts of amyl alcohol and strong hydrochloric acid added, mixed, the bottle filled nearly to the neck with concentrated sulphuric acid, and the liquids mixed by holding the bottle by the neck and giving it a gyratory motion. The neck is now filled to about the zero

[1] The Leffmann-Beam method is often erroneously called the "Beimling" method, but Beimling was merely the deviser of a cheap centrifuge (U. S. patents, Sept. 24, 1889; May 6, 1890). To protect the interest of a manufacturer who had invested in the Beimling machine under the impression that it was a practicable method for fat estimation, it became necessary for Leffmann and Beam to take out a patent and assign the same to this investor. U S. patent 479193, July 19, 1892 (now expired) was issued and assigned. The sum to be paid by the assignee was $500, but a parole agreement was made between the senior assignor and the assignee that the share of the former was not to be paid unless the patent yielded great profit. No payment was ever claimed under this agreement. The junior assignor received his full share ($250).

point with a mixture of sulphuric acid and water prepared at the time. It is then placed in the centrifugal machine, which is so arranged that when at rest the bottles are in a vertical position. If only 1 test is to be made, the equilibrium of the machine is maintained by means of a test-bottle, or bottles, filled with a mixture of equal parts of sulphuric acid and water. After rotation for from 1 to 2 minutes, the fat will collect in the neck of the bottle and the percentage may be read off. It is convenient to use a pair of dividers in making the reading. The legs of these are placed at the upper and lower limits respectively of the fat, allowance being made for the meniscus; one leg is then placed at the zero point and the reading made with the other. Experience by analysts in various parts of the world has shown that with properly graduated bottles the results are reliable. As a rule, they do not differ more than 0.1% from those obtained by the Adams process, and are generally even closer.

For great accuracy, the factor for correcting the reading on each of the bottles should be determined by comparison with the figures obtained by the Adams or other standard process.

Cream is to be diluted to exactly ten times its volume, the sp. gr. taken, and the liquid treated as a milk. Since in the graduation of the test-bottles a sp. gr. of 1030 is assumed, the reading must be increased in proportion.

A more accurate result may be obtained by weighing in the test-bottle about 2 c.c. of the cream and diluting to about 15 c.c. The reading obtained is to be multiplied by 15.45 and divided by the weight in grm. of cream taken.

The mixture of fusel oil and hydrochloric acid seems to become less satisfactory when long kept. It should be clear and not very dark in color. It is best kept in a bottle provided with a pipette which can be filled to the mark by dipping. Rigid accuracy in the measurement is not needed.

Babcock Method.—By means of a pipette that delivers 17.6 c.c., the approximate volume of 18 grm. milk, bottles suitably graduated are charged with this volume of the sample and 17.5 c.c. of sulphuric acid (sp. gr. 1.83) added by portions until the coagulum first formed is dissolved. The mixture is whirled for 5 minutes in a centrifuge, hot water added to fill the bottle to the beginning of the stem and again whirled for 2 minutes. Enough hot water is then added to bring the fat all into the graduated part of the neck, when after short whirling the volume is read off.

Gerber Method.—This is merely a modification of the Leffmann-Beam method (Richmond, *Anal.*, 1893, **18**, 137), and is now practically the only centrifugal method used in Europe. It presents features which make it the simplest and most satisfactory of all these methods. The necessary chemicals and butyrometers can be obtained anywhere to-day and detailed description is unnecessary.

The reagents required are:

(1) *Sulphuric Acid (Commercial).*—Sp. gr. 1.820–1.825. If the acid is not correct it must be adjusted to these limits.

(2) *Amyl Alcohol.*—The fraction boiling between 124° and 130° and having a sp. gr. of 0.815–0.818 is employed. It should be practically colourless and when tested in the butyrometer with the acid and water in place of milk, it should give no layer of insoluble matter at the surface of the mixture when heated and rotated after standing for 24 hours.

The test is carried out as follows:

10 c.c. of the acid are run into the butyrometer and 11 c.c. of the well-mixed sample run down the side onto the acid but without allowing admixture to take place. 1 c.c. of the amyl alcohol is then run carefully onto the milk, the butyrometer closed with a good india rubber stopper and the whole well shaken in an up and down direction till all the curd is dissolved.

The tube should then be placed in a water-bath at 70° for about 5 minutes (not more than 15) after which it is placed in the special centrifuge and whirled at a speed of about 2000 revolutions for 5 minutes. The tube is then removed from the machine and without warming the volume of fat which has collected in the graduated neck is read off in percentages. The graduations are from 0–90 and ten divisions represent 1% of butter fat by weight in the original sample. The fat layer should be perfectly clear and not coloured pink and there should not be a plug of fluffy matter beneath it. Any such appearances denote either too much or too little heating of the tube or that the tube has not been sufficiently shaken. It is advisable to check the readings of one tube against some standard method of fat estimation and to keep such a tube for comparison with other tubes. It is also advisable to roughly calibrate the graduated scale in order to see that the same reading is obtained in all parts of the scale.

Other Methods for Fat Estimation.—Many methods have been devised. Some employ the centrifuge with solvents not containing strong acid (so-called "sinacid" method), others use ether or similar

solvents. The methods above described in detail are in general use, and the less important ones do not need special description here.

Total Proteins.—3 types of processes are employed for this estimation: Calculation from the total nitrogen; precipitation and direct weighing; calculation from the "aldehyde-figure." Milk contains appreciable amounts of non-protein nitrogen, but the fact is usually disregarded. According to Munk, this may range, in cow's milk, from 0.022 to 0.034%, and from 0.014 to 0.026% in human milk. By these figures, the average protein nitrogen in cows' milk would be 94%, and in human milk 91%, of the total nitrogen.

Calculation from Nitrogen.—The nitrogen is estimated by the Kjeldahl-Gunning method.

5 c.c. of the sample are introduced into the digestion flask, 10 grm. of potassium sulphate and 20 c.c. of strong sulphuric acid added, the digestion and distillation being carried out as described in detail in Introduction, Vol. 1.

The nitrogen figure multiplied by 6.38 gives the figure for total proteins.

Ritthausen Method (*J. prakt. Chem.*, 1877, [ii], **15**, 329).—This method depends on precipitation by copper sulphate and sodium hydroxide. It is applicable only to fully developed milks; the proteins of colostrum and whey are only partially precipitated. The reagents are copper sulphate solution, 34.639 grm. of pure crystallised copper sulphate dissolved in water and made up to 500 c.c. and $N/10$ sodium hydroxide 10 grm. of milk are diluted with 100 c.c. of water, 5 c.c. of copper sulphate solution added, and well mixed. The sodium hydroxide solution is added drop by drop, with constant stirring, until the precipitate settles quickly and the liquid is neutral, or very feebly acid. An excess of alkali will prevent the precipitation of some of the proteins.

The reaction should be tested on a drop of the clear liquid, withdrawing it by means of a rod, taking care not to include any solid particles. When the operation is correctly performed, the precipitate, which includes the fat, settles quickly, and carries down all of the copper. It is washed by decantation with about 100 c.c. of water, and collected on a filter (previously dried at 130° and weighed in a weighing bottle). The portions adhering to the sides of the beaker are dislodged with the aid of a rubber-tipped rod. The contents of the filter are washed with water until 250 c.c. are collected, which may be mixed and reserved for the estimation of lactose. The water

in the precipitate is removed by washing once with strong alcohol, and the fat by 6 or 8 washings with ether. An extraction apparatus may be used for this purpose. The washings being received in a weighed flask, the estimation of the fat may be made by evaporating the ether, with the usual precautions.

The residue on the filter, which consists of the proteins in association with copper hydroxide, is washed with absolute alcohol, which renders it more granular, and then dried at 130° in the air-bath. It is weighed in a weighing bottle, transferred to a porcelain crucible, incinerated, and the residue again weighed. The weight of the filter and contents, less that of the filter and residue after ignition, gives the weight of the proteins. The results by this method are slightly high, since copper hydroxide does not become completely converted into copper oxide at 130°.

Richmond & Boseley have modified the process by diluting the milk to 200 c.c., adding a little phenolphthaleïn, and neutralising any acidity by the cautious addition of dilute sodium hydroxide solution, then adding from 2.0 to 2.5 c.c. of the copper sulphate solution. The precipitate is allowed to settle, washed, and estimated as above.

Aldehyde Number.—The addition of formaldehyde to milk increases the acidity by an action on the proteins. As commercial formaldehyde is always acid, this acidity must be either estimated or neutralised in applying the following method. The application of the reaction to the estimation of proteins in milk is due to Steinegger (*Zeitsch. Nahr. Genuss.*, 1905, **10**, 659). Richmond and Miller (*Anal.*, 1906, **31**, 224) investigated the method and suggested the use of strontium hydroxide instead of sodium hydroxide. Richmond has presented further experience and inferences lately. (*Anal.*, 1911, **36**, 9) and gives the following details:

To 10 c.c. of milk at least 1 c.c. of a 0.5% solution of phenolphthaleïn is added and the liquid then neutralised with standard strontium hydroxide solution. To the faintly pink liquid, 2 c.c. or more of 40% formaldehyde solution are added and the titration made to the same tint as the former. The strontium hydroxide required by the formaldehyde solution must be known, and this being deducted from that which was used in the titration and the remainder calculated to c.c. $N/1$ acid per 1,000 c.c. of milk will give the "aldehyde number." Richmond finds that this multiplied by 0.17 gives in most cases a close approximation to the total proteins obtained by the Kjeldahl method. The following are some of the test analyses he made:

Aldehyde number c.c. $N/1$ acid per 1,000 c.c.	Protein aldehyde number $\times$ 0.17	Protein $N \times 6.38$
6.6	1.12	1.16
16.1	2.74	2.76
19.0	3.23	3.22
20.2	3.43	3.44
20.3	3.45	3.44
21.4	3.64	3.73
24.0	4.08	3.95
25.0	4.25	4.15
23.7	4.03	4.54

The last sample was of abnormal composition and, as is seen, gave a divergent result. Richmond gives the following details of this sample:

Sp. gr.	Total solids	Fat	Lactose	Proteins	Ash
1.0283	13.20	5.00	2.87	4.54	0.89

Calculation Method.—Olson (*J. Ind. and Eng. Chem.*, 1909, **1**, 253) has shown that in normal milks the proteins may be calculated with close approximation by the formula

$$p = t - \frac{t}{1.34}$$

in which p is protein and t total solids. The table on page 156 has been calculated according to this formula.

Estimation of Individual Proteins.—Casein and albumin, dominating proteins of milk may be separated and estimated by several methods. That of Sébelein is among the earliest and is as follows: 20 c.c. of the sample are mixed with 40 c.c. of a saturated solution of magnesium sulphate and some of the powdered salt is also stirred in until no more will dissolve. The precipitate of casein, fat, and globulin, is allowed to settle, filtered, and washed several times with a saturated solution of magnesium sulphate. The filtrate and washings are saved for the estimation of albumin. The filter and contents are transferred to a flask, the nitrogen ascertained by the Kjeldahl method, and the figure multiplied by 6.38 gives casein.

The filtrate and washings from the estimation of casein are mixed, the albumin precipitated by *Almén's tannin reagent*, filtered, and the nitrogen in the precipitate estimated as above. The same factor is used.

Almén's reagent is prepared by dissolving 4 grm. of tannin in 190 c.c. of 50% alcohol and adding 8 c.c. of 25% acetic acid.

PROTEINS CALCULATED BY OLSON'S FORMULA.

Total solids	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85	0.90	0.95
15	3.81	3.82	3.83	3.85	3.86	3.87	3.88	3.89	3.90	3.92	3.94	3.95	3.96	3.98	3.99	4.00	4.01	4.03	4.04	4.05
14	3.56	3.57	3.58	3.59	3.60	3.62	3.63	3.64	3.65	3.67	3.69	3.70	3.71	3.72	3.73	3.75	3.76	3.77	3.79	3.80
13	3.30	3.32	3.33	3.34	3.35	3.37	3.38	3.39	3.40	3.42	3.43	3.44	3.45	3.47	3.48	3.49	3.50	3.52	3.53	3.54
12	3.05	3.06	3.08	3.09	3.10	3.11	3.13	3.14	3.15	3.17	3.18	3.19	3.20	3.21	3.23	3.24	3.25	3.27	3.28	3.29
11	2.80	2.81	2.82	2.83	2.86	2.86	2.87	2.88	2.89	2.91	2.92	2.93	2.95	2.96	2.98	2.99	3.01	3.02	3.03	3.04

The above figures multiplied by 0.8 give approximate *casein*.

In a mixture of milk and whey (prepared with rennet) in about equal parts, Richmond and Boseley found about 0.3% of albumoses not precipitated by copper sulphate nor magnesium sulphate, but precipitable, along with the albumin, by a solution of tannin. The separation may be effected by diluting the filtrate from the magnesium sulphate precipitation, acidifying slightly with acetic acid, and boiling, when the albumin will be coagulated and precipitated. The albumoses may be separated by filtering the solution and precipitating with tannin solution. The precipitated proteins are best estimated from the nitrogen of the moist precipitate. The separation of the proteins may be effected, though less accurately, by the use of acetic acid, as recommended by Hoppe-Seyler and Ritthausen.

Leffmann and Beam have modified the process to avoid the delay and trouble of washing the precipitate, as follows: 10 c.c. of the milk are mixed with saturated magnesium sulphate solution and the powdered salt added to saturation. The mixture is washed into a graduated measure with a small amount of the saturated solution, made up to 100 c.c. with the same solution, mixed, and allowed to stand until the separation takes place. As much as possible of the clear portion is drawn off with a pipette and passed through a dry filter. An aliquot portion of the filtrate is taken, the albumin precipitated by a solution of tannin, and the nitrogen in the precipitate ascertained as above.

The following are A. O. A. C. methods:

1. *Provisional Method for Casein.*—The estimation should be made when the milk is fresh, but if not practicable to make it within 24 hours, add one part of formaldehyde to 2,500 parts of milk and keep in a cool place. 10 grm. of the sample are diluted with about 90 c.c.

of water at between 40 and 42°, 1.5 c.c. of a solution containing 10% of acetic acid by weight added, allowed to stand for 5 minutes, washed three times by decantation, pouring the washings through a filter, and the precipitate transferred completely to the filter. If the filtrate is not clear at first, it will generally become so in two or three filtrations, after which the washing can be completed. The nitrogen in the washed precipitate and filter is ascertained by the Kjeldahl-Gunning method and multiplied by 6.38, to give the amount of casein.

With milk containing preservatives, the acetic acid should be added in small portions, a few drops at a time with stirring, and the addition continued until the liquid above the precipitate becomes clear or nearly so.

2. *Provisional Method for Albumin.*—The filtrate obtained in the above operation is neutralised with sodium hydroxide, 0.3 c.c. of the 10% solution of acetic acid added, and the mixture heated for 15 minutes. The precipitate is collected on a filter, washed, and the nitrogen ascertained.

Van Slyke has pointed out that the casein can be approximately ascertained by multiplying the figure for total proteins by 0.8.

Modified Proteins, Amino-derivatives and *Ammonium Compounds.*—The following procedures are given by Van Slyke (*Bull. New York Exp. Sta.*, **215**, 102). The filtrate from the albumin precipitate is heated to 70°, 1 c.c. of 5% sulphuric acid added, then solid zinc sulphate to saturation. The mixture is allowed to stand at 70° until the caseoses settle. The liquid is cooled, filtered, the precipitate washed with saturated solution of zinc sulphate slightly acidified with sulphuric acid and the nitrogen ascertained by the Kjeldahl method.

For amino-derivatives and ammonium compounds, 50 c.c. of the milk are mixed in a flask marked at 250 c.c. with 1 grm. of sodium chloride. A 12% solution of tannin is added, drop by drop, until no further precipitation occurs. The mixture is diluted to the mark, shaken and filtered through a dry filter. For amino-derivatives, 50 c.c. of the filtrate are treated for nitrogen in the usual way. For ammonium compounds, 100 c.c. of the filtrate are mixed with magnesium oxide and about 50 c.c. distilled, the distillate being received in a known volume of standard acid. Large excess of magnesium oxide must be avoided.

Lactose.—For gravimetric estimation, the A. O. A. C. employs Soxhlet's method with the following reagents:

Copper Sulphate Solution.—34.639 grm. of pure crystallised copper sulphate are dissolved in water and made up to 500 c.c.

Alkaline Tartrate Solution.—173 grm. of pure sodium potassium tartrate and 50 grm. of good sodium hydroxide are dissolved in water and the solution made up to 500 c.c.

N/2 sodium hydroxide.

25 c.c. of the sample are placed in a 500 c.c. flask and diluted with 400 c.c. of water and 10 c.c. of the copper sulphate solution and 8.8 c.c. *N/2* sodium hydroxide solution added. The mixture should still have an acid reaction and contain copper in solution. If this is not the case, the experiment must be repeated, using a little less of the alkali. The flask is filled to the mark with water, shaken, and the liquid passed through a dry filter. 50 c.c. of Fehling's solution, obtained by mixing equal parts of the copper sulphate and alkaline tartrate solutions, are heated to brisk boiling in a 300 c.c. beaker, 100 c.c. of the filtrate obtained as above added, and boiling continued for 6 minutes; the liquid is then promptly filtered through asbestos and the copper estimated by one of the methods described in detail in Vol. 1, pages 323–327.

Polarimetric Estimation.—For the routine work of milk inspection estimation by the polarimeter after removal of the fat and proteins is most convenient. This is usually carried out as recommended by Wiley, by a mercuric nitrate solution, prepared by dissolving mercury in twice its weight of nitric acid of 1.42 sp. gr. and adding to the solution 5 volumes of water. (Revis and Bolton find it better to use mercuric oxide for making this solution, see page 214.) The A. O. A. C. method is as follows:

For polarimeters (Ventzke scale) reading to 100 for 26.048 grm. sucrose (corresponding to 32.98 grm. lactose monohydrate), measure, in c.c., the amount obtained by dividing double this (*i.e.*, 65.96) by the sp. gr., add 10 c.c. mercuric nitrate solution, make up to 102.6 c.c., shake, filter through a dry filter and examine in a 200 mm. tube. Half the observed reading will be the percentage of lactose. For example, if the sp. gr. of the milk is 1.030, the amount taken will be $65.96 \div 1.030 = 64$ c.c.

The allowance for volume of precipitate by making up to 102.6 c.c. is not accurate, except with closely skimmed milks.

The correction may be made more closely by calculating the actual volume of the precipitate by multiplying the fat-percentage by 1.075 (average specific volume of fat) and the protein-percentage by 0.8 (average specific volume of coagulated proteins), deducting the sum of these products from 100 c.c. and correcting the observed reading by proportion. For ordinary milk, the volume of the proteins from

65.96 grm. may be taken at 1.68 c.c. Supposing the sample to contain 4.0% of fat and the polarimetric reading to be 10, the calculation would be thus:

65.96 × 0.04 = 2.63 Amount of fat in milk taken
2.63 × 1.075 = 2.82 c.c. Volume of fat in precipitate
1.68 c.c. Estimated volume of proteins in precipitate
4.50 c.c. Total volume of precipitate
100 − 4.50 = 95.5 c.c. Actual volume of liquid.
100 : 95.5 :: 10 : 9.55 9.55 ÷ 2 = 4.75, % lactose

Richmond gives the following simple method of calculating the correction for the volume of fat and protein:

The correction for 100 c.c. of milk is the % of fat × 1.1 and expressed as c.c. + the excess of the gravity over 1000 divided by 10 + 3 c.c. (to compensate for vol. of protein) + a correction to reduce scale readings of polarimeter to % of milk sugar, which with instruments graduated in the Ventzke scale is 6.63.

For instance in the case of a milk containing 3.6% fat and gravity 1032.2, the correction is calculated as follows:

$$3.6 \times 1.1 = 3.96$$
$$\frac{1032.2 - 1000}{10} = 3.2$$
Protein correction = 3.0
Scale factor = 6.63
Total correction = 16.79 c.c.
$$\frac{\text{Readings}}{3} = \text{anhydrous lactose per 100 grm. of milk}$$

The employment of a factor for correcting for the volume of precipitate may be avoided by Scheibler's method of "double dilution," in which two solutions of different volume are compared. The following is a summary of the method given by Wiley & Ewell: For polarimeters adapted to a normal weight of 26.048 sucrose, 65.82 grm. of milk are placed in a 100 c.c. flask, 10 c.c. of the acid mercuric nitrate added, the flask filled to the mark, the contents well mixed, filtered, and a reading taken. A similar quantity of the milk is placed in a 200 c.c. flask and treated in the same way. The true reading is obtained by dividing the product of the two readings by their difference. If the observations are made in a 200 mm. tube the percentage is half the true reading.

The instrument should be accurate, and great care taken in the work, or results will be less satisfactory than by the method in which an allowance is made for the volume of the precipitate.

In testing human milk by this method some difficulty may be found in getting a clear filtrate, owing to the small proportion of protein. The liquid may be cleared by moderate centrifuging or by using very close filtering paper.

Mutarotation.—When freshly dissolved in cold water, lactose shows a higher rotation than that given above. By standing, or immediately on boiling, the rotatory power falls to the point mentioned. In preparing solutions from the solid lactose for test analyses, care must be taken to bring them to the b. p. previous to making up to a definite volume. This precaution is unnecessary when operating on milk.

Acidity.—Milk being often amphoteric to litmus, that indicator cannot be employed in estimating acidity. Phenolphthaleïn is usually employed. Several methods differing in details have been proposed. Probably the best is that of Thörner (*Chem. Zeit.* 1892, **16**, 1479, 1519). In this, 10 c.c. of milk are diluted with 20 c.c. of water, a few drops of a dilute alcoholic solution of phenolphthaleïn added and the titration made with $N/10$ alkali. Thörner proposes that the number of c.c. required should be multiplied by 10 and the result termed the "degree of acidity." Fresh normal milk will show figures ranging from 16 to 18. When the degree of acidity is 23 or over, the sample will coagulate on heating.

The process involves a slight error, in that the addition of a notable amount of water to a milk sample disturbs somewhat the relation of the phosphates and diminishes the acidity. It may be advisable to titrate the undiluted milk. If the number of c.c. used is multiplied by 0.9 the lactic acid equivalent to the acidity of the sample is given in grm. per 1,000 c.c.

Citrates.—Two methods entirely different in principle are used.

Desmoulière's Method (*Bull. Scien. Pharm.*, 1910, **17**, 588).—200 c.c. of the milk are mixed with 100 c.c. of 2% acetic acid and boiled for a short time under a reflux condenser. The liquid is cooled, filtered through a dry filter, 150 c.c. of the filtrate mixed with 3 grm. of diatomaceous earth, or similar inert powder, and evaporated on the water-bath to a pasty condition. The residue is cooled, 3 c.c. of dilute (1 : 5) sulphuric acid added and the mixture allowed to stand for several hours with occasional stirring. An additional portion of 3 grm. of the inert powder is then stirred in and the mass repeatedly extracted with cold ether saturated with water. The extracts, which may measure as much as 1,000 c.c. in all, are mixed and distilled to small bulk at as low a temperature as possible. The residue is treated with water, made up to a known volume and divided into three equal portions. In one portion the total acidity is estimated, in the second the volatile acid (acetic) and in the third the phosphoric acid. The last named may not be present, but the test must always be made for

it. The citric acid is found by taking the difference between the total acidity and the sum of the acidities due to the volatile acids and the phosphoric acid. In examining human milk, the amount of sulphuric acid may be advantageously reduced to even 2 c.c., but with cow's milk the full amount given above should be used.

Denigès' Method, modified by Beau.—The description of this is taken from C. Barthel's "*Die Methoden zur Untersuchung von Milch u. Molkereiproducten*," 1911.

50 c.c. of milk are placed in a 200 c.c. measuring flask, 75 c.c. of water added and then 50 c.c. of mercuric sulphate solution (see below). The mixture is well shaken; the casein is precipitated. The flask is filled to the mark with water, filtered through a dry filter, the liquid being returned until the filtrate shows only a faint opalescence.

100 c.c. of the filtrate (equivalent to 25 c.c. of the sample) are heated to boiling, the source of heat withdrawn and a 1 % solution of potassium permanganate added drop by drop with constant stirring until the precipitate settles very readily, leaving a clear supernatant liquid. A slight excess of the permanganate is not objectionable. The precipitate may have a brownish tinge from the presence of manganese dioxide. This may be removed by heating the mixture to boiling, withdrawing the heat and adding a few drops of hydrogen peroxide until the precipitate is bleached. About 10 drops of this reagent will usually be needed. The mixture is allowed to cool, filtered through an asbestos layer by means of a filter pump and washed with water until the washings no longer react with barium chloride. The flask containing the filtrate is removed, the precipitate dissolved in concentrated hydrochloric acid, using two portions of 10 c.c. each and washing the filter with 50 c.c. of water after each treatment with acid.

The solution, which will not be perfectly clear, is introduced into an Erlenmeyer flask, brought to boiling and passed through the asbestos filter that has just been used (the filter pump may be again used) as it is necessary to obtain a clear filtrate. After washing the filter, the liquid is again put into an Erlenmeyer flask, and to it are added 20 c.c. of ammonium hydroxide and 10 c.c. of $N/10$ potassium cyanide solution (13.04 grm. in 1,000 c.c.). The liquids are mixed, 10 drops of 10% solution of potassium iodide added as indicator and titrated with $N/10$ silver nitrate until the precipitate does not dissolve after shaking. The number of c.c used is deducted from 10 (that is, from the number that would be used if no mercury compound was present) and the remainder gives the c.c. corresponding to the mercury. Beau has calculated a table for convenience, in which the amount of citric acid is

ascertained from this difference; the table being, of course, only applicable to the procedure just given, in which 100 c.c. of the filtrate are taken (equivalent to 25 c.c. of milk). The results are given in centigrams per 1,000 c.c., that is, approximately parts per 100,000 (as the volume of 1,000 c.c. of milk weighs notably more then 100,000 centigrams). By shifting the decimal three figures to the left, the numbers will be approximate percentages. The table is transcribed from Barthel's book, a slight error in one of the figures having been corrected by the author of this article.

c.c. difference	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	0.0	3.0	6.0	9.0	12.0	15.0	17.5	20.5	23.5	26.5
1	29.5	32.5	35.5	38.5	41.5	44.5	47.0	50.0	53.0	56.0
2	59.5	62.0	65.0	68.0	71.0	74.0	76.5	79.5	82.5	85.5
3	88.5	91.5	94.5	97.5	100.5	103.5	106.0	109.0	112.0	115.0
4	118.0	121.0	124.0	127.0	130.0	133.0	135.5	138.5	141.5	144.5
5	147.5	150.5	153.5	156.5	159.5	162.5	165.0	169.0	172.0	175.0
6	178.0	181.0	184.0	187.0	191.0	194.0	197.0	200.0	203.0	207.0
7	210.0	213.0	216.	219.0	223.0	226.0	229.0	232.0	235.0	239.0
8	242.0	245.0	248.	251.0	255.0	258.0	261.0	264.0	267.0	270.0
9	273.5	276.5	279.5	282.5	285.5	289.5	292.5	295.5	298.5	301.5
10	305.5	307.5	311.5	314.5	317.5	321.5	325.0	328.0	331.0	334.5

The method depends on reactions discovered by Denigès and set forth in an elaborate paper (*Ann. phys. chim.*, 1899, **18**, 382). The citric acid is converted by the permanganate into acetone dicarbonic acid, and this, by the mercuric sulphate, into a complex compound which contains mercury in the proportion of 1 part to 0.384 parts of citric acid in the original liquid. This compound is insoluble in water but soluble in strong hydrochloric acid and the amount of mercury is ascertained as given above by titration with silver nitrate.

The mercuric sulphate solution is prepared as follows: 50 grm. of mercuric oxide are shaken with 500 c.c. of water and concentrated sulphuric acid added in small portions until the oxide is all dissolved. About 75 c.c. of acid will be required. The liquid is made up to 1,000 c.c., boiled and filtered.

Lecithin.—The simplest method is that of Nerking and Haensel (*Biochem. Zeitschr.*, 1908, **13**, 348) as follows:

100 c.c. of milk are mixed with 200 c.c. of strong alcohol, the mixture well stirred and the precipitate then allowed to settle, collected in an extraction thimble and extracted for 30 hours with chloroform in the usual manner. The alcoholic filtrate is evaporated to dryness at about 55°, the residue exhausted with chloroform, the chloroform extracts mixed, evaporated to dryness in a platinum basin, the residue

ignited with the usual sodium carbonate—sodium nitrate oxidising mixture, the fused mass taken up with nitric acid and the phosphate present estimated by precipitation with ammonium molybdate and conversion into magnesium pyrophosphate in the usual manner. The magnesium pyrophosphate multiplied by 7.27 will give the lecithin.

As the phosphorus may not be all in the form of orthophosphate after the ignition, it is advisable to boil the nitric acid solution for a few minutes before adding the molybdate solution.

General Calculation Methods.—In 1879, Behrend and Morgen (*J. Landw.* 1879, **27**, 250) pointed out a relation between the sp. gr., fat and total solids of milk and published a table of calculations. Clausnitzer and Mayer (*Forschung. auf Gebiete Agric.*, 1879, **2**, 265) reported further on the method. Hehner (*Anal.*, 1883, **8**, 129) compiled a formula based on analyses by Wanklyn's method. He took the sp. gr. of fat as 0.9278 and assumed that 1% of this decreased the sp. gr. of milk by 0.725 (water being 1,000) while each unit of solids not fat increased the sp. gr. by 3.6. Fleischmann and Morgen (*J. Landw.*, 1882, **30**, 293) constructed a formula from data obtained by extracting fat from the residue after drying the milk absorbed by calcium sulphate. They assumed the sp. gr. of milk-fat as 0.940 at 15°, but Fleischmann subsequently found 0.930 to be more nearly correct and altered the formula accordingly. Hehner and Richmond (*Anal.*, 1888, **13**, 523) established a formula based on fat extraction by the Adams method. Later, Richmond (*Anal.*, 1889, **14**, 121) revised this and gave the following,

$$t = 0.25\ g + 1.2\ f + 0.14$$

in which t is total solids; f, fat; g, the last two integers of the sp. gr. and any decimals (water being 1,000). Fleischmann's formula, as given in German works, is

$$t = 1.2f + 2.665\left(\frac{100g - 100}{100}\right)$$

the letters having the same significance. Babcock's formula has been much used in the United States. It is adapted to calculating the solids not fat. In this formula g is the entire figure for sp. gr. referred to water as 1.

$$S.N.F = \left(\frac{100g - fg}{100 - 1.0753fg} - 1\right) \times 2.5(100 - f)$$

Babcock has also given a much simpler form adapted for total solids. This differs but slightly from Richmond's. A detailed investigation of the relations between the gravimetric results and the figures obtained with these formulæ has been published by Shaw and Eckles (*Bull.* 134, *Bur. An. Indust., U. S. Dep't. Agric.*).

Detection of Adulterations.

By far the larger part of the laboratory work on milk is for assistance in the sanitary control of the supply, and the analyses are principally directed to the detection of the ordinary forms of adulterations. The most important of these are: skimming, watering and addition of colouring, thickening and preserving agents. Skimming and watering are detected by the estimation of fat and total solids; from these data the solids not fat are calculated. For the ordinary purposes of milk control, fat can be estimated with quite sufficient accuracy by centrifugal methods. The total solids may be estimated directly as described on page 147, or after carefully ascertaining the sp. gr. by consulting the table on page 146.

Judgment whether a given sample has been skimmed or watered depends in many cases upon the standard for whole milk (*i.e.*, milk in the condition in which it is drawn from the animal). Great irregularity of standards for fat and solids not fat exists, and the opinion of the analyst will be determined, therefore, by the standard of the locality. In most cases the standard for fat is between 3 and 4%, and that for total solids about 8.50%.

As fat diminishes the sp. gr. of milk, and the other solids increase it, it is possible to take off a small amount of the former and add some water without disturbing the sp. gr., but, of course, the above analytical methods will detect this procedure. It is now admitted that, except in cases of wide departure from the usual limits, the adulteration of milk cannot be detected by the sp. gr. alone but the employment of a carefully graduated lactometer is of use in routine milk inspection.

Direct Detection of Added Water. Serum-refraction.—Of late years several methods have been proposed for this purpose. Some of them depend on the fact that natural waters contain substances not in milk; others again upon the different ratio in which some substances exist in natural waters and in milk respectively. Thus, attempt has been made to detect added water by estimating the nitrates in the sample. These methods have no positive value and have not come into general

use, especially as methods of precision based on a different principle have been devised. The employment of the Zeiss immersion refractometer for ascertaining the refractive index of the whey (milk-serum) offers a rapid and satisfactory method for detecting watering. Several methods of preparing this whey have been proposed, but Lythgoe (*Rep. S. B. of Health of Mass.*, 1909) has found as the result of extended experience the following to be the best yet suggested.

Dissolve 72.5 grm. of crystallised copper sulphate in water and dilute to 1,000 c.c. If this solution does not refract 36 on the scale of the immersion refractometer at 20°, add water or copper sulphate until the desired result is obtained. To 8 c.c. of the copper solution add 32 c.c. of milk. Shake well and pour upon a dry filter. When the filtrate begins to come through clear, change the receiver, pour the small quantity of cloudy filtrate upon the filter and continue the filtration as usual. Refract the clear filtrate at 20°, by means of the immersion refractometer. A reading below 36 indicates added water. The advantages of this method over the acetic acid method are as follows: It is quicker, heating of the samples is unnecessary, consequently there is no error due to evaporation. The range of differences in the refraction of pure milk is less. 10% of added water will reduce the refraction of high-grade milk below the minimum, but it takes 15% in the acetic acid method. Analyses have been made by this method of 150 samples of milk of known purity. The total solids ranged from 17.17 to 10.40%, the fat from 7.7 to 2.45%, the solids not fat from 10.50 to 7.5% and the refraction of the copper serum from 36.1 to 39.5. These refractions were distributed as follows:

Refraction	Number of samples
39.0 to 39.5	6
38.0 to 38.9	66
37.0 to 37.9	65
36.1 to 36.9	13
	150

See also table of refractions on page 143.

Richmond suggested that the amount of water added to milk may be calculated from the figures obtained by adding the difference between the sp. gr. of the sample and 1,000 to the figure representing the percentage of the fat. Thus, a milk of sp. gr. of 1029.2, containing 3.27% of fat, the figure from which the water is calculated is 29.2 + 3.27 = 32.47. The mean figure from unadulterated milks was found to be 36.0, but Richmond proposed 34.5 as a limit. Accepting this

figure, the percentage of added water in the sample given will be found by the proportion 34.5 : 32.47 : 100 : : 94.1, *i.e.*, it contains 5.9% of water. Experiments on milks which had been diluted with known proportions of water showed that this method gave closer approximations than by calculating from the figure for non-fatty solids.

Freezing-point.—It is stated that the watering of milk can be detected by the lowering of the freezing-point. According to Atkins (*Chem. News*, 1908, **97**, 241) the freezing-point of whole milk is −0.55 and never differs by 0.03 from this, fat having no influence. Bomstein claims that as little as 5% added water can be detected by this method. The special apparatus devised for cryoscopy must be used, and the data must be determined by each observer in order to be safely comparable.

Thickening Agents.—To conceal skimming and watering many thickening agents have been used. At least two instances of the use of brain matter have been reported (Leffmann, *J. Amer. Chem. Soc.*, 1900, **22**, 356). Dextrin, starch, sugar, salt, gelatin, saccharate of lime, and agar have all been used.

Brain matter can be easily detected by the microscope, starch jelly by the iodine test, dextrin by increased polarimetric reading, sodium chloride by the increased chlorides in the ash. Agar is frequently used in certain milk products, especially the cheap ice-cream sold in American cities. For its detection see under Milk Products.

Gelatin.—Stokes detects the presence of gelatin in cream or milk as follows: 10 c.c. of the sample, 20 c.c. of cold water, and 10 c.c. of acid mercuric nitrate solution (page 158) are mixed, shaken vigourously, allowed to stand for 5 minutes, and filtered. If much gelatin is present, it will be impossible to get a clear filtrate. A portion of the filtrate is mixed with an equal bulk of saturated aqueous solution of picric acid. Gelatin produces a yellow precipitate. Picric acid will detect the presence of 1 part of gelatin in 10,000 parts of water. The picric acid solution should give no turbidity with the nitrate solution.

Sucrose.—Cotton has devised the following test: 10 c.c. of the sample are mixed with 0.5 grm. of powdered ammonium molybdate, and 10 c.c. of dilute hydrochloric acid (1 to 10) are added. In a second tube 10 c.c. of milk of known purity or 10 c.c. of a 6% solution of lactose are similarly treated. The tubes are then placed in the water-bath and the temperature gradually raised to about 80°. If sucrose is present, the milk will assume an intense blue colour, while genuine milk or milk-sugar remains unaltered unless the temperature be raised to the boiling-point. According to Cotton, the reaction is well

marked in the presence of as little as 1 grm. of sucrose to 1,000 c.c. of the milk. For the detection of other organic thickening agents, such as pectoses, agar and mixtures of agar and gelatin, see under "Cream," page 193.

Calcium Saccharate (*Saccharate of Lime*).—A compound produced by the action of lime on sucrose has been used as a thickening agent. A test due to Bauer and Neumann is recommended by Lythgoe, from whose description (*Rep. S. B. Health, Mass.*, 1909) the following is taken:

To 25 c.c. of milk (or cream) add 10 c.c of 5% solution of uranium acetate, shake well, allow to stand for 5 minutes and filter. To 10 c.c. of the clear filtrate (in the case of cream use the total filtrate, which will be less than 10 c.c.) add a mixture of 2 c.c. saturated ammonium molybdate and 8 c.c. dilute hydrochloric acid (1 part 25% acid and 7 parts water), and place in a water bath at a temperature of 80° for 5 minutes. If the sample contains sugar the solution will have a prussian blue tint. This should always be compared in a colourimeter with the standard prussian blue solution prepared by adding a few drops of potassium ferrocyanide and 5 drops of 10% hydrochloric acid to a solution of 1 c.c. of 0.1% ferric chloride in 20 c.c. of water.

It has been claimed that pure milk will give this test. Occasionally samples of pure milk will give a pale blue, but this can be entirely removed by filtration, and the filtrate will be green; while the colour due to sucrose will pass through the filter, giving the blue solution characteristic of adulterated samples. The colour is due to reduction of molybdic acid, and is caused by levulose and dextrose as well as by sucrose. Solutions of 1 grm. of lactose, levulose, dextrose and sucrose in 35 c.c. of water were used in comparing the amount of colour produced when heated with the molybdenum reagent for 5 minutes. Lactose produced no colour, levulose gave a heavy blue, sucrose a weaker blue and dextrose the weakest blue, corresponding in intensity as 10 : 3 : 1.

Stannous chloride and ferrous sulphate give this colour, but the reaction takes place in the cold, and with small quantities the colour disappears on heating. In order for the colour to persist after heating the sample of cream must contain these substances to the extent of 1% calculated as the metal. In this case the sample will be completely coagulated and the taste will be disagreeable. Hydrogen sulphide will also give the blue, but it will disappear on heating. If the solution does not show blue before heating, it is free from hydrogen sulphide, ferrous sulphate or stannous chloride.

As a confirmatory test for sugar, the resorcinol test may be applied

to the serum prepared with uranium as described above. This test is given by sucrose and lævulose, but not by dextrose or lactose.

The quantitative estimation of sucrose in milk is given under Milk Products (page 213).

Detection of Heated Milk.—Fresh milk contains one or more enzymes of the "peroxydase" type, that is, having power to bring about transfer of oxygen from peroxides to oxidisable substances. As the function of these enzymes is destroyed by temperatures near 100°, it becomes possible to utilize the reaction for determining whether a given sample has been thus heated. In most cases the action of the enzyme is indicated by the production of a colour, no colour change occurring when the enzyme has been heated. Hydrogen peroxide is commonly employed for furnishing the oxygen. A considerable number of substances have been found to be susceptible to oxidation under the influence of the milk enzymes. Benzene derivatives, commonly used as photographic developers are especially susceptible. Guaiacum was first used, however. This was employed by Arnold (*Jahresber. d. Königl. Tierärztl. Hochsch.*, 1880–82, 161).

Arnold's Method.—A solution of guaiacum in acetone is, according to Arnold and Menzel (*Milch Zeit.*, 1902, 247) better than the ordinary tincture. The test is applied by adding to a small amount of the sample in a test tube, about 10 drops of the guaiacum solution, to which a drop or two of hydrogen peroxide solution has just been added, so that the reagent will float on the milk. If the sample has not been heated above 80°, the point of contact of the liquids will show a deep blue ring.

As guaiacum is liable to changes both in the solid form and in solution it is important to determine if the reagent is sensitive to raw milk, hence a control test should always be made. Other reagents are, however, now available which are, in the main, more trustworthy.

Dupouy's Method (*J. Pharm. Chim.*, 1897, **7**, 397).—In this method, 1–4 diamino-benzene is used. The reagent is dissolved in water (a weak solution will suffice), a few drops added to the sample, then a few drops of hydrogen dioxide solution, and the liquids shaken gently. Milk that has not been heated above 80° gives immediately a bright blue. Milk that has been heated above this temperature shows no colour change at first but may slowly acquire a bluish tint. This test is much in favor, but it is open to the objection that the solution of the reagent does not keep more than a few hours, and even in the solid state some commercial samples soon decompose.

Benzidine Method.—Wilkinson and Peters (*Zeit. Nahr. u. Genuss.*,

1908, **16**, 172) suggested this reagent, employing a solution of it with a few drops of acetic acid followed as usual by the oxidising agent. Leffmann finds that the commercial benzidin hydrochloride (furnished for volumetric estimation of sulphates) acts satisfactorily without acetic acid.

Wilkinson and Peters' test is performed similarly to those just described, and has a similar significance. They give experiments to show that the method is rather more delicate than with diaminobenzene or guaiacum. The solution of the benzidin compound keeps better. They found that milk heated to 77° had lost its reactivity to guaiacum but retained reactivity to the other two reagents. Heated to 78° the reactivity was also lost to these.

Leffmann has found the following well-known photographic developers to be applicable to the detection of raw milk with about the same limitations as the reagents just noted: metoquinone (Lumière) and amidol (Hauff). Dianol (Lumière) is not satisfactory.

At critical temperatures, however, the results with all the reagents depend materially on the length of the heating.

Colours.—Annatto, turmeric, and some coal-tar colours are much used. Caramel is occasionally used, saffron and carotin but rarely. *Annatto* may be detected by rendering the sample slightly alkaline by acid sodium carbonate, immersing a slip of filter paper, and allowing it to remain overnight. Annatto will cause a reddish-yellow stain on the paper.

Leys gives the following method for detecting annatto: 50 c.c. of the sample are shaken with 40 c.c. of 95% alcohol, 50 c.c. of ether, 3 c.c. of water, and 1.5 c.c. of ammonium hydroxide solution (sp. gr. 0.900), and allowed to stand for 20 minutes. The lower layer, which in presence of annatto will be greenish-yellow, is tapped off and gradually treated with half its volume of 10% solution of sodium sulphate, the separator being inverted without shaking, after each addition. When the casein separates in flakes that gather at the surface, the liquid is tapped off, strained through wire gauze, and placed in four test-tubes. To each of these amyl alcohol is added, and the tubes shaken and immersed in cold water, which is gradually raised to 80°. The emulsion breaks up, and the alcohol, holding the annatto in solution, comes to the surface. The alcoholic layer is separated from the lower stratum, evaporated to dryness, and the residue dissolved in warm water containing a little alcohol and ammonium hydroxide. Clean white cotton is introduced and the liquid evaporated nearly to dryness on the water-bath. The cotton, which is coloured

a pale yellow, even with pure milk, is washed and immersed in a solution of citric acid, when it will be immediately coloured rose-red if the milk contained annatto. Saffron, turmeric, and the colouring-matter of the marigold do not give a similar reaction.

Coal-tar colours may often be detected by dyeing wool, but Lythgoe has devised the following method, which is satisfactory: 15 c.c. of the sample are mixed in a porcelain basin with an equal volume of hydrochloric acid (sp. gr. 1.20), and the mass shaken gently so as to break the curd into coarse lumps. If the milk contains an azo-colour, the curd will be pink; with normal milk the curd will be white or yellowish.

General Method for Colours in Milk.—Leach has devised a general method. 150 c.c. of the sample are coagulated in a porcelain basin, with the addition of acetic acid and heating, and the curd separated from the whey. The curd will often collect in a mass; but if this does not occur, it must be freed from whey by straining through muslin. The curd is macerated for several hours in a closed flask, with occasional shaking, with ether to extract fat. Annatto will also be removed by it. The ether and curd are separated and treated as follows:

The ether is evaporated, the residue mixed with a little weak solution of sodium hydroxide, and passed through a wet filter; and when this has drained, the fat is washed off and the paper dried. An orange tint shows annatto, which may be confirmed by a drop of solution of stannous chloride, which makes a pink spot.

If the curd is colourless, no foreign colouring-matter is in it; if orange or brown, it should be shaken with strong hydrochloric acid in a test-tube.

If the mass turns blue gradually, caramel is probably present. The whey should be examined for caramel.

If the mass turns pink at once, an azo-colour is indicated.

Preservatives.[1]—These are largely used, especially in the warmer season, as a substitute for refrigeration. Many of them are sold under proprietary names which give no indication of their composition. Preparations of boric acid and borax were at one time the most frequent in use, but at present *formalin*, a 40% solution of formaldehyde, has come into favour. Sodium benzoate is now in common use as a preservative of cider, fruit-jellies, and similar articles, and may, therefore, be found in milk. Salicylic acid is not so much employed. Sodium carbonate is occasionally used to prevent coagulation due to slight souring. Fluorides and "abrastol" may be used. A mixture of boric

[1] Owing to rigid inspection the addition of preservatives to milk is little practiced in the United States, but analysts must nevertheless be continually on the lookout as any laxity on the part of inspectors and analysts would result in the extended use of such substances.

acid and borax is more efficient than either alone. The quantity generally used is equivalent to about 0.5 grm. of boric acid per 1,000 c.c. Formaldehyde is an efficient antiseptic. In the proportion of 0.125 grm. to 1,000 c.c., it will keep milk sweet for a week. Hydrogen peroxide, ozone and dichromates have been used. The almost universal decree of sanitary authorities is that milk must be free from any added material, but owing to its comparatively high cost, liability to decomposition and the marked characters of even incipient decomposition, great temptation to use preservatives exists and any antiseptic, not actively poisonous, may be used. It has been found that milk drawn and marketed under strict sanitary precautions will keep for 12 hours or even longer at moderate temperatures. The only permissible method of preserving milk is refrigeration. (See Appendix.)

In addition to the descriptions of the detection and estimation of preservatives given below, see also under "Cream," page 190.

Formaldehyde. *Hehner's Test.*—Hehner found that when milk containing formaldehyde is mixed with sulphuric acid containing a trace of a ferric compound, a distinct blue appears. Richmond and Boseley showed that the delicacy of the test is much increased if the milk is diluted with an equal volume of water and sulphuric acid of 90 to 94%, added so that it forms a layer underneath the milk. Under these conditions, milk, in the absence of formaldehyde, gives a slight greenish tinge at the junction of the two liquids, while a violet ring is formed when formaldehyde is present even in so small a quantity as 1 part in 200,000 of milk. The colour is permanent for 2 or 3 days. In the absence of formaldehyde, a brown ring may form in the course of a few hours, but it is below the junction line of the two liquids.

Phenylhydrazine Test.—The following test avoids the fallacy of some other tests. A pinch of phenylhydrazine hydrochloride is added to a few cubic centimetres of the sample, the liquid shaken, then a drop of a fresh solution of sodium nitroprusside and a few drops of sodium hydroxide solution. A greenish tint is at once produced if formaldehyde is present. If the test is applied to the liquid obtained by distilling some of the milk as described on page 172 the colour will be deep blue.

Phloroglucinol Test.—A small amount of a 1% solution of phloroglucol is added to the sample and then a considerable volume of sodium hydroxide solution. In the presence of formaldehyde a distinct rose tint will be produced. It is best to add the phloroglucinol by means of a tube passed to the bottom of the test-tube.

Bonnet's test utilizes the vapor of formaldehyde, and avoids the fallacies of some of the older tests. A solution is made by dissolving

0.035 grm. pure morphin sulphate in 10 c.c. of sulphuric acid. This solution does not keep well. A convenient amount of the sample is placed in a dish or beaker, a watch-glass containing 1 c.c. of the above solution is floated on it, and the dish covered with a glass plate. The materials are allowed to remain undisturbed at room-temperature for several hours. Formaldehyde is indicated by the development of a colour ranging from pink to dark blue. A black discolouration is disregarded. Bonnet found that with 1 part of formaldehyde to 25,000 parts of sample a distinct colour appeared in 1 hour.

In testing ice-cream and similar articles it must be borne in mind that some of the flavouring materials being aldehyde in nature may simulate formaldehyde. LaWall has found that vanillin may act thus. The phenylhydrazine and Bonnet tests are least liable to fallacy in this respect.

Nitrites and Formaldehyde.—Mixtures of these substances are now sold under fanciful and misleading names for milk preservatives, as a nitrite prevents the reactions of formaldehyde with some of the tests.

Leffmann has found that the phenylhydrazine test will react promptly with formaldehyde in presence of notable amount of nitrite and also that the well-known test for nitrites (sulphanilic acid and α-naphthylamine) reacts in the presence of formaldehyde. The reactions are obtained in fresh samples and in those that have stood for 24 hours.

Estimation of Formaldehyde.—The trustworthy methods for estimating formaldehyde are fully set forth in Vol. 1. It is there pointed out that a choice of method will depend on whether strong or weak solutions are to be examined. In the case of milk the proportion is almost always quite small. Formaldehyde may be in great part removed from milk by distillation especially in a current of steam. B. H. Smith found that if 100 c.c. of the sample are distilled with 1 c.c. of dilute sulphuric acid (1:3), one-third of the formaldehyde present will come over with the first 20 c.c. Distillation of milk is troublesome owing to bumping, but Smith found that it could be safely conducted with a flat evaporating burner. It is advisable to put a few pieces of pumice into the flask.

Shrewsbury and Knapp (*Anal.*, 1909, **34**, 12) recommend the following method for estimation of formaldehyde. An oxidising reagent is prepared by mixing 0.1 grm. of pure nitric acid (sp. gr. 1.52) with 100 c.c. of strong hydrochloric acid are mixed. This mixture should be freshly made.

Five cubic centimetres of milk are treated with 10 c.c. of the reagent, the mixture well shaken and kept for 10 minutes in a water bath at 50°. The depth of colour is proportional to the amount of formaldehyde present and by means of milk containing known amounts of the preservative estimations may be made.

Hydrogen Peroxide.—Many tests have been devised for detection of this substance. Among the most convenient and satisfactory is the reaction with vanadic acid first given by Werther (*J. prakt. Chem.*, 1861, **83**, 195). It may be carried out by adding to 10 c.c. of the milk, 10 drops of a 1% solution of vanadic acid in dilute sulphuric acid. This solution may be conveniently made by dissolved commercial sodium orthovanadate in the dilute acid.

In the presence of hydrogen peroxide a distinct red will appear promptly. Barthel states that a proportion of 0.010 grm. of the peroxide in 100 c.c. of milk can be detected positively using only 10 c.c. of the sample.

Benzoates and Salicylates.—The following method (*Ann. chim. Anal.*, 1909, **14**, 53) covers both these preservatives.

Ten cubic centimetres of dilute sulphuric acid (5%) are added to 20 c.c. of 95% alcohol and into this 50 c.c. of the milk are poured in a fine stream with constant stirring. After a few moments, the mixture is filtered, the filtrate being returned until it passes clear. A sufficient volume of the filtrate is extracted in the usual manner with an equal volume of ether or similar solvent. The solvent is divided into two portions that are separately evaporated and tested for benzoic and salicylic acids respectively as given below.

Benzoic Acid.—This is detected by a modification of Mohler's method by Von der Heide and Jakob (*Zeitsch. Nahr. Genuss*, 1910, **19**, 137). The text is from *Cir.* 136, *Bur. Chem. U. S. Dept. Agric.*

The residue that is to be tested for benzoic acid is dissolved in a little water, the solution mixed with from 1 to 3 c.c. of $N/1$ sodium hydroxide and evaporated to dryness. To this residue is added from 5 to 10 c.c. of concentrated sulphuric acid and a small crystal of potassium nitrate and the mixture heated either for 10 minutes in a glycerol bath between 120° and 130° or for 20 minutes in boiling water. If heated in the glycerol bath the temperature must not be permitted to go over 130°. Metadinitrobenzoic acid is formed. After cooling 1 c.c. of water is added, the liquid made decidedly ammoniacal, boiled to break up ammonium nitrite, and some fresh colourless ammonium sulphide solution added so that the liquids do not mix. A brown ring at junction indicates benzoic acid. The liquids being mixed, the colour diffuses

and on heating changes to greenish-yellow. The last reaction distinguishes benzoic acid from salicylic and cinnamic acid as these latter form amino-derivatives which are not destroyed by heating. Phenolphthaleïn interferes with this process.

Salicylic Acid.—The other portion of the ether-extract may be evaporated and tested for salicylic acid in the usual manner with a ferric compound (see page 191).

Sodium Carbonate and Sodium Hydrogen Carbonate.—These substances are occasionally added to milk to prevent acidity due to decomposition. Barthel recommends a test devised by Hilger. 50 c.c. of the milk are diluted with 250 c.c. of water, the mixture is heated, precipitated with a small amount of alcohol and a convenient volume filtered. The filtrate is evaporated to half its bulk. The presence of an alkali-carbonate is easily ascertained by the usual tests.

Borates.—Jenkins' method (*Rep. State Expt. Sta.*, Conn., 1901, 106) is convenient and reasonably delicate. 10 c.c. of milk are mixed with 7 c.c. of hydrochloric acid, filtered, a strip of turmeric paper dipped in the filtrate, and then dried on a watch-glass on the water-bath. The paper becomes red in the presence of borates.

A simple test is to mix in a porcelain basin a drop or two of the milk, a drop of hydrochloric acid and a drop of alcoholic solution of turmeric and evaporate to dryness on the water-bath. The residue touched with ammonium hydroxide will show a distinct greenish stain in the presence of very small amounts of borates.

It is obvious that the delicacy of both these tests may be materially increased so that they will suffice for all purposes by concentrating the sample. As boric acid is volatile with steam it is best to render the sample slightly alkaline with sodium hydroxide before evaporating.

Estimation of Borates, Richmond and Miller (*Anal.* 1907. **32**, 144) have developed a process originally suggested by Jörgenson (*Zeitsch. ang. Chem.* 1897, **10**). A known amount of the sample is mixed with half its volume of 0.5% alcoholic solution of phenolphthaleïn, and sodium hydroxide solution added until the liquid is alkaline. It is brought to boiling, and while boiling titrated with standard acid until the red disappears, and then with $N/10$ sodium hydroxide until a faint red is again obtained. 30% by volume of glycerol is added and the titration with sodium hydroxide continued. After deducting the alkali that would be required for the glycerol (ascertained by a blank test); the number of cubic centimeters of alkali required in the last titration multiplied by 0.0062 gives the amount of orthoboric acid. (See page 194.)

Abrastol (Asaprol).—This is a calcium β-naphthol sulphonate that has marked antiseptic powers and has been used as a food preservative. The following test suggested by Leffmann (*Chem. Zeit.*, 1905, **29**, 1086) will detect very small amounts. 10 c.c. of the sample are mixed with 0.5 c.c. of the solution of mercuric nitrate described on page 158. In the presence of abrastol a distinct yellow tint is produced in a few minutes. Greater delicacy can be obtained by using the same proportion of the reagent with 10 c.c. of milk known to be pure. (See also under "Cream," page 192.)

Organic Pollutions.—It is now well recognized that the greatest danger in the use of milk of domestic animals as food for human beings arises from the contamination of it with living organisms and decaying organic matter. These dangerous materials may be derived from the animal, from persons engaged in collecting and distributing the milk and from the mere increase under favorable conditions of temperature of the microbes ordinarily present. A very large proportion of the summer mortality of infants is due to the last-mentioned condition. The efforts of sanitary authorities in all civilised countries are now directed toward safeguarding market-milk throughout the entire cycle of its collection and distribution. When collected and distributed in the careless manner still common in most places, milk may contain millions of microbes to the cubic centimetre. By strict precautions the number may be kept within a few thousands. The recent report of the Philadelphia Milk Commission (of which the writer of this article was a member) recommended that the highest grade of milk (Certified Milk) should not contain more than 10,000 microbes per c.c.

Sanitary control of market-milk also involves tests for animal products, such as pus cells, and the identification of specific microbes, such as those causing tuberculosis and typhoid fever. These investigations, however, are mostly outside of the scope of a work on chemical analysis, and for information thereon recourse must be had to works on pathology and bacteriology.

Several chemical tests have been published by which it is claimed that approximate estimation of these contaminating organisms and substances can be made but they are not capable of replacing the exact methods of the pathologic and bacteriologic laboratory. One of these is given in *Zeits. Unt. Nahr. Genuss.*, 1908, 15. A dilute solution of methylene blue is prepared by adding 5 c.c. of a saturated alcoholic solution of the colour to 200 c.c. of water. 0.5 c.c. of this solution is added to 10 c.c. of the sample. If the colour is discharged

promptly, the sample contains over 100,000,000 bacteria per cubic centimetre.

Bacteriologic Examination.—This is usually limited to a count of colonies obtained after culture in standard media. A method much used is described in *Amer. J. Pub. Hyg.*, 1907, **17**, 338. The following summary is from Williams' Bacteriology, 5th edition.

Dilutions of 1/10, 1/100, 1/1,000, 1/10,000, 1/100,000, 1/1,000,000 are recommended. The 1/10 dilution is prepared by shaking the sample 25 times and taking 1 c.c. and mixing with 9 c.c. of sterile water. The 1/100 dilution is prepared in the same way, adding 1 c.c. of the milk to 99 c.c. of sterile water. The other dilutions are prepared from the 1/1,000 dilution. The plate giving from 40 to 400 colonies should be selected for counting. All the colonies on the plate should be counted in preference to counting any part of a plate and calculating the total number. The colonies are counted after 24 hours culture at 37° in a moist atmosphere.

As a routine procedure, in cold weather, satisfactory results may be obtained by adding 1 c.c. of the sample after it is thoroughly mixed, to 9 c.c. of sterile water mixing and adding 1 c.c. of this solution to 9 c.c. of sterile water. Plates made from this dilution using 1/10 c.c. and 1 c.c. respectively, have been found to give closely corresponding results. Unless the milk is badly contaminated it is always possible to count the colonies readily. In warm weather and in the case of cream, a third or even a fourth degree of dilution should be made. If milk or cream is mixed with the medium in the tube, the colonies are apt to be more uniformly distributed on the plate than if the milk is put first into the Petri dish and the medium added. The number of bacteria remaining in the test-tube in the former method must be very few if the medium is quite fluid when poured. The objection to this method would appear to be purely theoretical, and counterbalanced by its advantages.

Preservation of Samples for Analysis. Calculation of Original Solids in Decomposed Samples.

For the preservation of milk samples for a day or two, refrigeration is the best method. Sterilisation in the ordinary steam steriliser used in preparing culture media, will enable milk to be kept for a considerable time, especially if in a flask closed with a cotton plug. Several preservatives have been proposed for keeping samples. Richmond found small amounts of hydrofluoric acid effective, but it has been but little used. Formaldehyde is very efficient; in large amount it in-

creases the total solids, interferes with the reactions of the proteins and simulates some reactions of the carbohydrates. A couple drops of commercial formalin to 25 c.c. will preserve a sample for several days.

In consequence of requirements of the British Food and Drugs Act, milk samples are liable to be referred to the government analysts long after having been taken, and as the conditions at the time of taking are usually such that no method of preservation can be employed, the government analysts have been obliged to devise methods that permit of calculation of the original solids. After much experience, the following process has been adopted. It is given in the form set forth by T. E. Thorpe (*Trans.*, 1905, **87**, 206).

Fat. Solids not Fat.—Sour milk samples are thoroughly mixed with a wire whisk. Portions of 10 grm. each are accurately weighed in platinum basins each containing a tared glass rod flattened at one end. These quantities are accurately neutralized with $N/10$ strontium hydroxide using phenolphthaleïn as indicator. The samples are evaporated on a water-bath until the residue is apparently dry, a very gentle heat being used toward the end of the process. The residue should have the consistency of dry cheese. 20 c.c. of anhydrous ether are poured on and the mixture carefully stirred with the rod. The ether is poured off through a dried tared filter. The residue in dish is macerated in the same way with 8 successive portions of ether. The non-fatty solids should then be like prepared chalk. Before becoming quite dry, the solids with the filter are transferred to a weighing bottle, and this with the platinum capsule is dried at 100° for 3 hours, weighed, dried further for 2 hours, weighed again, and then, as a check, dried for 1 hour. The last weight should not differ from the second by more than 0.001. For each c.c. of strontium solution used 0.00428 should be deducted; the remainder will be the solids not fat actually in the sample. The ether-solutions are mixed, evaporated in a small tared dish and weighed as usual.

Alcohol.—The decomposition of lactose in ordinary cases gives rise to a little alcohol and from the amount of the alcohol the lactose thus lost can be calculated. For estimation of alcohol, Thorpe recommends the following method:

A known weight (50, 75 or 100 grm.) according to circumstances are distilled, the distillate neutralised with $N/10$ sodium hydroxide, re-distilled, made up to the original or some convenient volume and the sp. gr. taken with a pycnometer. The percentage by weight being ascertained, this multiplied by 1.956 will give the lactose lost by conversion into alcohol.

The immersion refractometer could probably be used with advantage for the estimation of alcohol in this distillate.

Volatile Acids.—10 grm. placed in a platinum capsule are one-half neutralised (see below) using phenolphthaleïn, the mixture evaporated to dryness on the water-bath with frequent stirring, treated with about 20 c.c. of water so as to break up and detach the solids from the dish, and $N/10$ sodium hydroxide added until the mass is neutral. The difference between the original acidity and that of the evaporated portion is called acetic acid, of which 6 parts equal 6.2 of lactose.

The amount required to half neutralise the original portion is ascertained by titration of an equal portion to neutrality with phenolphthaleïn.

Butyric Acid.—The volatile acids are separated from the portion taken for the estimation of alcohol (see above), a known portion of the mixed aqueous solution of acids is neutralised with barium hydroxide and the mixture evaporated until the weight is constant. The percentage of barium in the mixed salt is ascertained and the proportion of the two acids are calculated (see page 517, Vol. 1); 88 parts of butyric acid equal 92 of lactose.

Ammonium Compounds.—2 grm. are made up to 100 c.c. and filtered through a carefully washed filter. 10 c.c. of this filtrate are made up to 50 c.c. with water and nesslerised in the usual manner, comparing with a solution of ammonium chloride of which 1 c.c. is equivalent to 0.00001 ammonia (NH_3). Of course, the water used in diluting must give no colour with Nessler's reagent.

Milks other than Cows' Milk.—The processes of analysis given in this article are in general applicable to all forms of milk using the term in the sense in which it is given at the beginning of the article. It must, however, be borne in mind that much remains to be done in the isolating and identifying the ingredients in less-used milks and that it is not unlikely that the protein and carbohydrate constituents are materially different in the milks of animals of widely different nature.

In analytic methods involving coagulation especially with a view of enclosing the fat and thus obtaining a clear filtrate for examining the carbohydrates, it must be noted that with milks low in proteins, such as human milk, the coagulum may be too scanty to accomplish the purpose and filtration through very close filters or clarification by the use of the centrifuge may be needed. It is possible that the addition of a small volume of fresh white of egg may aid the operation or the addition of a little china clay.

MILK PRODUCTS.

By CECIL REVIS, A. C. G. I. and E. RICHARDS BOLTON.

The products obtained by treating milk in various ways are only second in importance to milk itself. In the following section, the chemistry of the chief of these is considered, together with details of manufacture, etc., in so far they are of interest to the analyst. The following products are dealt with:

Section A.—Cream, clotted cream and skim milk.
Section B.—Butter milk. Whey and milk sugar.
Section C.—Condensed milk.
Section D.—Humanised, diabetic, peptonised and homogenised milks.
Section E.—Sour and fermented milks.
Section F.—Infants' foods.
Section G.—Dried milks.
Section H.—Cheese.

A. CREAM, CLOTTED CREAM, SKIM MILK.

Cream is the product consisting of an artificial agglomeration of the fat corpuscles of the original milk, brought about either by gravitation or mechanical (centrifugal) power. The colour varies from pure white to a deep yellow, according to the breed of cattle and time of year, the usual tint being a very faint yellow.

Cream obtained by gravitation is now practically an obsolete substance commercially and is only so produced where mechanical power is unobtainable, or for certain milk preparations (infants' foods).

The setting of milk in pans, either shallow or deep, never results in any complete separation of the butter fat. When milk is allowed to stand in cylindrical vessels, it is found that after a definite cream layer is formed, the milk below has at all points a practically constant composition at any moment. To illustrate this the following experiment was carried out by the writers in such a manner that samples of milk

could be drawn off at various levels in a cylindrical vessel after various periods.

The sampling tubes were placed so that samples from immediately below the cream layer and at three equal distances between it and the bottom, could be obtained. The following table gives the results:

Fat in original sample	Time	Three lower sampling tubes		
		A	B	C
3.70	2½ hours.	2.70	2.65	2.70
3.70	19 hours.	2.15	2.15	2.20

Another experiment gave similar results:

Fat in original milk	Time	Sampling tubes			
		A	B	C	D
3.40	4 hours.	2.70	2.70	2.75	2.75
3.40	6 hours.	2.70	2.45	2.45	2.55
3.40	24 hours.	.....	2.20	2.20	1.95

The similarity of the fat percentage at the different levels suggested that milks containing an augmented fat content could be easily obtained by gravitation, the process involving only two rough fat estimations. The following figures show this to be the case:

Fat in original sample (a)	Fat in lower layers (b)	Fat in enriched milk		Ratio $\frac{a}{b}$
		Found	Calculated	
3.67	2.45	7.1	7.0	1.50
3.62	2.37	6.25	6.0	1.53
3.55	2.20	8.15	8.0	1.61
3.80	2.57	6.65	6.5	1.47
3.60	2.50	7.40	7.0	1.44
3.55	2.47	7.05	7.0	1.44

Advantage of this has been taken by the writers in the preparation of modified milk for infant feeding.

The rate at which cream rises in milk is dependent on several different factors. This point has been carefully investigated by one of us (*The Dairy*, 1904, **16**, 296) in connection with the use of the creamometer as a test for the fat content of milk. The factors are principally (a) the temperature of setting, (b) the effect of mechanical manipulation; both of which tend to decrease the apparent volume of cream

thrown up. The addition of water has no effect on the volume of cream, no greater proportion being obtained by so doing. The following table exemplifies the effect of temperature.

Volume of cream at 13–18°	Volume of cream at 36°	Percentage reduction
9.3	4.9	47.3
17.3	11.6	32.9
10.9	5.7	47.7
6.2	4.0	35.5
5.9	3.6	39.0
9.0	6.2	31.1
8.9	5.6	37.1
6.0	5.0	16.6

The want of uniformity between the ratio (R) of percentage volume of cream to actual percentage of fat is very marked. The following figures were obtained in milk set for 4 hours at 25°:

Values of R	Mean	
1.78		
2.73		Farmers' milk on arrival at depôt after train journey.
2.22		
1.46		
1.42		
2.24		Mixed milks before delivery to customers.
2.17	2.23	
2.28		
1.67		Mixed milks on return from rounds.
1.92	1.86	
2.00		

It will be noted that R becomes much more uniform after mechanical treatment (cooling, cleaning, etc.).

Hunziker has compared the following methods of obtaining cream:

(1) By the hand separator.
(2) By deep setting.
(3) By shallow setting.
(4) By dilution with water.

He concludes that the loss in butter fat by the last 3 methods as compared with the first, is respectively 6, 9 and 10 times.

The plea of this natural rising of cream is often submitted as a defence of the deficiency of fat in samples of milk taken by inspectors for Public Health Control. Undoubtedly such deficiency is often so caused in milk allowed to stand in tall vessels on the counters of shops, where the milk is drawn off from time to time by means of a tap at the

bottom and for this reason such vessels should be fitted with a suitable stirrer, so that proper admixture may be made at intervals. In the case of milk carried in tall "churns" on ordinary delivery rounds, there is much less tendency, on account of their shape, to the separation of cream and there is little difficulty in delivering a uniform milk, even on rounds of 3 to 4 hours.

The ascent of the fat globules when milk is allowed to stand causes no alteration in the composition of the milk, other than the separation of the fat. It has often been erroneously stated that the fat globules carry up with them certain protein constituents, an opinion which has been held to support the view that these globules are surrounded by a membrane of protein. Careful experiments on the composition of milk and of the cream and skim milk after separation, show that the ratio of solids not fat to water is the same in each case.

The following exact experiments carried out by H. D. Richmond fully confirm this view:

	Milk	Skim milk	Cream
Sp. gr.	1.0308	1.0349	.....
Total solids	12.75	9.27	34.61
Fat	4.10	0.28	28.09
Ash	0.71	0.78	0.58
Non-fatty solids	8.65	8.99	6.52
Ratio of non-fatty solids to 100 of water.	9.91	9.91	9.97
Ratio of ash to 100 of water.	0.81	0.86	0.89

It is necessary to point out that there are several sources of error in connection with such experiments, which seem to have been overlooked by some investigators. These are: (a) the use of too small quantities of milk, (b) loss of water by evaporation during the passage through the separator, (c) "caking" in the pasteurisers, etc.

All cream offered for sale to-day is the product of mechanical separators, and of such machines there are now a very great variety on the market and the adoption of any type of machine is largely a question of the taste and convenience of the purchaser. The earlier "open bowl" type has now been almost entirely displaced by "disc" machines, as this latter type admits of a much more rapid and perfect separation than was possible with the former, though in the opinion of the writers the "open bowl" type threw a cream of smoother consistency and greater freedom from lumpiness. The greater capacity of the disc machine is easily understood from the following illustrations:

In the "open bowl" type, particles of cream or skim milk at the point A would have to travel half the radius of the bowl (in their

respective directions) under the effect of the rotation; but in the "disc" type the effective diameter is reduced to the distance between two discs. The fat globule on reaching the upper surface of the disc below then slides to the centre without encountering the resistance of skim milk passing in the other direction, while the corresponding particle of skim milk on reaching the under surface of the disc above, similarly slides down until it reaches the periphery of the separator.

All separators at present on the market are of the central-feed type,

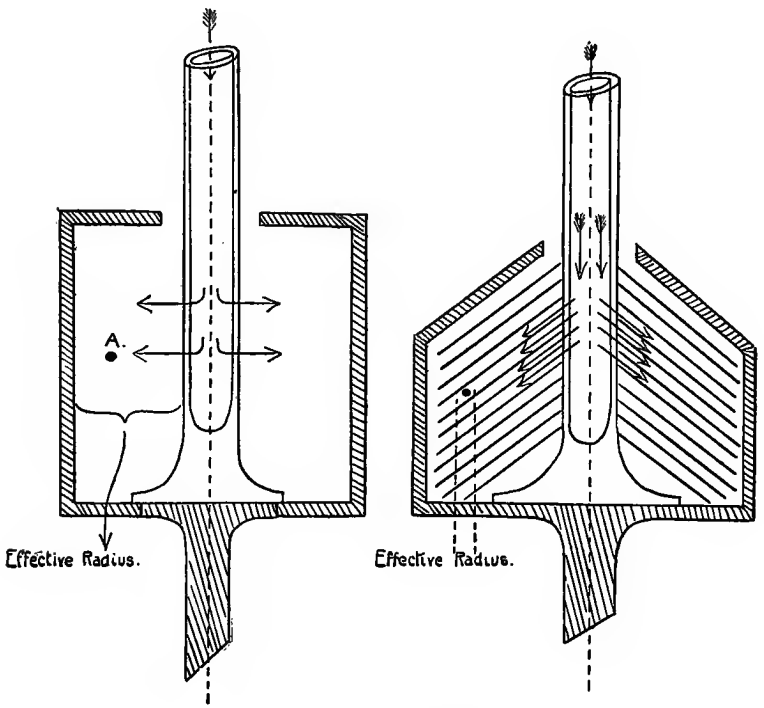


FIG. 5.—Cream separator.

but the ideal form would be a separator which fed at the periphery. The writers have carried out experiments with such a separator and have ascertained that the capacity is enormously increased thereby.

A by-product always obtained during the use of mechanical separators is a substance known as "separator slime." This substance has been credited with the most extraordinary compositions, but consists of quite harmless and natural ingredients. It contains any dirt and cellular elements present in the original milk, but the chief component

is casein, which, being present in a colloidal state, suffers a slight precipitation under high centrifugal speed. It has been recently shown by Friedenthal (*Ber.*, 1911, **44**, 904) that casein can be separated quantitatively from milk by a speed of 10,000 revolutions per minute.

The following analysis has been published by H. D. Richmond (*Analyst*, 1894, **19**, 86).

Total solids	33.76%
Fat	0.50%
Lactose	0.50%
Casein	22.00%
Ash	3.01%

Olson (*J. Biol. Chem.*, 1908–09, **5**, 261) finds a new protein in the slime after removal of casein and albumin with dilute acids and alkalis (see page 140). It is very rich in nitrogen (18.93%) and gives the biuret reaction and apparently has the properties of an enzyme.

The quantity of fat lost in the separator slime has been investigated by Gordan (*Milch Zentralblatt*, 1905, **1**, 599) with the following results:

Milk in litres	Weight of slime in grm.	Grm. of fat per 1,000 grm. of slime
2,823 unstrained	1,200	15.0
2,795 unstrained	1,185	15.0
2,794 unstrained	980	10.0
3,900 strained	715	10.0
3,627 strained	720	19.0
3,710 strained	740	12.0

Modern mechanical separators are so arranged that a cream of any desired fat percentage from 10 to nearly 70% can be obtained. There is absolutely no standard for the percentage of fat[1] which should be present in marketable cream, the question being entirely settled by the taste of the consumer and the custom of various countries. On the Continent of Europe, cream with low percentage of fat (20%) is usual, but in England, a percentage of from 40 to 50 is expected in good thick cream; while in America standard cream must not contain less than 18%, though there is an artificial distinction made between heavy and light creams.

The quality of cream is largely judged by the public by its thickness. This, however, is no true guide to the actual fat percentage. The consistency of cream is dependent on many factors, one of the most important of these being the temperature to which the milk has been exposed before and during separation, and as a preliminary pasteurisation is almost always resorted to in modern separating, it is by no means un-

[1] The regulations of the Local Govt. Board (England) 1912, state that "preserved" cream shall contain not less than 35% by weight of milk fat.

common to obtain cream, made by an unskilful and negligent operator, containing a large percentage of fat with an appearance sufficiently thin to suggest a much lower percentage, and for this there is little legitimate remedy beyond an excessive and immediate cooling of the cream as it leaves the separator.

Various practices which are in vogue for the artificial thickening of cream must be looked upon in the light of an adulteration if their effect is to produce the appearance of a higher fat content than that actually present. These will be dealt with under methods of analysis.

The writers have examined recently a sample of so-called "Jernut Cream," which closely resembles natural cream and gives the following figures: Total solids 38.01, fat 32.85, protein 2.57, lactose 2.04 and ash 0.55%. The fat on examination proved to be a mixture of 85% coconut oil and 15% butter fat. There was present also some substance, probably gelatin, for the purpose of keeping the emulsion.

It is an interesting fact that the first portion of any milking, usually termed "fore milk" is less rich in fat than the last portions called the "strippings." This is well seen in the following table of results obtained by the writers.

	Quantity of milk in cubic centimetres	Fat, %	Total solids, %
Experiment I.—Fore milk.....	170	3.95	13.64
	340	4.60	14.56
	284	5.60	15.20
Strippings.....	284	6.30	16.01
Experiment II.—Fore milk.....	260	1.80	11.12
	300	2.52	11.80
	300	3.20	12.32
Strippings.....	170	4.00	13.02
Experiment III.—Fore milk.....	150	0.80	10.24
	150	0.70	10.18
	170	0.70	10.16
Strippings.....	240	1.42	10.72
Experiment IV.—Fore milk.....	200	2.22	11.86
	400	2.60	12.24
	450	2.40	12.03
Strippings.....	460	4.37	13.76
Experiment V.—Fore milk.....	160	1.90	10.48
	430	3.00	12.12
	240	3.05	12.00
Strippings.....	130	4.45	13.16
Experiment VI.—Fore milk.....	330	0.68	10.44
	990	0.55	10.31
	415	0.60	10.33
Strippings.....	460	0.62	10.32

Clotted Cream.—This product is largely associated in England with Devonshire and Cornwall, though there is no difficulty in preparing the

substance anywhere provided milk fresh from the cow can be obtained. The mode of preparation is usually as follows: Whole milk, warm from the cow, is placed in pans about 15 in. in diameter and 6–7 in. in depth, with sloping sides; the milk is allowed to stand in these for 12–24 hours, and they are then placed in the scalding stove and brought to a temperature of 175–180° F. in not less than half an hour. They are then removed and allowed to cool naturally. The pans are then placed on a revolving table, and the cream removed with a "slice."

From the method of preparation it follows that the ratio of solids not fat to water is altered by the evaporation that takes place, the solids not fat being higher than in separated cream.

The results of Vieth and Richmond, for an aggregate number of 463 samples of clotted cream analysed during the series of years 1886 to 1893 inclusive, show the following range of composition:

	Water	Total solids	Fat	Solids not fat	Ash
	%	%	%	%	%
Maximum	45.57	74.84	68.59	11.70	1.17
Minimum	25.16	54.43	45.78	5.26	0.42
Average	34.56	65.44	58.05	7.39	0.58

The average figures found by Richmond for clotted cream produced in 1896 were: total solids, 67.64; fat, 59.16; solids not fat, 8.48; and ash, 0.68%.

The analytical methods for clotted cream are exactly similar to those employed for the analysis of cream (page 187). Search should be made for preservatives and thickening agents as these are now sometimes employed though quite unnecessary in properly prepared clotted cream.

Skim Milk.—Skim milk is the lower layer, comparatively poor in fat, which remains when the cream is removed by skimming or similar means. It may be regarded as essentially new milk deprived of the greater part of its fat.

Some analysts have attempted to draw a sharp distinction between skimmed milk and separated milk, on the ground that the latter product commonly contains a smaller proportion of residual fat than hand-skimmed milk. The distinction should be borne in mind, but the two products are merely varieties of the same article produced by different processes.

The composition of skim milk, as affected by the various processes for its production, is shown by the following results of Vieth (*Analyst*, 1884, **9**, 63).

No.	Sp. gr.	Total solids, %	Fat, %	Solids not fat, %	Remarks on system
1	1.0350	9.75	0.55	9.20	Shallow pans.
2	1.0355	9.90	0.54	9.36	
3	1.0340	10.10	1.00	9.10	
4	1.0355	10.43	0.98	9.45	Deep pans.
5	1.0335	9.68	1.05	8.63	
6	1.0345	9.70	0.60	9.10	
7	1.0355	9.81	0.43	9.38	
8	1.0350	10.26	0.88	9.38	
9	1.0365	9.96	0.46	9.50	Centrifugal system.
10	1.0350	9.28	0.34	8.94	
11	1.0370	9.94	0.34	9.60	
12	1.0370	9.80	0.35	9.45	

A much greater skimming effect is brought about with modern separators, and in good working not more than 0.08–0.10% of fat should be found. It may be observed here that in testing the efficiency of separators, a centrifugal method of estimating the fat is alone allowable, as these processes do not estimate the fat (the very fine globules) which the separator will not remove, as is the case with extraction methods, which latter give a false idea of the efficiency of the machine.

Analysis of Cream.—The analysis of cream is undertaken (1) for the detection of added substances, in which case exact methods are required, or (2) for control of separators for which simpler methods are employed. It is also examined for the presence of preservatives and thickening agents.

Fat.—*Rapid estimation for control.*

(1) *The Babcock Method.*—Approximately 10 grm. of well-mixed cream are placed in a special "bottle," a convenient balance being made for the purpose and usually employed. 5 to 6 c.c. of water are added, and then 17.5 c.c. of the regular sulphuric acid (sp. gr. 1.82–1.84) measured in and the test continued as under milk (page 151). The percentage of fat is calculated by the following formula:

$$\%\,\text{Fat} = \frac{\text{Reading} \times 18}{\text{weight of cream taken}}$$

(2) *The Gerber Process.*—This is similar to the above in certain ways, a different bottle being employed. The writers recommend the use of the ordinary Gerber-test bottle and not the special form made for the purpose. About 1 grm. of cream is weighed out in a small funnel fitted with a ground glass rod and suspended from the balance hook (see Fig. 6). The cream is washed into the bottle with 2 quantities of 5 c.c. of warm water, and the bottle being held in a *slanting* direction, the liquid is caused to fill the bulb by tapping, if necessary. The contents are cooled and 10 c.c. of sulphuric acid (sp. gr. 1.820–1.825) run

in and 1 c.c. of amyl alcohol. The bottle is shaken as usual and allowed to stand in water at 65–70° for 5 minutes. After rotation the volume of fat is read *without* warming the tube. The percentage of fat is calculated by the following formula:

$$\% \text{ Fat} = \frac{\text{Reading (cold)} \times \text{factor}}{\text{weight of cream used.}}$$

The usual factor given = 11.22, but the writers find that the factor is variable and dependent on the fat percentage. They have estimated the following factors:

55 % fat	10.81
45 % fat	11.23
20 % fat	11.41

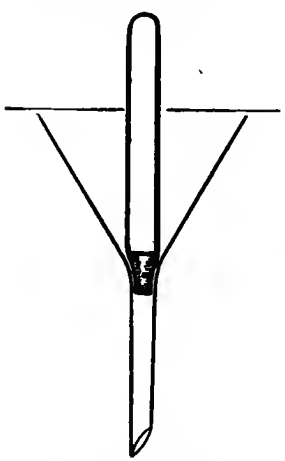


Fig. 6.—Cream funnel.

It is, however, advisable to determine a personal factor if many estimations are being made.

(3) *The Mats-Weibull Method.*—For the rapid control of cream the method of Mats-Weibull as modified by L. Fr. Rosengrew is both useful and satisfactory. The total solids are estimated and the fat is calculated from the formula,

$$f = 1.1t - 9.5$$

where f is the required percentage of fat and t is the total solids as estimated. The total solids may be estimated sufficiently exactly for this purpose by carefully boiling off the water in a porcelain dish over a free flame exactly as in the rapid estimation of water in butter by Patrick's method (Vol. 2, page 306). When carefully carried out, the results are quite concordant with those obtained by drying in a water-oven. This of course assumes that no water has been added to the cream. If water has been added, the amount can be calculated fairly exactly from the formula,

$$\% \text{ added water} = \frac{100f + 950 - 110t}{1.1t - f}$$

where f= the estimated percentage of fat
and t= the estimated total solids.

Fat.—*Exact estimation by the Gottlieb process.*

From 0.5 to 1.0 grm. ot the cream are weighed out direct into the apparatus shown in the sketch.

The total weight is made up to 5.2 grm. with water. To the mixture is added 0.5 c.c. of ammonia (sp. gr. 0.925), and the contents mixed *by rotation*, 5 c.c. strong alcohol (95% by volume) are added and the contents again mixed by rotation after which about 12.5 c.c. of methylated ether are added and the contents of the flask mixed *by inversion* 3 times after closing with a tight fitting india-rubber stopper; 12.5 c.c. of petroleum ether (redistilled and boiling below 40°) are then added and the contents mixed by inversion. The flask is allowed to stand for 15 minutes and the mixed ethers down to within 0.5 cm. of the lower layer, blown off into a tared flask with the wash bottle arrangement shown. This is then carefully removed and the under surface of the india-rubber stopper and the sides of the flask washed down with about 10 c.c. of mixed ethers (reserved from previous tests), the apparatus being rotated to mix up the ether. After a few minutes settling the ether is blown off as before into the flask and the washing repeated once more with 10 c.c. of mixed ethers. (It is unnecessary and even inadvisable to mix the contents of the apparatus with the washings *by inversion*.) The ether in the tared flask is distilled off and the fat dried at a temperature not exceeding 100° to constant weight, a state usually attained in half an hour.

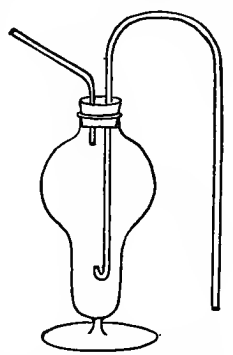


FIG. 7.—Röse-Gottlieb apparatus.

The following is the form in which this method is recommended by the A.O.A.C. (Bulletin 107):

One grm. of cream is placed in a glass cylinder about 2 cm. diameter and 36 cm. long (a 100 c.c. burette or eudiometer will do), 1 c.c. of concentrated ammonium hydroxide added and mixed. Then in succession are added 10 c.c. alcohol (92%), 25 c.c. washed ether, and 25 c.c. petroleum spirit (b. p. below 80°). The cylinder is closed with a moistened cork and shaken after each addition of the above liquids. It is allowed to stand 6 hours. The clear liquid is pipetted off into a tared flask by means of a siphon, with the end drawn to a fine point and lowered into the solution within 0.5 cm. of the bottom layer. After evaporating the solution in a hood the flask is dried in a steam oven for 2 or 3 hours and weighed. When the sample is high in fat a second treatment with 10 c.c. of ether and petroleum spirit is advisable.

Total Solids.—About 1–2 grm. are weighed out in a flat porcelain dish mixed with 1–2 c.c. of water and 1 c.c. of acetone and placed on a

rapidly boiling water-bath for 10–20 minutes, and afterwards in the water-oven for 2 hours.

Nitrogen.—This is best estimated by Kjeldahl's method in the usual way; but a preliminary extraction of the fat is necessary or severe charring results. To effect this, the water in the cream is rapidly removed, by placing the digestion flask in warm water and connecting to a vacuum pump till the water is removed. Ether is then added and poured off through a small filter paper, the treatment being repcated and the filter paper finally dropped back into the flask.

Lactose and ash are estimated as in Milk (page 158 and 148).

Preservatives.—Cream is examined for preservatives in the following manner: (See also under "Milk," page 171).

Boron Compounds.—About 20 c.c. or less of the cream are made slightly alkaline with sodium carbonate and boiled down rapidly in a platinum dish till deep brown, and the fat poured off. The residue is burnt till only carbon remains, acidified with hydrochloric acid and after insertion of turmeric paper, carried to dryness. In the presence of boron compounds the paper will take on shades of pink to rose red, varying with the quantity present, the colour being changed to indigo blue by a drop of strong ammonia.

Fluorides. (a) *In the Presence of Boron Compounds* (*Hehner's Method*).—50 grm. of cream are made just alkaline with sodium carbonate and rapidly boiled down in a platinum dish, the fat poured off and the residue charred well. The contents of the dish are taken up with hot water and 1 grm. of calcium chloride dissolved in a little water added and a moderate excess of sodium carbonate then run into the hot solution. The contents of the dish are filtered and the filter dried and burned completely in a platinum dish. The residue is taken up with acetic acid (1 in 3) and well boiled for 5 minutes, the dish being covered. The residue is filtered off and ignited, moistened with a few drops of conc. sulphuric acid, and the dish, covered with a waxed watch-glass having some marks scratched through the wax, is heated on a hot plate (80°) for half an hour, the watch-glass being filled with water or ice as necessary. After removal of the wax, the glass is examined for etching.

(b) *In Absence of Boron Compounds.*—The residue after burning off the fat, is burned nearly white and treated direct with the sulphuric acid as under (a). (See also Appendix.)

Benzoic and Salicylic Acids.—50 grm. of the cream are diluted in a flask with 100 c.c. of water and made alkaline with sodium carbonate. The flask is heated in boiling water for 5 minutes and 5 c.c. of

10% calcium chloride added to the hot liquid (the heating being continued till the protein and fat separate). The flask is cooled and the contents without mixing filtered into another flask. The filtrate is neutralised to litmus paper and treated with 10 c.c. of Fehling's copper sulphate solution and then with 10 c.c. of potassium hydroxide solution (31.15 grm. per 1,000 c.c.), mixed and filtered into a separating funnel. The clear solution in the funnel is acidified with dilute sulphuric acid and extracted with 20 c.c. of ether, adding a few drops of alcohol if necessary. The ether is separated and washed twice with a little water and then, after the addition of 5 c.c. of water and a drop of phenolphthaleïn, saturated solution of barium hydroxide is run in till, on shaking well, the pink colour is permanent. The aqueous layer is filtered off and concentrated to 2–3 c.c., filtered again if necessary into a small test-tube, sufficient very dilute acetic acid added to discharge the pink colour and then 1 drop of neutral ferric chloride solution. A violet colour indicates salicylic acid, while in the presence of benzoic acid, a flesh-coloured turbidity or precipitate forms, which does not dissolve on the addition of a further small quantity of ferric chloride solution.

Peroxides.—Solutions of hydrogen peroxide are used for preserving cream, but usually the amount added will have completely decomposed before the cream is examined. If present it may be detected by the addition of a 1% solution of titanic acid in 1:3 sulphuric acid. In the presence of peroxides a yellow colour is developed, which is best seen if the reagent be allowed to flow down the side of the test-tube containing the cream.

Formic Acid.—As preservatives containing formic acid mixed usually with glucose syrup are on the market, the examination for this substance is important. 100 grm. of cream are mixed with 100 c.c. of water and 20 c.c. of 20% phosphoric acid and steam distilled till 200 c.c. approximately have passed over, the end of the condenser being allowed to dip under the surface of a mixture of 20 c.c. of lime water (containing at least 1 gm. of lime in suspension) to which has been added 2 c.c. of 3% acetic acid free from formic acid. The distillate is evaporated to dryness and scraped into a small hard glass tube about 1/2 in. in diameter, closed at one end, the other being drawn out and passed into one limb of a miniature U-tube containing 1 or 2 c.c. of water, care being taken that none of the water can be sucked up into the hard glass tube; the contents of the tube are then heated strongly till nothing more distils. The water in the U-tube is then mixed with 2–3 c.c. of Schiff's reagent, when a violet colour will appear either at once or within

30 minutes if formates were present in the cream. A very slight colour is often given by pure cream but could not be mistaken for the colour produced even by 1 part in 1,000 of formic acid. Schiff's reagent is prepared by dissolving 0.5 grm. of rosaniline hydrochloride in about 50 c.c. of water. A mixture of 10 c.c. of saturated sodium hydrogen sulphite solution, and 5 c.c. hydrochloric acid (conc.) are then added and the whole made up to 500 c.c. This solution keeps well in the dark.

α- and β-Naphthol.—A few cubic centimetres of cream are diluted somewhat and cleared with calcium chloride as in the case of benzoic acid and the filtrate, after being made slightly alkaline with sodium hydrogen carbonate, mixed with a few drops of an emulsion of α-naphthionic acid, when in the case of α- or β-naphthol or Abrastol a fine crimson colour *immediately* develops (only an immediate colour is of any importance). (See page 175; also Vol. 3, page 311.)

The α-naphthionic acid is prepared as follows: About 0.2 grm. of 1–4 naphthylamine sulphonic acid are boiled with 10 c.c. of 50% alcohol, cooled in ice, 1 c.c. of 1 in 3 sulphuric acid added *gradually*, and then slowly 1 grm. of potassium nitrite dissolved in 10 c.c. of water (the suspension turns a yellowish colour—if pink, the nitrite is being added too fast). The mixture is allowed to stand for 2 or 3 minutes in the ice water, the precipitate filtered off, washed with 2 or 3 c.c. of water and then emulsified with about 5 c.c. of water, and used as above directed.

Formaldehyde and Hexamethylene Tetramine.—These are tested for by Hehner's method (see page 171). In the presence of small quantities of formalin a violet ring develops at the junction of the acid and the cream at once, and after a short interval in the case of hexamethylene tetramine. (See also Vol. 1, page 259.)

Mystin.—Quite recently a preservative under the name of "Mystin," and which seems to be a mixture or compound of formalin and a nitrite, has been offered to the Dairy Trade (see page 172).

It does not give the ordinary reactions for formalin. The method proposed by Monier-Williams in a report on this preservative to the Local Govt. Board (London) is perhaps most reliable and simple. 5 c.c. of the cream (or milk) are mixed with 5 c.c. of water and to it are added 0.5 c.c. of a 10% solution of urea, then 1 c.c. of N/H_2SO_4 and the mixture heated in boiling water for 2 minutes. The mixture is then cooled and the ordinary test for formaldehyde made with sulphuric acid (p. 171). If the preservative has been recently added—

whey obtained from the cream on acidification with sulphuric acid will give a blue colour with diphenylamine.*

Thickening agents. (1) **Saccharate of Lime.**—To 10 c.c. of the cream in a test-tube add 0.5 c.c. of strong hydrochloric acid and 0.1 grm. of resorcinol dissolved in 5 c.c. of water. The mixture after shaking is placed in boiling water for 5 minutes, when in the presence of saccharate of lime a pink to deep red colour gradually develops. Pure cream turns yellowish or brown. This test is strictly one for the presence of cane sugar, but any attempt to determine the presence of lime saccharate by an increase in the calcium content of the ash is likely to be erroneous (see also page 167).

(2) **Gelatin** (Stokes' Method).—10 c.c of cream are mixed with an equal quantity of Wiley's dilute acid mercuric nitrate solution which has been diluted with twice its volume of water, and the test finished as on page 166.

(3) **Agar-agar.**—About 50 grm. of cream are diluted with 100 c.c. of water, heated in boiling water, and cleared by the addition of 5 c.c. of 10% calcium chloride solution.

The mixture is filtered clear, for preference in a hot water funnel, cooled, and from one-half to two-thirds of its volume of strong alcohol added. The precipitate is filtered off and boiled with a small quantity of water till no more dissolves, filtered and evaporated down to 5 c.c. when the solution will gelatinise on cooling in the presence of agar-agar. An examination for diatoms is not satisfactory.

If a mixture of gelatin and agar-agar are suspected, they may be separated as follows:

The *boiling* dilution of the cream is cleared as before, and filtered *clear*, the filtrate precipitated with alcohol and a solution of the precipitate obtained as above. To a few cubic centimetres of the filtrate picric acid solution is added, when a turbidity or precipitate is produced if gelatin is present. In this case the remainder of the filtrate from the cream is evaporated to about 25 c.c. and a 10% solution of tannin added, until a precipitate ceases to form. To the mixture, which must be under 60° are added 5 to 10 c.c. of white of egg and the whole heated in boiling water for 30 minutes. The solution is filtered hot and concentrated on the water bath to a small bulk, when the presence of agar-agar is indicated by gelatinisation.

(4) **Casein, Dried and Condensed Milks.**—The addition of these substances can usually be detected by an unusually high solids not fat. Casein, however, may generally be found by diluting the cream with an equal volume of boiling water and strongly centrifugalising the mix-

* A solution of formaldehyde containing potassium chlorate is also sold.

ture, when a gummy mass of undissolved casein is usually obtained. Cream thus treated is in most cases frothy, on account of the agitation employed in incorporating the thickening material.

(5) **Pectoses.**—This class of substances may be detected in the following manner. 10 to 20 grm. of the sample are treated exactly as in the Gottlieb process for fat estimation, increasing the reagents in proper proportion. The process is best carried out in a graduated cylinder. The aqueous layer, which, if pectoses are present is usually characterised by containing flocculent particles, is filtered, or better centrifugalised, and the gummy deposit washed well with water, either on the filter or if a rotator be used, by shaking with water and repeated rotation. The deposit is then mixed with water to a volume of 5 c.c., one c.c. of conc. hydrochloric acid added and the mixture heated in boiling water for 45 minutes. The mixture is then cooled and neutralised with solid sodium carbonate, heated to boiling and poured into 5 c.c. of boiling Fehling's solution. If any appreciable amount of reduction takes place the presence of pectoses in the original sample may be taken as certain. It is essential that all lactose shall have been washed out of the deposit before the acid conversion.

(6) **Starch.**—This substance is easily detected in the usual manner by iodine solution, care being taken to add sufficient iodine to develop a distinct yellow colour before deciding on the absence of starch.

(7) **Homogenisation.**—This is a form of mechanical thickening sometimes resorted to especially in the case of sterilized creams; it is, however, neither permanent nor satisfactory. It can be detected by microscopical examination after well diluting the cream.

Quantitative Estimation of Preservatives.—The only preservatives that can be estimated with accuracy are boron compounds and salicylic acid.

Boron Compounds.—20 grm. of the *well mixed* cream are placed in a 100 c.c. flask and mixed with 2 c.c. of 10% sodium carbonate and diluted to the mark with water and 10% calcium chloride solution added in the following proportions,

For 55% fat	12.5 c.c.
For 50% fat	11.4 c.c.
For 40% fat	9.3 c.c.
For 30% fat	7.2 c.c.

these volumes compensating for the precipitated fat and protein. The mixture is warmed till precipitation has taken place, cooled and filtered. 20 c.c. of the filtrate are mixed with 10 c.c. of a 0.5% solution of phenolphthaleïn in 50% alcohol. The mixture is boiled and

titrated while boiling with $N/10$ sulphuric acid till distinctly acid, and then with $N/10$ sodium hydroxide till pink and the end point finally adjusted, while boiling, by the alternate use of the acid and alkali. To the solution are now added 2 grm. of mannitol (or glycerol to make one-third of the final volume) and the solution titrated with $N/10$ sodium hydroxide till pink.

If x = c.c. of alkali used for the final titration and y = c.c. required by the mannitol or glycerol then

$$(x-y)\times 0.0062\times 25 = \text{boric acid in } \%.$$

(The above is a modification of the method of Richmond and Miller, *Analyst*, 1907, **32**, 144.) (See page 174.)

Salicylic Acid.—The estimation of this preservative is difficult on account of the solubility of the acid in fat, and any method of clearing the cream in acid solution gives erroneous results. It may be estimated with accuracy by the method of Revis and Payne (*Analyst*, 1907, **32**, 286).

B. BUTTER MILK.

This term is applied to the liquid which runs away from the churn after butter formation has taken place. Its constituents are naturally those of the original sour milk or cream employed for the churning less the greater percentage of the fat. The amount of the latter varies somewhat with the skill of the operator and the perfection of the souring process, but 0.5% should not be exceeded in proper working.

Butter milk at the present day is only obtained from the churning of cream, as whole milk is scarcely ever used for the production of butter, and as the cream will in all probability have been "soured" it contains variable quantities of lactic acid and consequently has a distinct acid flavour. Sweet cream is but rarely churned as butter formation is in such cases incomplete and only obtained with difficulty. The composition of butter milk closely follows that of milk, except that a portion of the lactose has been converted into lactic acid and there may be a certain amount of protein degradation. On analysis butter milk often appears to be watered owing to the fact that the "butter grains" are washed in the churn with cold water which is usually run off into the butter milk. The legality of this operation must be left an open question, as the butter factory looks on butter milk as a waste product.

The writers are of the opinion that in cases where butter milk is sold ostensibly for dietetic or toilet purposes, it should be undiluted. The analytical methods are those of peptonised milk (see page 218).

Siegfeld and Keisten (*Molkereizeitung Hildesheim*, 1910, **24**, No. 48) draw attention to the fact that the Gottlieb process yields usually 0.15–0.25% more fat than the Gerber process and attribute it to a partial homogenisation of the fat during churning.

The following analyses of butter milk are due to Vieth (*Analyst*, 1884, **9**, 63):

No.	Total solids, %	Fat, %	Non-fatty solids, %	Ash, %
1	9.77	1.09	8.68	0.69
2	9.03	0.63	8.40	0.70
3	10.39	0.78	9.61	.....
4	8.02	0.65	7.37	1.29
5	9.64	2.51	7.13	0.64
6	8.13	0.82	7.31	0.64
7	10.14	0.92	9.22	0.73
8	8.91	0.50	8.41	0.71
9	8.98	0.49	8.49	1.32
10	10.70	0.54	10.16	0.82
11	9.80	0.76	9.04	0.73
12	9.72	0.80	8.92	0.73

WHEY.

This term is strictly applied to the liquid obtained by straining off the curd produced in milk by the action of rennet. A similar liquid is also obtained when the curd is precipitated by acids or natural souring but the chemical composition of this latter differs from that of a rennet whey.

Rennet, containing as it does both chymosin and pepsin, not only precipitates the curd as paracasein, but produces a partial though slight digestion and a certain proportion of a soluble caseose appears consequently in the whey. The amount of this latter will depend on the time allowed before removing the curd and also on the temperature at which the curd is kept.

In the case of acid precipitation, the casein alone is removed and no caseoses are found. Further, in rennet coagulation a considerable quantity of the calcium of the original milk is left bound with the curd, while in acid precipitation, the curd is practically free from mineral constituents.

The following is a collection of analyses of pure acid and rennet curds given by Burr (*Milch. Zentralblatt*, 1910, **6**, 383):

ACID CASEINS.

No.	Water, %	Total solids, %	Fat, %	Ash, %	N_2 in total solids, %	Calc. factor for nitrogen to casein
1	7.89	92.11	0.075	0.00	14.37	6.40
2	1.65	98.35	0.005	0.00	15.33	6.41
3	6.73	93.27	0.055	0.00	14.56	6.45
4	8.65	91.35	0.005	0.00	14.28	6.40
5	5.55	94.45	0.005	0.00	14.71	6.41
6	9.62	90.38	0.090	0.49	14.03	6.40

All were snow-white powders.

RENNET CASEINS (Paracaseins).

No.	Water, %	Total solids, %	Fat, %	Ash, %	N_2 in total solids, %	Calc. factor for N_2 into casein
1	0.61	99.39	0.13	6.85	14.53	6.35
2	0.60	99.40	0.55	7.35	14.31	6.39
3	10.85	89.15	0.25	7.75	12.77	6.35
4	2.97	97.03	0.08	8.55	13.91	6.35
5	7.30	92.70	0.12	6.90	13.42	6.38
6	0.70	99.30	0.10	5.00	14.83	6.38

All were white powders.

One hundred parts of rennet curd ash gave as a mean value—P_2O_5 60.64%, Ca 37.44%, Mg 0.088%. The ash in the paracaseins is naturally higher as some of the ash constituents of the milk are in chemical combination with it.

It must be pointed out that in whey made from very fresh milk and in which the curd is separated at once after coagulation, such as would be the case in the manufacture of sweet whey for dietetic purposes, the percentage of proteins and fat is generally higher than that of whey obtained in the manufacture of cheese.

The constituents of whey are: lactose, lactalbumin, caseoses, generally a small proportion of lactic acid and the bulk of the salts of the milk, and traces of fat. The analytical methods are those of peptonised milk (page 218). For a dissertation on sp. gr. and refraction of serum of milk obtained by precipitation of the curd with calcium chloride see Weigner (*Milch. Zentralblatt*, 1909, **5**, 473 *et seq.*).

Milk Sugar (Lactose).

Milk sugar is usually manufactured from the whey obtained in the process of cheese manufacture. The following details give the outline of the method usually employed: The whey is acidified with about 2 or 3% of lactic acid and heated to boiling, when the albumin is precipitated and is filtered off, and a clear filtrate containing the sugar and

milk salts is obtained. The clarified whey is concentrated in vacuum pans at about 60°, till the solids are nearly 60%, when the pans are emptied into crystallising vats cooled by water. After several hours' standing the thick mass is stirred and again left when a granular yellow pulp settles out. This is freed from the mother-liquor in a hydro-extractor and the liquors are heated to boiling, strained and again evaporated in the vacuum pan and allowed to crystallise, the first and second crops of crystals being mixed. The crude sugar or "sand" is refined by dissolving the mass in water till a liquor of 13–15° Baumé is obtained, mixed with bone-black and a small quantity (0.2%) of acetic acid and heated to 90°, a little magnesium sulphate added and the whole heated to boiling. The liquid is then passed through a filter-press, concentrated in the vacuum pan to 35° Baumé and run into crystallising tanks, the mother liquors being worked up again for fresh crops of crystals. The mass of crystals is freed from liquid in the hydro-extractor and dried by warm air. The yield is usually about 2.5% of the whey used. Sometimes the crystallisation is hastened by continuous stirring of the concentrated liquors, the sugar being then obtained as a powder.

Milk sugar is still, however, manufactured in certain places by boiling down the whey in open vessels to the crystallising point, a very impure "sand" being so obtained which has to be refined.

The chemistry of milk sugar is fully dealt with in Vol. 1.

Raw Milk Sugar.—The composition of raw milk sugar is distinctly variable. The following figures have been published by Burr and Berberich (*Milch. Zentralblatt*, 1911, **7**, 241).

RAW MILK SUGAR FROM ONE FACTORY.

	Water, %	Total solids, %	Lactose, %	Total ash, %	Protein, $N \times 6.3$, %	Fat, %	Acidity as lactic acid, %	Other constituents, %
Mean.....	1.07	98.93	92.50	1.66	2.07	0.52	0.40	1.81
Maximum	2.33	99.82	95.29	2.21	2.95	0.80	0.59	5.09
Minimum.	0.18	97.67	89.25	1.19	1.32	0.25	0.09	0.03

RAW SUGAR FROM VARIOUS SOURCES.

	Water, %	Lactose, %	Protein $N \times 6.3$, %	Fat, %	Total ash, %	Water soluble ash, %	Water insoluble ash, %	Acidity as lactic acid, %
Mean.....	2.34	89.60	1.96	0.19	2.70	0.65	2.06	0.44
Maximum..	14.07	95.95	3.22	0.42	5.44	1.42	4.02	2.14
Minimum..	0.25	78.45	0.62	0.08	1.23	0.06	1.08	0.07

The ash of raw milk sugar contains: calcium, magnesium, phosphoric and sulphuric acids, but of course will vary slightly with the method of manufacture. The following percentage composition is given by the above authors (mean of 15 analyses):

CaO	MgO	Fe_2O_3	P_2O_5	SO_4	Cl
30.5	4.5	0.55	3.72	6.10	0.99

Refined Milk Sugar.—For the control of refined milk sugar the following examinations are recommended by them:

First.—For the presence of cheaper sugars, such as cane, beet, invert and starch.

Second.—For the presence in the ash of weighty inorganic substances, such as chalk and gypsum.

Third.—For its solubility in hot water—a 1 : 1 solution of refined milk sugar should be clear and colourless—if turbid, impurities are present.

Fourth.—For its nitrogen content.

Fifth.—For free-acid or alkali.

Sixth.—For the presence of other soluble substances.

Burr and Berberich give the following analyses of refined milk sugar from one manufactory:

	Water	Total solids, %	Total ash, %	Fat, %	Nitrogenous substances, %	Solubility tests at 22 to 23° according to the German Pharm., IV (see below)	Solubility in hot water 1 in 1
Mean.....	0.07	99.93	0.08	Negligible	Negligible	0.0358	Clear and colourless.
Maximum	0.11	99.98	0.13	Negligible	Negligible	0.0380	Clear and colourless.
Minimum.	0.02	99.89	0.05	Negligible	Negligible	0.0330	Clear and colourless.

The mean of 15 ash-constituent estimations gave

CaO	MgO	Fe_2O_3	P_2O_5	SO_4	Cl
22.50%	0.08%	0.00%	3.59%	20.57%	0.00%

An examination of a number of samples from other factories gave very similar results, except that the nitrogen varied from traces to 0.06% and with some samples there was a slight opalescence of the

1 in 1 hot water solution and the aqueous solution of the sugar when mixed with ammonium sulphide varied in colour from colourless to dark brown. It must be understood that slight traces of iron and copper may appear in refined milk sugar derived from the piping and vacuum pans.

The presence of more than a trace of nitrogenous substances must be looked upon as deleterious, as milk sugar is used simply in solution for infants' feeding in cases of acute intestinal toxæmia, and organisms readily develop in solutions containing more than traces of protein, which would vitiate the effect of the treatment.

Pharmacopœial Tests.—The following are some of the tests which have been suggested from time to time for examining milk sugar for pharmaceutical purposes.

The edition of the British Pharmacopœia still in force gives the following tests for examining milk sugar:

It should be soluble in 7 parts of cold water and about 1 part of boiling water. It should not leave more than 0.25% of ash when incinerated with free access of air. 1 grm. dissolved in 10 c.c. of water should give a red colour with a solution of phenolphthaleïn after adding 3 drops of the volumetric solution of sodium hydroxide (this being defined as a solution of sodium hydroxide containing 39.76 grm. NaOH in 1,000 c.c.).

In the third report of the Committee of Reference in Pharmacy to the Pharmacopœia Committee of the General Medical Council, published May, 1911, the following is recommended in place of the above:

"After free access of air" read "5 grm. dissolved in water should not require for neutralisation more than 1.5 c.c. of $N/10$ volumetric solution of sodium hydroxide (*limit of lactic acid*). If 5 grm. be well shaken with 20 c.c. of alcohol (90%) and filtered off, the filtrate on evaporation should leave no residue (*absence of sucrose*)." The $N/10$ solution of sodium hydroxide is one-tenth the strength of the solution mentioned above.

The following details are from the French Codex 1908:

Sp. gr. 1.534. The commercial product does not lose water of crystallisation at 150°, but does at 170°, becoming coloured. Melts at 203.5° with decomposition. Soluble in 6 parts cold and 2.5 parts of boiling water. Insoluble in alcohol and in ether. Notes on birotation are given. Aqueous solution reduces hot Fehling's solution. Lactose dissolved in twice its weight of nitric acid and heated, oxidises, and the products of oxidation when mixed with water separate in a crystalline

powder. Lactose should be completely soluble in water, and leave no ash.

The last edition (1900) of the United States Pharmacopœia has the following:

Soluble in 4.79 parts of water at 25°, and in 1 part of boiling water. Water solution neutral to litmus, and dextrorotatory.

On adding to a hot saturated aqueous solution an equal volume of sodium hydroxide test solution and gently warming, the liquid will turn yellow and finally brownish-red and on further adding a few drops of copper sulphate solution will give a brick-red precipitate.

If 1 grm. powdered lactose is placed on 5 c.c. of sulphuric acid (1.84) in a flat dish covered with a watch-glass, the acid margin acquires a greenish or reddish, but no brownish-black, colour in half an hour.

A 1 in 10 solution with a few drops of hydrochloric acid should not respond to the time limit test for heavy metals.

One gram boiled for 5 minutes with 50 c.c. of water and cooled should not give any colour with 1 drop of iodine solution.

The German Pharmacopœia *II* states that if 0.2 grm. of the milk sugar be spread on 1 grm. of conc. sulphuric acid no colour or at least only a very slight red should appear—on no account should it go black (*v. infra*).

Similar tests are recorded in other Pharmacopœias. Another useful test is the Resorcinol test, which has been given under many forms. The following is one of the simplest methods of application: 1 grm. of milk sugar is dissolved in 10 c.c. of water and mixed with 0.1 grm. of resorcinol and 1 c.c. of conc. hydrochloric acid and the mixture boiled for 5 minutes, within which time no red colour of the mixture should appear. Pinoff has given the following modification: 0.05 grm. of the sugar are mixed with 5 c.c. of alcoholic sulphuric acid (750 c.c. 96% alcohol, 200 c.c. of conc. sulphuric acid)—together with 5 c.c. of alcohol—and 5 c.c. of an alcoholic solution of resorcinol and the whole warmed to 95 to 98°. Saccharose, raffinose, fructose and sorbose within 1 minute give a deep red colour, but 30 minutes heating is necessary to give a colour with lactose, dextrose and maltose. The writers are of the opinion that the resorcinol test is not always reliable.

Anselmino has suggested fermentation of a 10% solution with a small quantity of freshly washed beer yeast at 20 to 30° for 2 days, within which time no gas should develop, if sucrose or invert sugar is absent.

The Pharm. Germanica IV, 1st Jan., 1901, gives the following test for examining milk sugar: 15 grm. of powdered milk sugar are mixed with 50 c.c. of *spiritus dilutus*, and allowed to stand for 1/2 hour with repeated shaking. The liquid is then filtered and the filtrate should neither give any turbidity when mixed with an equal volume of absolute alcohol, nor should 10 c.c. when evaporated on the water-bath give a residue of more than 0.04 grm. The test to be carried out at 15°.

Spiritus dilutus is defined as having a sp. gr. of 0.892–0.896, and contains 68 to 69 parts of alcohol by volume or 60 to 61% by weight.

The 5th edition of the German Pharmacopœia returns to the sulphuric acid test for the presence of sucrose. The method there prescribed being as follows: 0.5 grm. of finely powdered milk sugar is mixed with 10 c.c. of conc. sulphuric acid (1.836 to 1.841) in a test-tube previously washed with the acid, when the mixture should only be slightly yellowish within 1 hour, and on no account brown.

It also provides that a hot aqueous 1:1 solution of the sugar should be clear and at the most slightly yellow. The saturated aqueous solution should scarcely affect litmus paper and should not show any colouration with hydrogen sulphide after the addition of ammonia. There should not be more than 0.25% of ash.

Burr and Berberich, working under the conditions of this latest sulphuric acid test, find the following times and colours for various percentages of sucrose.

Sucrose %	5 minutes	10 minutes	15 minutes
1	Slightly yellow.	.....	Dark yellow.
2	Yellow.	Dark yellow.	Brown.
3	Yellow.	Dark yellow.	Brown.
4	Yellow.	Dark yellow.	Deep brown.
5	Brownish-yellow.	Brownish-black.	Blackish-brown.

For the gravimetric estimation of lactose, see page 158.

By-products of Milk Sugar Manufacture.—During the clarification of the whey a considerable quantity of protein matter is obtained as a by-product, and is used either as a manure, or fodder, or for making into cheese, or for incorporating with bread. The composition varies considerably, but the following analyses of the by-product (Ziger) from cow's milk whey have been published respectively by Klenze-enging and Fleischmann.

	%	%
Water	68.47	69.02
Total solids	31.53	30.98
Fat	5.22	0.47
Protein	18.72	13.18
Lactose	3.97	12.39
Ash	3.62	4.94

The analysis of the ash of some pressed Ziger was as follows:

CaO	MgO	Fe_2O_3	P_2O_5	SO_4	Cl	Alkali
22.2%	3.6%	1.5%	25.52%	0.58%	17.77%	balance %

Milk Sugar Molasses.—The following mean analyses of the uncrystallisable residues are given by Burr and Berberich: water, 72.67; total solids, 27.33; total ash, 6.03; water soluble ash, 3.98; water insoluble ash, 2.05; fat (Gottlieb), 0.09; nitrogen, 0.63; acidity (as lactic acid), 1.47; reducing substances (calculated as lactose), 22.29.

Analysis of Milk Sugar.—Beyond the rough tests for other sugars described above, various estimations are sometimes required in judging the purity of milk sugar.

Moisture.—This is usually estimated by drying 5 grm. to constant weight in the water oven, preferably in a wide flat weighing bottle. As it must be borne in mind that commercial milk sugar is usually a mixture of monohydrate and anhydrous milk sugar, the writers prefer to estimate the actual moisture in a vacuum desiccator; the combined water is then estimated on the same sample by heating at 140° to constant weight.

Fat.—This may be estimated on 2.5 grm. by the Gottlieb method.

Nitrogen.—5 grm. are treated by the Kjeldahl-Gunning method, or if great accuracy is required it is preferable to dissolve a larger quantity in water, precipitate the protein with phosphotungstic acid and to estimate the nitrogen in the precipitate after filtration.

Acidity.—10 grm. dissolved in 50 c.c. of water are titrated with $N/10$ sodium hydroxide to phenolphthaleïn. Each cubic centimetre equals 0.009 grm. of lactic acid.

The estimation of acidity is important, as any excess may cause trouble when used for modified milks.

Sugar. (1) *By the Polarimeter.*—The writers prefer to make a 5% solution of the sample, adding 7 c.c. of Wiley's dilute acid mercuric nitrate solution before making up to the mark. A direct reading is made at once, and the remainder of the solution counterpoised, then heated for 7 minutes in boiling water, cooled, readjusted to the original weight and again polarized. The two readings should be practically

identical. This procedure allows of the estimation of any appreciable quantity of cane sugar which may be present (see condensed milk), while the production of turbidity or an actual precipitate gives an indication of protein present. 1° Ventzke=0.31213 grm. of anhydrous lactose dissolved in 100 Möhr c.c. (see page 158).

(2) *By Fehling's method* in the usual manner, and in the case of the volumetric method, the solution should be standardised against pure lactose.

Ash.—The ash is best estimated in the following manner: 10 grm. are incinerated at a low temperature until completely carbonised, the char then extracted with water, the filtrate evaporated and very gently ignited, the result being the *soluble* ash. The extracted char is ignited till completely incinerated, giving the *insoluble* ash. The sum of the two equals the *total* ash.

Analysis of the Ash.—It is often of importance to know the proportion of the constituents of the ash, which may be estimated by the well-known methods of mineral analysis. As this involves the incineration of very large quantities of milk sugar, the writers desire to draw attention to the value of the methods described by Thresh (*The examination of water and water supplies*, 1904, page 238) for the examination of the saline constituents of water, for this purpose.

Fifty grams of the sugar are ignited, and the char after extraction with not more than 40 c.c. of water, is burnt completely, and its ash dissolved in a few drops of hot dilute hydrochloric acid, and added to the solution of the soluble ash. The whole solution, which should not exceed 50 c.c., is heated to boiling and 2 c.c. of a saturated solution of ammonium oxalate added and then dilute ammonia till just alkaline to litmus; the solution is then made distinctly acid with acetic acid and the whole allowed to stand covered on the water-bath for about an hour. The calcium oxalate is then filtered off on a small hardened filter and the flask and precipitate washed free from chlorides—the washings should not exceed 50 c.c. The calcium oxalate is then washed off the filter back into the original flask, the filter being finally washed with 10 c.c. of boiling 10% sulphuric acid. The solution of the oxalate is then heated to 50° and titrated with permanganate solution (1 c.c. equals 0.0025 Ca).

The filtrate from the calcium oxalate is cooled and made up to 55 c.c. To 4 c.c. add 2 to 3 drops of a solution of ammonium phosphate (made by dissolving 10 grm. of ammonium phosphate in 100 c.c. of water and adding ammonia (0.88) until a permanent turbidity results; the solution is then filtered). If a heavy precipitate is produced on shaking

or scratching the sides of the test-tube, 25 c.c. or less of the solution may be used, but if only a slight turbidity is formed 50 c.c. will be required. The necessary amount is placed in a 50 c.c. Nessler tube, and made up to the mark if required. 1 c.c. of the ammoniacal solution of ammonium phosphate is added and the whole *vigorously* stirred for 2 minutes with a flat disc plunger of nearly the same diameter as the tube.

A graduated 50 c.c. Nessler glass is then placed in a good diffused light, on a disc of white cardboard thickly covered with black dots. The test solution is then slowly poured in until the dots can be no longer distinguished and the number of cubic centimetres required is noted, and the process repeated 2 or 3 times, the mean of the readings being then taken. A test is then carried out in an exactly similar manner with a solution of magnesium chloride containing 0.00025 of magnesium per cubic centimetre (made by dissolving 0.025 grm. of pure magnesium wire in dilute hydrochloric acid and diluting to 1,000 c.c.). A sufficient quantity of this is diluted to 50 c.c. and treated exactly as the test solution, in order to give a turbidity which will obscure the dots in practically the same amount as the test solution. By comparison, the amount of magnesium in the test solution is easily ascertained. The method is exceedingly accurate and more reliable for these small quantities than the usual gravimetric method.

Sulphates are quite easily estimated in a similar manner. About 20 to 25 grm. of the sample are ashed as above described, and dissolved in a small quantity of hydrochloric acid and made up to 100 c.c. To 50 c.c. of this solution in a Nessler tube are added 1 c.c. of dilute hydrochloric acid (1 in 3) and 0.5 c.c. of an acid solution of barium chloride (made by dissolving 10 grm. of barium chloride in 80 c.c. of water and adding 20 c.c. of hydrochloric acid) stirred vigorously and the turbidity point ascertained, as in the case of magnesium. This is then compared with that of a standard solution of sulphuric acid containing 0.0032 grm. of SO_4 (made by diluting 100 c.c. $N/10$ sulphuric acid to 150 c.c.). The test must be carried out rapidly owing to the tendency of barium sulphate to settle out. If the amount of sulphate is at all excessive the sulphate is more accurately estimated after the above approximate estimation, in the following way: the reserved 50 c.c. is boiled and a standard solution of barium chloride (containing 5.071 grm. pure cryst. barium chloride in 1,000 c.c. of water, of which solution 1 c.c. = 0.002 grm. of SO_4), added in slight excess of that required to precipitate the whole of the SO_4 as already estimated. The liquid is allowed to stand 10 minutes and a dilute solution of ammonia (free

from carbonate) added till the solution is faintly alkaline. It is again boiled and a standard solution of potassium chromate (containing 4.05 grm. per 1,000 c.c. which is equivalent to the barium chloride solution) added, 0.5 c.c. at a time, until the supernatant liquid is distinctly yellow. The whole is then filtered into a Nessler glass and made up to 50 c.c. and the colour matched by running the standard chromate solution into 50 c.c. of distilled water placed by the side. The excess of chromate over that required to precipitate the excess of barium chloride solution is thus found, and so the SO_4 in the ash obtained.

Phosphates can be estimated on another portion of the ash. The ash is dissolved in dilute nitric acid and evaporated to dryness (*in a porcelain dish*) heated to 120° for 15 minutes and dissolved in 20 c.c. of distilled water, containing 2% of nitric acid. 5 c.c. of ammonium molybdate solution are placed in a test-tube and heated to 60° and 2 to 3 c.c. of the test liquid added, and the whole allowed to stand for 15 minutes in a warm place—this preliminary test will show how much of the test solution must be used to obtain a good yellow colour without actual precipitation. The colour is then matched by that given by a standard solution of sodium phosphate, containing 0.0001 grm. of PO_4 per cubic centimetre.

The writers have the utmost confidence in the above methods and in cases in which estimation of the above constituents have to be made for control purposes, the saving of time over the ordinary gravimetric methods is enormous.

C. CONDENSED MILK

This form of milk product has of late years been brought to a great state of perfection and has in consequence acquired a considerable and well-deserved popularity. It appears on the market in two forms: (1) Sweetened condensed milk, whole and skim, and (2) unsweetened condensed whole milk. The chief difference between the two varieties is the presence of a considerable percentage of cane sugar in the former, the addition of which obviates the rigorous sterilisation to which the latter has to be subjected.

The sugar added has not probably in itself any preservative action, but the high density so produced causes plasmolysis of the organisms present and so prevents their growth. As the temperature of the vacuum pan has little sterilising effect, organisms would develop in the unsweetened variety, if rigorous sterilisation were not carried out. It is to the growth of organisms of a sporogeneous type that the bitter-

ness and protein degradation so characteristic of some of the earlier unsweetened milks must be attributed.

The process of treatment of the milk is practically the same in either case. The following is the procedure at one of the largest and best known makers of condensed milks: The fresh milk is brought each day to the factory by farmers in the vicinity and is run into large tanks after passing through strainers (centrifugal cleaners are often used). From the tanks the milk runs through a pasteuriser, both for the purpose of heating the milk sufficiently to dissolve the sugar when added and also to obviate all risk from pathogenic organisms.

The hot milk is run from the pasteurisers into tanks in which the fine cane sugar is mixed with the milk by means of stirrers. The sweetened milk is run direct into the vacuum pans, in which it is carried down to the desired concentration, the process taking about 3 hours. The condensed milk is run off into drums placed in cold water and fitted with a stirring device driven from beneath, the cooled milk being then filled into the tins.

In the case of unsweetened condensed milk the milk is run from the pasteurisers after suitable cooling to the vacuum pans. After condensation the warm milk is homogenised (see page 220), as this process by its effect on the cream imparts a stability to the product which enables it to stand storage and transit in a way otherwise impossible. The homogenised condensed milk is filled into tins which are then packed into large rotating sterilisers and subjected to a temperature of about 105°.

The strictest cleanliness has to be observed in every stage of the process and there are many technical details involved in the production of a high-class product especially in regard to the temperatures employed. In a high-class condensed milk of either variety, there is not the least need for the addition of any preservative, such as was the common practice in earlier days of milk condensation.

The degree of condensation of unsweetened condensed milk is such that 3 parts by volume of the original milk produces 1 part by volume of condensed product. In the case of sweetened condensed milk 1 part by weight is obtained from 3 parts by weight of original milk, about 1 1/4 lb. of cane sugar being added to each gallon of fresh milk.

A serious consequence arising from the addition of a large quantity of sugar to condensed milk is that the preparation becomes unsuitable for ordinary purposes unless mixed with such a proportion of water as to dilute it beyond the bulk of the milk before concentration.

Some labels bear a statement that "if mixed with 3 to 5 volumes of

water" the milk may be used as a substitute for cream. As a matter of fact, the article thus diluted will contain less fat than is present in ordinary uncondensed new milk, instead of 30% of fat which may be taken as the minimum proportion present in true cream. A highly reprehensible statement which is made on the labels of many brands of condensed milk is that "for infants' use the preparation should be diluted with from 6 to 14 parts of water." This direction if carried out to the extreme limit, which will naturally be the case, would yield a fluid containing only 3–4% of milk-solids, and in some cases less than 1% of fat. On the other hand, the writers must clearly point out that condensed milk is often (when intelligently used) of immense value in infant feeding, being found suitable even when the most scientific mixtures have failed. A similar reservation must be made in favour of machine-skimmed condensed milk, which while quite unsuitable for infant feeding, has a high nutritive value and may be used with advantage as a substitute for jam.

The writers agree with McGill that the concentration of condensed milk should be based on the percentage of non-fatty solids. They find the sp. gr. of sweetened condensed milk in the most important brands to be usually 1.32 *circa.*

The density is estimated in a 10% solution and from this the density of the original condensed milk is calculated from the following formula:

$$Sp.\ gr.\ cond. = \frac{1}{11 - 10\ \text{Sp. gr. diluted milk.}}$$

In the case of unsweetened condensed milk, the extent to which the concentration has been carried may be judged from the proportion of non-fatty solids. The percentage of ash affords an independent criterion, but this is liable to be vitiated if mineral preservatives have been added. Further, a deposition of certain salts is liable to occur during the evaporation of the milk, and this circumstance tends to diminish the proportion of ash in the finished product.

As the fat of cows' milk is usually in excess of the protein it will be at least as high as the protein in the condensed preparation, provided that none of it has been removed. There is considerable inducement to remove part of the milk fat prior to concentration, as a portion is liable to separate from very rich milk, and this difficulty has only been overcome of late years by homogenisation.

Besides preservatives, certain materials such as glucose syrup, wheat flour and starch are sometimes added in low-grade products in order to

create thickness and to prevent solidification of the condensed product. Saccharate of lime is also used for this purpose.

The following requirements were suggested by a Departmental Committee (England) in their Report of 1901:

(1) The fat must not be less than 10%, and must be true butter fat.

(2) The protein (estimated by multiplying the nitrogen by 6.39) must not exceed the figure obtained for the fat.

(3) The sample must be free from preservatives, starch, and all other foreign matters.

In the case of sweetened condensed milk the percentage of protein affords the most reliable method of calculating the concentration which has been effected.

The number of volumes of water required to reduce 1 volume of sweetened condensed milk to its original concentration may be found by the following equation:

$$W = \frac{C \times G}{8.8} - 1$$

Where:

W = required volumes of water.

C = milk solids not fat in condensed milk.

G = sp. gr. of the condensed milk (see above).

The solids not fat in normal milk being assumed to be 8.8% the percentage of solids not fat in a diluted condensed milk (D) therefore is given by the formula:

$$D = \frac{G \times C}{W + 1}$$

If for C, in the above formula the percentage of fat in the condensed milk be substituted, the proportion of fat in the diluted milk may be found. The foregoing equations give the dilutions necessary to bring the parts of milk solids not fat per 100 volumes of the diluted condensed milk to those contained in the original milk before concentration.

The following standards are in force in the United States for condensed milk products (1906):

Condensed or evaporated milk is milk from which a considerable portion of water has been evaporated and contains not less than 28% of milk solids of which not less than 27.5% is to be milk fat.

Sweetened condensed milk is milk from which a considerable portion of water has been evaporated and to which sucrose has been added

and contains not less than 28% of milk solids of which not less than 27.5% is milk fat.

Condensed skim milk is skim milk from which a considerable portion of water has been evaporated.

Canada prescribes a minimum of 26% of milk solids and 7.2% of fat for evaporated milk and 28% of milk solids and 7.7% of fat for sweetened condensed milk.

The following analyses of unsweetened condensed milk are by Hunziker and Spitzer (1909):

Brand	Total solids, %	Fat, %	Protein, %	Sugar, %	Ash, %
Goldmilk	29.25	9.42	8.44	9.75	1.54
Columbine	24.63	7.45	7.41	8.56	1.36
Every Day	26.20	8.07	7.54	9.10	1.47
Star	29.04	8.35	7.86	10.37	1.62
Morning Glory	31.08	10.48	8.26	10.47	1.67
Carnation	23.81	8.05	6.49	7.55	1.24
Beauty	23.38	8.47	8.39	9.94	1.56
Van Camp's	27.89	8.69	7.52	9.66	1.54
Wilson's	25.23	8.70	6.53	8.68	1.37
Monarch	26.70	8.09	6.77	10.35	1.44
Diadem	24.96	8.16	7.06	7.92	1.33
Reindeer	26.66	8.08	6.88	10.21	1.45
Dundee	27.04	8.73	7.21	9.36	1.48

One of these authors (1910) is of the opinion that unsweetened condensed milk should not contain less than 24% total solids and not less than 7.5% fat. Sweetened condensed milk should not contain less than 28% total milk solids and not less than 8.5% fat.

The following analyses were made (1907) at the request of the British Dairy Farmers' Association by F. J. Lloyd:

Nature	Machine skimmed	Machine skimmed	Machine skimmed	Machine skimmed	Machine skimmed
Prepared in	Holland	Holland	Limerick	Limerick	Holland
Price of tin	2d	2½d	3d	2½d	3d
Contents of tin	10 oz.	12 oz.	16 oz.	11¾oz.	18 oz.
Analysis—water, %	21.76	24.66	20.72	20.26	22.44
Fat, %	0.62	1.26	0.80	0.30	1.20
Casein, %	9.60	9.05	10.50	11.10	11.00
Sugar, etc., %	65.68	62.77	65.50	65.94	62.92
Ash, %	2.34	2.26	2.48	2.40	2.44
	100.00	100.00	100.00	100.00	100.00
Containing boric acid	0.28	0.43	0.34	0.25	0.25
Parts of milk contained in 1 of condensed milk.	3.3	3.2	3.5	3.4	3.5

All samples were tested for formalin, salicylic acid, starch and viscogen, none of which were present.

Nature	Full cream	Unskimmed swiss	Full cream	Machine skimmed	Full cream unsweetened
Prepared in	England	Switzerland	Switzerland	Holland	Norway
Contents of tin	15 oz.	15 oz.	14½ oz.	13 oz.	12 oz.
Price of tin	5½d.	5½d.	5½d.	2d.	4½d.
Analysis—water, %	18.70	13.04	16.52	26.14	67.72
Fat, %	9.94	11.13	8.20	1.02	10.13
Casein, %	10.95	8.37	9.90	8.43	10.86
Sugar, etc., %	58.21	65.46	62.54	62.41	9.55
Ash, %	2.20	2.00	2.84	2.00	1.74
	100.00	100.00	100.00	100.00	100.00
Containing boric acid	0.17	0.10	none	none	none

The following analyses made by the writers are typical of those commercially supplied to the English market:

	Whole milks			Machine skimmed	
	I %	II %	III %	IV %	V %
Fat	8.83	10.32	13.88	0.21	0.39
Protein (N×6.39)	9.19	9.72	9.72	10.15	10.10
Lactose	13.15	11.59	14.59	14.30	15.90
Ash	1.67	1.88	2.03	2.47	2.11
Sucrose	40.76	40.80	34.90	46.82	43.23
Water	26.40	25.69	24.88	26.05	28.27
Total milk solids	32.84	32.51	40.2	27.13	28.50
Remarks	Poor	Good	Very good		

The following unpublished analyses have been kindly supplied to us by Mr. T. Macara.

The analyses in the first three sections show the variations which may take place in the product of one maker:

Source	Sample	Fat, %	Lactose, %	Protein, %	Ash, %	Sucrose, %	Water, %	Total milk solids, %
Maker A Holland.	1	8.00	14.21	8.80	2.20	41.77	25.02	33.20
	2	7.52	13.30	8.87	2.06	43.00	25.25	31.75
	3	11.00	13.29	8.74	2.08	39.65	25.24	35.11
	4	12.58	13.20	9.00	1.90	38.00	25.32	36.68
Maker B Holland.	1	9.94	13.20	9.57	2.00	39.30	25.99	34.71
	2	8.94	14.24	9.02	2.00	41.20	24.60	34.20
	3	9.64	14.00	8.87	1.90	40.76	24.83	34.41
	4	9.30	14.40	8.93	1.93	41.95	23.49	34.56
	5	9.72	13.95	8.30	1.89	40.97	25.17	33.86
	6	10.50	14.20	8.04	1.90	40.20	25.16	34.64
Maker C Holland.	1	9.40	13.86	9.20	2.10	40.86	24.58	34.56
	2	9.56	13.00	8.68	2.00	40.20	26.56	32.24
	3	9.50	13.54	8.22	2.00	41.10	25.64	30.26
Various makes.	1	9.7	13.40	9.00	1.90	41.60	24.40	34.00
	2	8.7	13.30	9.20	1.80	42.30	24.70	33.00
	3	7.70	10.00	8.12	1.93	38.95	30.30	30.75
	4	8.66	11.00	7.41	1.65	43.50	27.78	28.72
	5	11.26	14.50	8.49	1.85	39.80	24.10	36.10
Machine skimmed. Various makes.	1	0.34	13.66	9.64	2.20	44.90	29.26	23.64
	2	0.70	14.94	9.57	2.34	44.27	28.18	27.55
	3	0.38	14.60	10.21	2.37	43.52	28.92	27.56
	4	0.34	13.75	8.31	2.04	41.96	33.60	24.44

None of these contained boron compounds.

All the above constituents were estimated practically as described in the text, except that the protein was precipitated with phosphotungstic acid, the filtered precipitate being treated by the Kjeldahl method, and the lactose was estimated by Fehling's method. Total solids in all cases were estimated to check the calculated water. Macara is of the opinion that the ash is quite unreliable for determining the concentration. Of the 4 milks under Maker "A" Holland, the first 2 were condemned by him as made from partially skimmed milk, whereupon milks giving the analytical figures of 3 and 4 were received from the same maker.

The following are the limits of variation found in a large number of analyses collected by Dr. Coutts for a report to the Local Government Board (England), 1911.

	Full cream				Machine skimmed	
	Sweetened		Unsweetened		Sweetened	
	Lowest	Highest	Lowest	Highest	Lowest	Highest
Total solids, %...	68.1	83.6	29.2	38.0	56.9	79.1
Fat, %.....	8.0	13.7	8.2	11.9	0.1	6.5[2]
Protein, %.....	7.3	11.4	8.0	10.0	7.6	12.3
Ash, %.....	1.6	3.4[1]	1.6	2.5	1.6	2.9
Lactose, %.....	11.6	17.6	11.1	16.0	10.9	17.0
Cane sugar, %....	36.1	44.6	.....	.....	30.4	52.6

The above report also contains much useful information on the subject of condensed milk.

Analysis of Condensed Milk.

The sample is well mixed, care being taken that no crystalline sugar is left, and 60–65 grm. are weighed into a large beaker, dissolved in boiling water and the whole washed into a 250 c.c. flask, cooled and made up to the mark. The solution is used for the following estimations.

Total Solids.—Dilute 10 c.c. of the above solution with water to 100 c.c. and measure out 25 c.c. of the dilution into a platinum or porcelain dish, add 1 to 2 c.c. of acetone and place on a water-bath which must be in a state of strong ebullition before the dish is placed on it. Evaporate to dryness, and place in the water-oven till the weight is practically constant (about 2 hours).

Ash.—The residue from the total solids is carefully burnt at a low

[1] Probably contains sodium hydrogen carbonate or boric acid.
[2] Only partially skimmed.

red heat in a muffle or over a burner, the incineration in the latter case being materially hastened by placing another platinum dish partially over the original dish. If special accuracy in the ash figure is required, a larger quantity of the sample should be taken.

Protein.—8 or 10 c.c. of the original dilution are placed in a digestion flask and the water rapidly boiled off and the nitrogen estimated by the Kjeldahl-Gunning method.

If only an approximate estimation of the protein is required, the aldehyde figure of Steinegger may be employed. For this purpose 20 c.c. of the dilution are titrated with $N/10$ sodium hydroxide to the neutral point using phenolphthaleïn; to the neutralised solution 5 c.c. of 40% formaldehyde solution (previously neutralised) are added and the titration continued till a second neutral point is obtained. The difference between these 2 titrations is expressed as c.c. of $N/1$ NaOH per 1,000 c.c. of milk, and is the aldehyde figure. The percentage of protein in the dilution employed is calculated from the following formula:

$$\text{Protein} = \text{Aldehyde figure} \times 0.225$$

The above method is useful for correcting for the volume of protein when estimating the amount of sugars. Richmond uses strontium hydroxide (see page 154).

Fat.—5 c.c. of the dilution are measured into the Gottlieb apparatus and treated exactly as described under Cream. A rough estimation of the fat may be obtained by the Gerber or Babcock process, exactly as in the case of milk. These processes are inclined to give erroneous results with sweetened condensed milk and to deal with these Leach has devised a special process (*J. Amer. Chem. Soc.*, 1900, **22**, 589), but the writers are of opinion that the extreme rapidity and accuracy of the Gottlieb process renders its use preferable.

Sugars.—For sweetened condensed milks as made now-a-days the polarimetric method is the most accurate and reliable, but in the case of unsweetened condensed milks, there appears to be some difference of opinion as to the reliability of optical methods, as the temperatures employed in the sterilisation of unsweetened condensed milks leads to slight modification of the specific rotation of the milk sugar; in such cases the method of Fehling is preferably employed.

The following polarimetric method is originally due to Harrison (*Analyst*, 1904, **29**, 248). For saccharimeters reading in the Ventzke scale, weigh out the normal weight and dilute with sufficient water to make fluid, and heat to boiling, cool and make up to 100 c.c.,

reduce the temperature to 10° and add 15 c.c. of Wiley's dilute acid mercuric nitrate solution.[1]

Shake well and after standing 5 to 10 minutes, filter through a dry fluted filter. The filtrate must be perfectly clear, and the temperature must not be allowed to rise before the reading is taken, as otherwise slight inversion of the sugar may take place.

A direct polarisation is taken at about 20°. Another portion of the filtrate is placed in a flask and counterpoised. The flask is then placed in boiling water for 8 minutes, rapidly cooled, the loss in weight rectified, the solution filtered if necessary and polarised, carefully noting the temperature. The sugars are calculated in the following way:

$$S = \frac{(D - I) \times 100}{142.68 - \frac{T}{2}}$$

Where
- S = Cane Sugar %.
- D = Direct reading.
- I = Invert reading.
- T = Temperature of invert reading.

$$\text{Lactose (anhydrous) } \% = (D - S) \times \frac{0.31213}{\text{Normal Wt.}}$$

As 15 c.c. of acid mercuric nitrate are usually more than necessary to compensate for the volume of the precipitated fat and protein, the readings must be multiplied by $\frac{115-c}{100}$ to give the true readings for the milk, where c = the correction for the volume of fat and protein. The value of c is obtained as follows: The weight of fat in grams in the normal weight of the condensed milk is multiplied by 1.11 and expressed as cubic centimetres, plus the weight of protein in grams in the normal weight multiplied by 0.85 and expressed as cubic centimetres.

If a polarimeter is not available or in the case of unsweetened condensed milk the original solution is cleared by the addition of acid mercuric nitrate as for the optical method, and 10 c.c. of the filtrate diluted to about 70 c.c. in a 100 c.c. flask with water, about 0.5 grm. of a soluble phosphate added, and the solution neutralised with sodium hydroxide to phenolphthaleïn, made up to the mark and filtered. In

[1] *Note.*—The method of making this solution as slightly modified by the writers is as follows: Pure mercuric oxide is dissolved in twice its weight of strong nitric acid (sp. gr. 1.42) and the resulting solution diluted to 5 times its volume with water; mercuric oxide being preferable to mercury, as its solution does not produce Millon's reaction in the sugar solution.

this solution the lactose is determined by Fehling's solution in the usual manner (see Vol. 1), and if cane sugar be present 5 c.c. of the solution (cleared and inverted as under the optical method) is freed from mercury in a similar manner and titrated against Fehling's solution; and from the 2 titrations the amounts of cane sugar and lactose respectively are calculated. The figures so obtained being corrected by the formula:

$$\frac{115-c}{100} \text{ as before.}$$

The examination of condensed milk for preservatives and the constituents of the ash may be carried out as in the case of milk.

D. HUMANISED MILK.

On account of the increasing demand for modified milks for the artificial feeding of children, there are at the present time a very great number of such modifications. They may be divided roughly into two classes: (1) sterilised, (2) unsterilised. The first class simply aims at a reduction in the percentage of casein, combined with an increase in the percentage of milk sugar and fat, to amounts approximating to what is called "average human milk," though what this last may be depends almost entirely on the opinion of the maker.

The process of sterilisation precludes any closer approximation in the nature of the proteins, but it may be well to point out that such sterilised preparations have proved perfectly satisfactory to those who have to undertake long voyages. To the second class belong those modified milks in which the closer approximation to the proteins of human milk is brought about by reducing the casein to a very low percentage and supplying the proteins of whey by the lactalbumin so characteristic of the natural product. Of such modifications a very large number of different prescriptions have been proposed from time to time; many of them effect a composition which appears to be exceedingly satisfactory, but as in some cases the prescriptions are based on erroneous ideas of the composition of the milk and cream employed, it often happens that the product is very different from that which it is intended to be. It is outside the regular province of the ordinary analyst to say that any particular modified milk is, or is not, fitted for the feeding of children, unless some ingredient be present which is glaringly contra-indicated, such as preservatives, etc.

An excellent example of this second type of milk prescription is afforded by the methods of the Walker Gordon Laboratories, where

fresh milk obtained under the strictest hygienic conditions is immediately separated so as to give a cream of known fat percentage, while at the same time whey is produced by the use of rennet. The ingredients, namely, cream, separated milk, whey, together with milk-sugar and water are then combined in varying proportions according to the prescription of the physician.

The following table due to Ladd shows how these mixtures are used (page 217):

A humanised milk exceedingly well-known, especially in Europe, is called *"Backhaus"* milk, in the preparation of which trypsin is combined with rennet. The milk is separated and the standardised mixture of rennet and trypsin allowed to act for 30 minutes at 40° when it is supposed that 1.25% of soluble albumin is present. The curd is filtered off and the whey recombined with the cream and dried casein and lactose added to give the requisite formula. The product is then sterilised.

The analyses of the milk approximate to the following: Fat, 3.1%, lactose 6.0%, casein 0.6%, albumin 1.0%, ash 0.4%.

The Gärtner "Fett Milch" is made simply by diluting milk and then adding cream. The composition is usually as follows: Fat 3.0%, lactose 1.9%, protein 1.4%.

It has also been proposed to add saccharate of lime in small proportions to diluted or modified milk. Sodium citrate is similarly employed in order to prevent the coagulation of the casein in the stomach.

Quite recently a soluble albumin called *Albulactin* has been brought forward to supply the deficiency of lactalbumin in ordinary modified milk. The substance in question is soluble in water and does not coagulate on boiling the solution. It is prepared from milk proteins.

Many milk preparations have been described of which the following are a selection:

Mansfeld (Ref. *Zeit. Unters. Nahr. Genussm*, 1911, **21**, 424) describes the following:

Galafer.—Water 34.22, ash 1.96, fat 3.50, casein 9.46, lactose 11.74, sucrose 39.42, iron oxide 0.19.

It is made by concentrating partially skimmed milk to half its volume with the addition of sugar and ferric-hydroxide.

Leciplasma.—Water 9.80, ash 9.45, fat 3.76, casein 29.06, lactose 42.98 nitrogen free substances 4.95, phosphoric acid 3.12, of which 0.34% is present as lecithin phosphoric acid. It is made by drying a milk poor in fat after addition of lecithin.

Lacto-milk-food.—Peptone and other digestion products of milk, casein 36.03, tyrosine 1.9, lecithin 0.3, fat 0.67, lactose 3.21, lactic

THE PRACTICAL COMPOSITION OF CERTAIN PERCENTAGE MIXTURES.

No.	20-ounce mixtures, percentage of				Ounces of cream				Ounces of fat-free milk, used with creams of				Ounces		Lactose measure	Lactose, % without dry lactose
	Fat	Lactose	Proteins	Alkalinity	10%	12%	16%	20%	10%	12%	16%	20%	Lime-water	Boiled water		
1	1.50	4.50	0.25	5	*	*	*	1 1/2	*	*	*	0	1	17 1/2	2	0.33
2	1.50	4.50	0.50	5	3	2 1/2	2	1 1/2	0	1/2	1	1 1/2	1	16	2	0.61
3	2.00	5.00	0.25	5	*	*	*	2	*	*	*	0	1	17	2 1/4	0.75
4	2.00	5.00	0.50	5	*	3 1/4	2 1/2	2	*	0	1/2	1	1	15 3/4	2 1/4	0.73
5	2.00	5.00	0.75	5	4	3 1/4	2 1/2	2	3/4	1 1/2	2 1/4	2 3/4	1	14 1/2	2	1.01
6	2.00	5.50	1.00	5	4	3 1/4	2 1/2	2	1 3/4	2 1/2	3 1/4	3 1/3	1	13 1/4	2 1/4	1.30
7	2.50	5.00	0.50	5	*	*	3 1/4	2 1/2	*	*	0	3/4	1	15 3/4	2 1/4	0.73
8	2.50	5.50	0.75	5	*	4 1/4	3 1/4	2 1/2	*	1/4	1 1/4	2	1	14 1/2	2 1/4	1.01
9	2.50	6.00	1.00	5	5	4 1/4	3 1/4	2 1/2	1	1 3/4	2 3/4	3 1/2	1	13 1/2	2 1/2	1.23
10	3.00	6.00	0.50	5	*	*	3 3/4	3	*	*	0	3/4	1	15 1/4	2 1/2	0.84
11	3.00	6.00	0.75	5	*	5	3 3/4	3	*	0	1 1/4	2	1	14	2 1/2	1.12
12	3.00	6.00	1.00	5	6	5	3 3/4	3	0	1	2 1/4	3	1	13	2 1/4	1.35
13	3.00	6.00	1.25	5	6	5	3 3/4	3	1 1/4	2 1/4	3 1/2	4 1/4	1	11 3/4	2 1/4	1.35
14	3.00	6.50	1.50	5	6	5	3 3/4	3	2 1/2	3 1/2	4 3/4	5 1/2	1	10 1/2	2 1/4	1.91
15	3.00	6.50	2.00	5	6	5	3 3/4	3	5 1/2	6 1/2	7 3/4	8 1/2	1	7 1/2	2	2.68
16	3.50	6.00	0.50	5	*	*	*	3 1/2	*	*	*	0	1	15 1/2	2 1/2	3.78
17	3.50	6.00	0.75	5	*	*	4 1/2	3 1/2	*	*	0	1	1	14 1/2	2 1/2	1.01
18	3.50	6.50	1.00	5	*	5 3/4	4 1/2	3 1/2	*	0	1 1/4	2 1/4	1	13 1/4	2 1/2	1.26
19	3.50	6.50	1.25	5	7	5 3/4	4 1/2	3 1/2	1/2	1 3/4	3	4	1	11 1/2	2 1/2	1.68
20	3.50	6.50	1.50	5	7	5 3/4	4 1/2	3 1/2	2	3 1/4	4 1/2	5 1/2	1	10	2 1/4	2.02
21	4.00	6.00	0.60	5	*	*	*	4	*	*	*	0	1	15	2 1/2	0.78
22	4.00	6.00	0.75	5	*	*	5	4	*	*	0	1	1	14	2 1/2	1.12
23	4.00	7.00	1.00	5	*	*	5	4	*	*	1	2	1	13	2 3/4	1.35
24	4.00	7.00	1.25	5	*	6 3/4	5	4	*	3/4	2 1/2	3 1/2	1	11 1/2	2 1/2	1.68
25	4.00	7.00	1.50	5	8	6 3/4	5	4	1	2 1/4	4	5	1	10	2 1/2	2.02
26	4.00	7.00	2.00	5	8	6 3/4	5	4	3 1/2	4 3/4	6 1/2	7 1/2	1	7 1/2	2 1/4	2.56
27	4.00	7.00	2.50	5	8	6 3/4	5	4	6 1/4	7 1/2	9 1/4	10 1/4	1	4 3/4	2	3.20
28	4.00	7.00	3.00	5	8	6 3/4	5	4	9 1/4	10 1/2	12 1/4	13 1/4	1	1 3/4	1 1/2	3.88
29	4.00	6.00	3.00	5	8	6 3/4	5	4	9 1/4	10 1/2	12 1/4	13 1/4	1	1 3/4	1	3.88
30	4.00	5.50	3.00	5	8	6 3/4	5	4	9 1/4	10 1/2	12 1/4	13 1/4	1	1 3/4	3/4	3.88

* Combination impossible with strength of cream indicated.

acid 0.75, caramel and nitrogen free extractives 13.66, soluble salts (containing 9.01 KH_2PO_4) 17.38, insoluble salts 5.82, water 20.27.

Milchlin.—Fat 0.30%, protein 4.61%, lactose 6.10%, ash 0.87%. Is said to be treated so that it tastes like whole milk.

Full-cream Lactogen.—Soluble in water at 60–70° and feebly alkaline to litmus. It gives the following figures: Water 5.65%, fat 23.42%, protein 25.48%, ash 6.46%, lactose 38.99%. The ash contained 20.32% sodium chloride and 36.17% phosphoric acid. 93% of the protein was digested by pepsin and hydrochloric acid.

Diabetic Milks.—These preparations have been put on the market in order to supply a fluid resembling milk but practically free from milk sugar. They are usually patented preparations; one of the best-known is prepared by precipitating the casein of skim milk with acid, washing and treating with alkali. This solution of the casein is recombined with cream and certain salts, such as sodium phosphate, etc., the resulting liquid usually being prepared of such a composition that the proportions of fat, protein, etc., are about twice those of normal milk. This liquid can be sterilised without alteration and needs only to be diluted with water for use.

Peptonised Milk.—This form of milk product is quite easily prepared by the action of tryptic ferments on milk. The milk is usually diluted about one-third with water and treated with the requisite quantities of sodium hydrogen carbonate and the pancreatic preparation, the milk being kept at 37° during the digestion. The treatment is naturally only very partial as, if the peptonisation be pushed too far, the milk is rendered distinctly bitter and unpalatable.

Analysis of Peptonised Milk.—*Qualitative test* for peptonisation: 10 c.c. of milk are mixed with 1 drop of pure acetic acid in a test-tube and heated for 5 minutes in boiling water. The curd is broken up and filtered and the filtrate neutralised to litmus with dilute sodium hydroxide. The liquid is again filtered and 0.1 c.c. of 1% copper sulphate added, and 5 c.c. of 10% sodium hydroxide; a rose-red colour indicates the presence of caseoses and peptones (Biuret reaction). The writers have found that in perfectly fresh milk a slight rose-red colour is always given, even though caseoses are absent. This seems to be due to the presence of traces of lactalbumin which in their experience gives a reddish colour with the biuret test, and not the usually bluish-violet of native albumins. Cobalt sulphate may be substituted for the copper sulphate and gives a yellowish colour in the presence of caseoses and peptones, but in the opinion of the writers the original reaction is more characteristic.

Test for the Nature of the Caseoses Present.—20 c.c. of the milk are cleared from casein and albumin as in the above test, and the neutralised filtrate treated with an acid solution of zinc sulphate (made by saturating with zinc sulphate a mixture of 100 parts water containing 2 parts of 20% sulphuric acid) as follows:

(a) To 8 c.c. of the neutralised filtrate add 2 c.c. of the reagent. A precipitate indicates *acid albumin* which is usually absent in these cases. Filter.

(b) To 6.25 c.c. of the filtrate from (a) add 3.75 c.c. of the reagent. A precipitate = *proto-* and *hetero-caseoses.* Filter.

(c) The filtrate from (b) is saturated with zinc sulphate. Precipitate = *Deutero-caseoses.* Filter.

(d) The filtrate is treated with phosphotungstic acid (4% solution). A precipitate = *peptones and nitrogenous bases.*

Quantitative Examination.—The estimations of total solids, fat, ash, etc., are carried out as in the case of milk (*q. v.*).

Estimation of Total Protein, Caseoses, Peptones, Etc.—It must be pointed out that Ritthausen's copper precipitation method for total protein is not applicable in the case of peptonised milk products.

Total protein may be estimated with sufficient accuracy as in the case of milk by the Kjeldahl-Gunning method using the factor 6.39, as in most peptonised milks, the digestion will not have proceeded beyond the peptone stage. If amino-acids are suspected, total nitrogen must be estimated and used in conjunction with the following estimations.

Casein.—20 grm. of the milk are diluted with 20 c.c. of water, raised to 40 to 50° and 5% solution of lactic acid (acetic acid may be used) added until the casein separates completely. The casein is filtered off and washed with water till free from acid. The filter paper and casein are dropped into a digestion flask and the nitrogen estimated by the Kjeldahl-Gunning method. The filtrate is tested with a few drops of lactic acid solution in order to see that precipitation is complete. In the filtrate:

Albumin is estimated by neutralisation with sodium hydroxide and heating in boiling water till coagulation is complete. The liquid is cooled and filtered and the precipitate washed with water, and the nitrogen estimated in it as before. The filtrate is concentrated if necessary and divided into 2 parts. One part is used for the estimation of:

Caseoses by saturation with zinc sulphate, the precipitate being filtered off, washed with saturated solution of zinc sulphate and the nitrogen estimated.

The other portion of the filtrate from the albumin should be diluted

to 100 c.c. and mixed with 1% of sodium chloride and a 12% solution of tannin added till precipitation is complete. The total volume is noted; the liquid is then filtered and the nitrogen estimated in 50 c.c. of the filtrate, the nitrogen so obtained being due to amino-acids. The method of applying the tannin-sodium chloride solution as used by Bigelow and Cook (*J. Amer. Chem. Soc.*, 1906, **28,** 1496) is more correct but the use of ice renders it less convenient.

Ammonia may be estimated if desired by distilling 25 c.c. of the filtrate from the tannin precipitation into $N/10$ acid.

In the above estimations if pure cellulose filters are used, no correction is necessary for them in the nitrogen estimations, but a blank estimation must be made of the same volume of tannin solution as is used in the actual test.

The sum of the various nitrogen estimations (including ammoniacal nitrogen) is subtracted from the total nitrogen, the difference being nitrogen due to peptones. In the case of casein, albumin, caseoses and peptones the factor 6.38 will give the protein content, but in the case of amino-acids the factor 3.19 is employed. The writers draw attention to the fact that though the above separations are theoretically correct, in practice a *complete* separation of lactalbumin is not possible by coagulation and a certain small proportion is estimated as albumose.

Homogenised Milk.—Recently there have appeared machines which are capable of so emulsifying the fat of milk *in situ* that it no longer rises as cream, but remains evenly distributed throughout the milk. The machines are of several types, the best known being the Gaulin, Hignette and Berberrich machines. The milk is forced under high pressure (about 2,500 to 4,000 lb. per square inch) by pumps either between closely approximated surfaces or through fine apertures placed opposite one another so that the streams of milk meet under high pressure. The result is much the same in either case.

The method is being extensively used in the preparation of sterilised milks, condensed milks, and sterilised creams. It has a limited use in ordinary milk supply, and would be more extensively used if the preliminary heating of the milk were not necessary.

By the use of the homogeniser, margarine fats may be introduced into skimmed milk and the resulting fluid has all the appearance and behaviour of ordinary milk. This is extensively employed for calf-feeding, and the writers have also examined such milk intended for human consumption.

It must be carefully remembered that the homogenisation of milk renders the centrifugal methods of estimating fat erroneous, unless

the rotation be repeated at least twice, heating the tubes between each rotation. The following comparative figures are given by Richmond (*Analyst*, 1906, **31**, 218).

Gottlieb	Werner Schmidt	Adams	Gerber
3.79	3.81	3.58	3.78
3.70	3.74	3.52	3.70
3.66	3.67	3.53	3.70
3.46	3.45	3.32	3.47
3.86	3.88	3.66	3.89
3.93	3.98	3.81	3.95

The Gottlieb and Werner Schmidt were not completely extracted, but an aliquot portion of the ether taken.

Homogenised milk may be recognised usually by its microscopical appearance, the fat globules being almost uniformly of very small size (about 0.5 to 1.0 μ diameter) though some larger globules are always present, and account for the very fine layer of cream that rises even on the best homogenised milk on long standing.

It is not easy to say that sterilised creams and dried milks have been homogenised as the heating process largely undoes the work of the homogeniser and produces aggregations of fat globules.

According to Buglia (*Zeits. Chem. u. Indus. d. Kolloide*, 1908, 2, 353) the electrical conductivity of homogenised milk is greater than that of normal milk, and its viscosity is also greater.

Sterilised Milk.—This product in itself calls for little comment. The colour of the milk is usually somewhat brownish if the sterilisation has been at all effective, though this is improved by a previous homogenisation of the milk. A sterilised milk should be practically sterile, or the remaining organisms should have been so attenuated by the heating as to be almost incapable of development. Many brands of sterilised milk show profound change after as short a sojourn as 72 hours in an incubator at 37°. Such milks must be looked upon with suspicion, as the organisms which develop in such cases are very often capable of producing toxic substances in the milk, and their growth is often only manifested by a slight bitterness.

It may be well to point out that "boiled" milk usually means milk raised to such a temperature that it froths up, and as a rule will then be much less than 100°. For this reason it preserves its white colour in contradistinction to sterilised milk (which has been maintained at 100° for a more or less prolonged period) in which a certain amount of caramelisation of the lactose has occurred. In fact, it may be taken as a general rule that "sterilised" milk which is white is not sterile and

for the reasons given above may prove dangerous. Sterilised milk has no longer life than ordinary milk when once the bottles have been opened. The acidity of milk falls slightly when heated, though at the same time the concentration of hydrions increases somewhat. $C_H \times 10^6$ is about 0.20 to 0.29, though this value returns again to close on the original figure if the milk be kept. The lowering of the acidity is not caused by action on the glass of the containing vessel. A plausible explanation is that the following reaction takes place:

$$\text{Casein Ca} + 2H_2O \rightleftarrows \text{Casein } H_2 + Ca(OH)_2$$

but Van Dam was unable to get experimental evidence in favour of this.

Analyses of the white sediment which always appears when sterilised milk is kept standing for long periods have been made by Siegfeld. The bottoms of the bottles were removed and the substance dried and washed well with ether. From 1000 c.c. 0.120 grm. were obtained and after ashing, this was reduced to 0.0235. This contained 42.4% CaO and 51.7% P_2O_5 (the last figure is probably slightly high). Another quantity gave CaO 51.7% and P_2O_5 37.2%. He concludes that it is $Ca_3(PO_4)_2$.

Buddeised Milk.—A process has been devised by Budde by which milk may be sterilised by the action of hydrogen peroxide. In its latest developments the milk is treated with the peroxide for 3 hours *in vacuo* at 50 to 55° and the excess of peroxide removed by the addition of a small quantity of catalase. By this process, milk may be freed from all pathogenic and non-sporing organisms, but spores are not destroyed and the milk has therefore only the life of ordinary pasteurised milk. When properly prepared it has the taste of normal milk and of course has undergone no destructive heating.

Specially Treated Milks.—From time to time various methods appear for the treatment of milk in order to improve its keeping properties. Many attempts have been made to achieve the desired result by exposing milk in thin films to the action of ultra-violet rays from quartz mercury vapour lamps, and though conflicting results are reported, the process does not seem very satisfactory. Experiments made by the writers, in an experimental apparatus with two lamps, only achieved a reduction of 88% of the organisms present, after two treatments. The milk does not appear to be altered by the treatment.

Treatment of milk with CO_2 has often been proposed, and produces certain enhanced keeping properties, but the chemical character of the milk is undoubtedly altered.

Toward the end of the year 1911, a method of reducing the lactic acid in milk was introduced by passing an electric current between special electrodes. Apart from the undesirability of obliterating lactic acid it seems that by the process the acidity of milk can be reduced below that of freshly drawn milk, from which fact it seems certain that chemical change in the milk must be brought about.

E. SOUR AND FERMENTED MILKS.

These important milk products, though in their inception of Eastern origin, from time to time create a vogue in other countries. While scarcely coming within the province of the analyst, it is certain that once and again he may be called upon to give an opinion concerning them, and for this reason a general description of their nomenclature, composition, modes of preparation and bacteriology is here given. This is rendered very much more easy to-day, on account of the careful and exact investigations which have been made recently in this subject, investigations which have successfully cleared up much that was hitherto but little understood.

Both fermented and sour milks are undoubtedly the result of natural causes in their own native home. In tropical climates it is quite impossible to keep milk fresh and sweet, and the combination of a high temperature and dirty handling would inevitably lead to a rapid and probably dangerous decomposition, had not nature provided a safeguard in the form of lactic acid organisms of considerable activity, by the growth of which all danger is avoided. The inclusion of yeast is probably quite as accidental in the beginning, but on account of the alcoholic content so produced in the milk commending itself to the native consumer, the activity of these yeasts has been sedulously cultivated, resulting in the well-known ferments used to-day for the production of fermented milks. The steady handing on of these "starters" for generations has undoubtedly produced by selection and environment a number of organisms which now form a class by themselves, though exhibiting considerable pleomorphism and biological eccentricities which have but recently been recognised.

Fermented milks are represented chiefly by Koumiss, Kephyr and Mazun, while soured milks are known as Yoghout (Bulgaria), Leben (Egypt), Gïoddu (Sardinia), and Dadhi (India).

The chief distinction between the two classes is the presence of of an active alcoholic fermentation of the milk sugar by yeasts present in the inoculating material, while the distinguishing characteristic of all classes of soured milks, fermented or otherwise, is the presence of

a long rod-shaped organism, known under many names such as *B. caucasicum*, *B. mazun* and more recently as *B. bulgaricus*.

This organism is distinguished by its exceptional power of converting lactose into lactic acid, its high optimum temperature of growth and its curious "root"-like colonies on suitable media. In properties it is closely allied to *B. acidophilus* and some other organisms which seem to be part of the natural flora of the intestines.

As isolated from the various kinds of fermented and soured milks, it exhibits certain differences of growth on media and in temperature limits and more particularly in the formation of granules in its protoplasm (whence the name "*Körnchen-bacillus*") and of producing a viscous state of the milk in which it grows. It is to this extreme pleomorphism that there must be attributed the varying names and species which have been allotted to it by different observers, but the writers, after a prolonged study of many varieties of this organism, are strongly of the opinion that *one* species and one only is really being dealt with. This view has been stated by Makrinoff (*Cent. f. Bakt.*, Abt. ii, 1910, **26**, 374) who proposes for the designation of the organism the term *B. lactic acidi leichmanni viscosus* and *non-viscosus* according as milk is or is not rendered slimy by it. The writers would, however, go further and delete this last appellation as there is no doubt from their own experiments that the production of slimy milk is variable. It is to this organism that Metchnikoff has of recent years drawn so much attention, on account of its powerful acid production and its undoubted ability to flourish in the intestine. Whether it be the organism itself or whether it be the lactic acid produced by it, and introduced usually at the same time with the organism, which is the effective agent, must be yet left an undecided question. The acid produced by it is stated by various observers to be inactive, dextro- and lævo-lactic acid; but there is little doubt that the predominant acid is *d*-lactic acid. Statements with regard to the nature of the acid must be always accepted with reserve on account of the extreme likelihood of conversion during isolation. For a discussion of this point see White and Avery (*Cent. f. Bakt.* Abt. ii, 1909, **25**, 161). The bacteriology of this organism will be found fully dealt with by the following authors:

Kuntze (*Cent. f. Bakt.*, Abt. ii, 1908, **21**, 737).

Luerssen and Kühn (*ibid.*, **20**, 234), also

Makrinoff (see above).

Together with this organism there is present in all native soured and fermented milk, the ordinary lactic acid organism known in

Europe generally as *Bacillus* or *Streptococcus Güntheri*, but more properly called, as by Heinemann, *Streptococcus lacticus*. This organism to which the ordinary coagulation of milk is usually due, has also on account of its great pleomorphism received a host of various names and shows a similar variation to *B. bulgaricus* in its temperature optima. It is probably of fæcal origin and is also closely allied to, if not identical with, *Streptococcus pyogenes*. It is very probable that it is an accidental contamination, but finding in milk its most suitable environment it is always present as a predominant organism and may be looked on as quite characteristic of these milk products.

The distinguishing organisms of the fermented milks then are, a yeast, *B. bulgaricus* and *Streptococcus lacticus*, the last two being also the necessary feature of soured milks. From the methods of production it follows that there are always present with these a very great variety of other organisms such as *B. acidi lactici* Hueppe, *Oidium lactis*, *Sarcina lutea*, etc., but these can only be looked upon in the light of accidental impurities and are of no value to the product.

The following brief accounts of the preparation of these products may be of interest, but for full descriptions, reference should be made to the interesting details given in a book recently published called "*The Bacillus of Long Life*" by Loudon M. Douglas.

Koumiss.—This is a fermented milk characteristic of the Steppes of Russia and Central Asia and is made from mare's milk. The milk is warmed, usually by the addition of warm water and some old koumiss is added as a starter, the heat being retained by carefully covering up the vessel. After standing 24 hours it is violently agitated, a process repeated once again after 24 hours.

In case old koumiss is not available as a starter, this is made from ground millet which is boiled with water to a thick paste. This is added to some boiled milk and kept at about 37–38° for 24 hours till active fermentation is in progress. More milk is then added, with constant stirring for 12 hours. The resulting fluid is a weak koumiss which may be used for making a fresh batch.

The use of such a starter will undoubtedly lead to butyric acid formation if care be not exercised, and for this reason mare's milk is preferable to cow's milk on account of its greater lactose content and smaller fat percentage. Undoubtedly casein degradation does take place and it is only the thorough aëration brought about by the beating that keeps the butyric acid change in check, while at the same time lactic acid development is favoured and the acid then protects the milk from undesirable changes.

The bacteriology of koumiss has been very carefully studied by Rubinsky (*Cent. f. Bakt.*, Abt. ii, **28**, 163). The necessary organisms are: (1) The koumiss yeast and (2) the koumiss bacterium. The yeast is of an exceptional nature. It ferments lactose readily forming lactic acid to the extent of 0.36% and the milk is coagulated and at the same time it creates a peptonising action on the casein and albumin. Free fatty acids also appear as part of the activity of this organism and consequently esters arise which give the peculiar odour so characteristic of koumiss.

The koumiss bacterium according to Rubinsky is not the same as *B. caucasicum*, as its optimum temperature lies about 32–36°, but from a general consideration of the facts, the writers are of opinion that this distinction cannot be upheld. The maximum acidity produced by it is 1.1% calculated as lactic acid.

These two organisms in pure culture will produce a normal koumiss with either mare's or camel's milk.

The product is usually styled "Weak" koumiss for 12 hours after the second beating up, after a further 12 hours' standing it is termed "medium" koumiss and finally "full" koumiss. These distinctions simply mark certain stages in the progress of the alcoholic fermentation and acid production.

Kephyr.—This fermented milk is characteristic of the Caucasus districts. It is notably distinguished from koumiss in that it may be made well from cow's milk, and that the temperature of preparation is markedly lower, viz.: about 11–15.5° whereas for koumiss a higher temperature is employed. The fermentation is started by adding to the milk the so-called "Kephyr grains" which have been well soaked in water and then several times in milk before use (each soaking taking about 2–3 hours). The milk to be fermented is boiled just before use and cooled and the grains added, a leathern bottle usually being employed as a receptacle and this is well shaken every 2 hours, the milk being ready in 24–48 hours. The casein should then be in a fine flocculent condition and not lumpy and show no tendency to conglomerate. A gradual degradation of the casein takes place on keeping. The "Kephyr grains" themselves are when dry, hard brownish granular masses. These are made up of a yeast generally confined to the rind and a mass of bacteria in the centre. With regard to the nature of the bacteria present and their necessity in the preparation of Kephyr there is much dispute. Nikolaiewa claims that only the yeast and an organism closely allied to *B. caucasicum* are essential. *Streptococcus lacticus* is always present but is

looked on as accidental. It may be remarked at this point that the streptococcus can survive the drying in the grains for a very long period. The most careful work, however, is that of Küntze (*Cent. f. Bakt.*, Abt., ii, 1909, 24, 101). He finds present, (1) *streptococcus lacticus*; (2) *B. acidi lactici* Hueppe and allied organisms; (3) yeasts; (4) butyric acid bacteria. Of these, the last seems to be essential. The yeast may be any ordinary variety as the lactose is attacked in presence of the lactic acid bacteria. The fermentation is a distinctly symbiotic one, the butyric acid bacteria start well at first, but are held in check by the development of the yeasts and the lactic acid bacteria, which finally are themselves checked by the yeasts leaving these predominant for a short time, though in the end the butyric acid organisms reassert themselves. Küntze describes a particular butyric acid organism which he terms *B. esterificans* or *B. Kephyr* and states that typical "grains" are formed by this organism. It is to this organism that the protein degradation must be attributed.

Mazun.—This fermented milk is characteristic of Armenia. In many ways it resembles the last, but has its own characteristic aroma. It is made from milk which has been boiled and cooled and inoculated with some old Mazun rubbed up with milk. A shallow dish is employed to hold the milk which is thoroughly stirred and closely covered with a thick cloth. Well-made Mazun shows no separation of whey. It is often used as a "starter" for making butter and the curd obtained from the butter milk after pressing is mixed with flour and dried in the sun and then often used as a "starter" for fresh Mazun. Three different organisms seem to be necessary: (1) yeasts, (2) long rod-shaped lactic acid bacteria and (3) *streptococcus lacticus*.

The yeasts like those of koumiss give rise to esters which give the characteristic odour, but protein degradation is slight as the lactic acid production is so powerful as to render such a change almost impossible.

The long rod-shaped lactic acid bacteria seem to belong to the *B. bulgaricus* group and have an optimum temperature of 37°.

For the general bacteriology see Düggeli (*Cent. f. Bakt.*, Abt. ii, 1905, 15, 577).

Yoghout.—This product may be taken as characteristic of the non-fermented soured milks. It must be borne in mind, however, that yeasts are always present in these milks but the alcoholic fermentation is never pronounced. The characteristic organism is the *B. bulgaricus*, though *Strept. lacticus* is always present and in pure cultivation products seems to produce a more finely flavoured result. As made in their native countries, the milks are always practically a solid curd, which,

however, is easily broken up on shaking. The milk used is boiled down to half its volume and being placed in wooden bowls is inoculated usually by rubbing the bowl with some old "soured milk." The bowl is then wrapped in cloths to maintain the necessary temperature (37–42°) till the milk has set to a firm curd. The starter used in Bulgaria is termed "*Maya.*" The bacterial flora of these products is very mixed, but there is little doubt that the necessary organisms are the *B. bulgaricus* and *streptococcus lacticus.*

The work of Metchnikoff has resulted in a very large number of more or less pure cultures of these organisms being placed on the market either as liquid milk cultures or in tablets of compressed dried milk or milk sugar for the home preparation of "soured" milk. It can scarcely be said that the result is altogether satisfactory except in skilled hands. The tablets are often quite useless, as the life of the *B. bulgaricus* in them is short and only the *Streptococcus* survives. The liquid cultures are much more reliable, but in them also the life of the *B. bulgaricus* is usually not more than 4 weeks. The chief danger however lies in the inefficient sterilisation of the milk used, which cannot be considered safe unless boiled for at least three-quarters of an hour before use, otherwise spore-bearing organisms present in the milk develop readily, and outgrowing the organisms introduced in the culture, produce a result which may be very prejudicial to the consumer.

The great difference between the milks so produced and the native article is that in the former the milk is not concentrated as a rule and for convenience of transport, the curd is often beaten into a cream.

The *B. bulgaricus* seems to attack the milk sugar only, and the statement that butyric acid is formed by it from the butterfat is in the opinion of the writers erroneous.

Further, it is necessary to clearly realise that such products as koumiss and soured milks prepared on the home market seldom resemble very closely the native-made article. In soured milks practically the only change is that of lactose to lactic acid and protein degradation is practically nil. Koumiss is also often made by the addition of glucose to separated cow's milk in order that alcoholic fermentation may be brought about by ordinary distiller's yeast. Such an article probably fulfils all necessary requirements and cannot be looked on as an adulterated product.

The following photographs[1] give some typical forms of *B. bulgaricus* and *Streptococcus lacticus.*

[1] The writers are indebted to the kindness of Dr. Ralph Vincent, Director of the Bacteriological Laboratory of the Infants Hospital, Vincent Sq., London, for these photographs which have been specially made from cultures in the writers' possession.

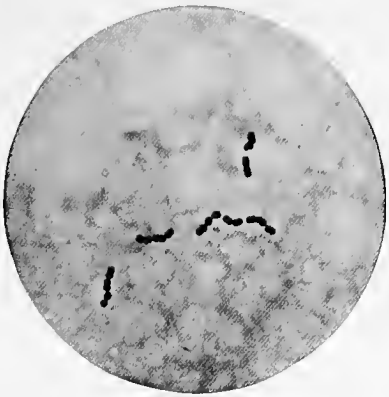


FIG. 8.—Streptococcus lacticus.

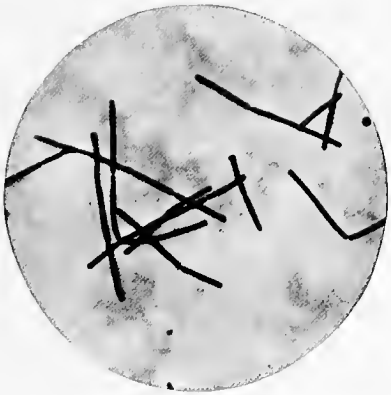


FIG. 9.—B. bulgaricus.

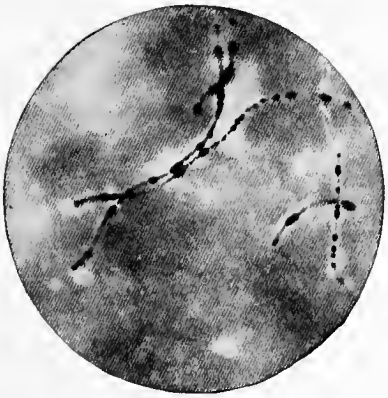


FIG. 10.—B. bulgaricus.

The following analyses are by Veith (*Analyst*, 10, 24, 11, 69, 12, 43):

%	Full kourniss			Medium kourniss			Whey kourniss			Diabetic kourniss			Russian kourniss			Cows' milk kourniss		
	Days old			Days old			Days old			Days old			Days old			Days old		
	1	8	22	1	8	22	1	8	22	1	8	22	1	8	22	1	8	22	90
Water	88.90	90.35	90.57	87.55	88.39	88.62	89.74	90.63	91.07	92.24	92.38	92.55	91.87	92.26	92.52	88.50	90.15	90.31	90.36
Alcohol	0.15	0.94	1.04	0.29	0.97	1.05	0.30	1.03	1.38	0.28	0.35	0.57	0.22	0.45	0.57	0.17	0.92	1.03	1.12
Fat	1.35	1.36	1.38	1.54	1.56	1.58	0.11	0.13	0.15	0.51	0.52	0.51	0.34	0.33	0.33	1.65	1.48	1.58	1.57
Casein	2.01	1.96	1.88	1.46	1.40	1.30	0.15	0.14	0.11	2.19	2.13	2.05	2.32	2.17	2.03	2.06	2.00	1.93	1.70
Albumin	0.30	0.23	0.20	0.43	0.25	0.14	0.39	0.36	0.32	0.30	0.25	0.18	0.08	0.07	0.07	0.32	0.22	0.21	0.09
Lactoprotein and peptones	0.34	0.53	0.77	0.48	0.76	0.97	0.44	0.49	0.58	0.36	0.48	0.65	0.32	0.48	0.63	0.32	0.56	0.74	0.91
Lactic acid	0.34	0.96	1.40	0.68	1.20	1.67	0.60	0.91	1.26	0.75	0.86	1.22	0.06	0.31	0.56	0.26	0.97	1.39	1.94
Sugar	6.03	3.10	2.18	6.80	4.70	3.90	7.48	5.52	4.34	2.78	2.42	1.64	3.95	3.08	2.45	6.16	3.14	2.23	1.73
Ash:																			
Insoluble	0.41	0.34	0.35	0.49	0.45	0.44	0.42	0.42	0.42	0.37	0.37	0.37	0.38	0.36	0.35	0.42	0.34	0.35	0.33
Soluble	0.17	0.23	0.23	0.28	0.32	0.33	0.37	0.37	0.37	0.22	0.24	0.26	0.46	0.49	0.49	0.16	0.22	0.23	0.25

The following analyses of Koumiss have been published by Orloff (Russian Koumiss):

Age of koumiss	Alcohol, %	Sugar, %	Lactic acid, %	Fat, %	Protein not peptone, %	Peptone, %
12 hours.....	1.94	2.11	0.84	.....	2.24	0.28
30 hours.....	2.18	1.87	0.94	.....	2.20	0.30
40 hours.....	2.32	1.53	1.09	2.25	2.15	0.35

and the following by Allik (Caucasian Koumiss):

Age of koumiss	Alcohol, %	Lactic acid, %	Casein, %	Albumin, %	Acid albumin %	Hemi-albumose %	Sugar, %
62 hours.....	0.57	0.84	1.01	0.41	0.18	0.66	1.43
84 hours.....	0.67	0.91	0.95	0.40	0.19	0.56	0.98

And the following are analyses of Kephyr given by Spivak and show the alterations which take place during maturing:

	First day	Second day	Third day
	%	%	%
Fat.....	.....	1.75	1.70
Casein.....	3.34	2.87	2.99
Lactalbumin.....	0.11	0.03	0.00
Acid albumin.....	0.09	0.10	0.25
Hemi-albumose.....	0.09	0.28	0.40
Peptone.....	0.03	0.04	0.08
Lactose.....	3.75	3.22	3.09
Lactic acid.....	0.54	0.56	0.65
Alcohol.....	.....	0.80	1.00

Analysis of Fermented Milks.—The fat, total nitrogen, sugar and ash are estimated in the usual manner, except that in the case of the fat, if determinations are made by the Gottlieb process (which is the only satisfactory method for these substances), preliminary neutralisation of the acidity must be effected in the Gottlieb tube.

Lactic Acid.—There is no exact method of estimating lactic acid; but the amount present is usually arrived at by titrating a known weight of the sample with $N/2$ sodium hydroxide using litmus paper as an indicator. (1 c.c. of $N/2$ NaOH = 0.045 grm. lactic acid). (See also Vol. 7, page 437.)

Alcohol.—About 100 grm. of the fermented milk are diluted with an equal volume of water and 100 c.c. distilled off. The distillate is neutralised with $N/10$ barium hydroxide and redistilled till 50 c.c. have passed over, and the alcohol estimated from the gravity of this last distillate.

Volatile Acidity.—A further quantity of water is added to the residue from the *first* alcohol distillation and the distillation continued till 50 c.c. of the distillate require only about 0.1 c.c. $N/10$ sodium hydroxide for neutralisation. The total distillate is then titrated with $N/10$ barium hydroxide and to the amount so found are added the number of cubic centimetres required to neutralise the first alcoholic distillate. The volatile acidity is usually expressed as acetic acid.

Casein.—This can be directly filtered off, washed and the nitrogen estimated.

Lactalbumin.—This cannot be found very accurately, but is usually estimated by neutralising the filtrate from the casein, acidifying with 0.1% of acetic acid and boiling the solution. The precipitate is taken to be lactalbumin, the nitrogen being estimated in it after filtration and washing.

Albumoses and peptones are found by deducting the sum of the nitrogen in the above constituents from the total nitrogen, or the filtrate from the albumin may be neutralised, cautiously evaporated to a convenient bulk, saturated with zinc sulphate and the precipitate filtered off, washed with saturated zinc sulphate solution and the albumose nitrogen estimated in the precipitate. The peptone nitrogen is obtained by difference.

F. INFANTS' FOODS.

The great multiplicity of artificial preparations classed under Infants' Foods can hardly be included under the heading of milk products, as a very large number of them are produced from cereals and similar substances, and only occasionally contain actual milk derivatives.

The methods of analysis usually employed are simply those which estimate the various constituents in a similar manner as in the case of the analysis of feeding-stuffs and give no idea of the method of manufacture, or of the condition, or digestibility of the components. Quite recently a systematic investigation of a large number of infants' foods by methods which give to some extent the desired information have been carried out by Julian L. Baker and H. F. E. Hulton. These methods have not yet been published, and the writers are greatly indebted to these investigators for permission to publish the following details of the methods which they suggest. As the various estimations themselves are not pertinent to this section, only the lines of investigation are here given without entering into the full analytical details. In

the preparation of the sample and in the interpretation of the results of analysis care must always be taken to bear in mind the directions prescribed for making up the food as these may produce profound changes in its nature.

The following are some of the estimations suggested by Baker and Hulton:

Starch.—This is estimated by a direct polarimetric method, as originally devised by Lintner, and modified by Thorne and Jeffers (*Analyst*, 1909, **34**, 322). The readings must be corrected for other optically active substances found. In cases where much starch is present information may be obtained as to whether it has been heated or not, by estimating the starch as above, and also in that part of the food which is insoluble in warm water. In the *absence* of diastase, the difference gives information as to the amount of starch rendered soluble by heating. When diastase is present the material must be extracted with ammonia (80 c.c. of $N/10$ diluted to 1,000 c.c.) in order to stop its action.

Hydrolysed Starch Products.—These when present cannot be directly estimated, but from the sp. gr., copper reduction and rotation of the aqueous extract, a very fair idea can usually be obtained of the proportions of these substances after making allowance for other soluble substances, such as cane sugar, lactose and soluble proteins, when present. The aqueous extract of the food is made by shaking a suitable quantity—from which fat has been extracted if necessary—with distilled water for 2 or 3 hours.

Reducing Sugars. Dextrose.—In the absence of starch conversion products and lactose, the copper reduction of the cold water extract may be taken as due to dextrose or invert sugar. In the presence of maltose the estimation of dextrose becomes difficult, but there is little probability of dextrose being present in the ordinary cereal foods, except in very small quantities. In presence of lactose, the copper reduction should be corrected by separately estimating this sugar. Baker and Hulton have shown (*Analyst*, 1910, **35**, 512) that for the estimation of lactose very fair results can be obtained by fermenting away the other sugars by means of yeast, and estimating the lactose in the residual liquid. For this purpose the aqueous extract of the material is boiled with 2% of citric acid to invert any cane sugar, neutralised, and a small quantity of cold water extract of diastatic malt added. About 0.5 grm. of washed brewers' yeast is then added and the whole incubated for 3 days at 27°. The liquid is then cleared with alumina cream, filtered, boiled, and titrated

against Fehling's solution. When hydrolysed starch products are present the estimation of the lactose will give slightly too high a value on account of the presence of unfermentable reducing substances derived from malto-dextrins, but as a rule the error may be neglected.

Cane Sugar.—This may be estimated in the cold water extract by heating with 2% of citric acid in boiling water and the increase in the copper reduction estimated. But where any considerable amount of cane sugar is present it should be inverted by digestion with a little washed yeast for 4 hours at 55°, the solution cleared and polarised and the cane sugar calculated by the usual Clerget formula.

Protein.—The nitrogen is estimated in the usual manner by the Kjeldahl-Gunning method. The factor 6.25 is suitable though necessarily only approximate on account of the diversity of proteins which may be present. The identification of the various proteins is, owing to the nature of the case, very complex (see pages 153 and 113).

Fat.—This is estimated by the Röse-Gottlieb method, with all the precautions given under dried milk, or by the Bondyzynski method, as described under cheese (see page 253). Ether extraction is unsuitable in the case of foods containing dried milk.

"Cellulose."—Baker and Hulton include under this heading those substances of a carbohydrate nature, which are insoluble in boiling water and are not attacked by diastase. In order to estimate the actual indigestible substances a pancreatic digestion would be necessary, but to avoid this these investigators have devised the following method: 5 grm. of the material—from which the fat has been extracted if necessary—are made into a paste with cold water, 200 c.c. of water added, the whole brought to the boil, and the boiling maintained for 30 minutes. The solution is then cooled to 80° and 5 c.c. of malt extract (prepared by extracting a diastatic malt, with 3 times its weight of cold water for 1 hour and filtering clear) added, and after 5 minutes again boiled, cooled to 60°, 25 c.c. further of the malt extract added, and the whole digested at this temperature for 3 or 4 hours. The solution is then filtered through a tared filter paper, and the residue washed repeatedly with water at 60° till free from sugar, then with alcohol and ether and finally dried to constant weight. The nitrogen in it is then estimated on the residue, and the protein present calculated and deducted. In a parallel experiment the ash in the weighed residue is estimated and its amount is also deducted. The final residue is returned as "cellulose."

Moisture and Ash.—These are estimated in the ordinary way by drying and ignition.

Saccharifying Diastase.—This is determined by Lintner's method as modified by the Institute of Brewing (*J. Inst. Brewing*, 1906, 12, 6).

Liquefying Diastase.—This is estimated by observing the change in viscosity produced in 200 c.c. of a 2% potato starch paste in 10 minutes at 20° under the action of 10 c.c. of a 5% cold water extract of the sample.

Alkalinity.—A definite volume of the cold water extract is titrated with $N/50$ sulphuric acid, using methyl orange. An alkalinity of more than 0.15% calculated as acid sodium carbonate would appear to point to the addition of alkalis.

Nature of Starch Present.—This is determined by microscopical examination.

A consideration of the results so obtained will nearly always indicate the nature of the constituents of the food and whether the claims of the makers can be substantiated.

In those foods which consist entirely of milk constituents the methods given under dried milks will apply, and in many cases in which lactose is present, and there are no added proteins, the relation of fat, protein and lactose to one another will often be a guide to the presence of dried milk. In such cases the presence of casein may be easily demonstrated.

G. DRIED MILK.

This product which a few years ago was practically only of theoretical interest has now become an established and important industry chiefly through the great improvements in the manufacturing process.

There are really only two methods in use for effecting the production of dried milk; (1) drying on steam-heated rotating drums, (2) spraying into a chamber through which a current of warm air is passing.

The former, or drum process, was first introduced by Hatmaker, and all processes involving drying on drums are really modifications of the Just-Hatmaker process.

In the original process the milk is run between two drums separated about 1/8 in., heated to a temperature of 100° and the dried milk is scraped off in a thin film by cutting edges after the film has passed over about 3/4 of the surface of the drum. In the Ekenburg-Passburg method the drum rotates *in vacuo* and a temperature of 110–120° is employed. In the Nicolai method, the milk is first condensed and

run in a fine film on to a rotating drum heated by steam and the film is continuously removed, raised by an elevator and allowed to fall into an apparatus which completes the removal of the water. The product is then sieved. In the Gabler process (much used in Switzerland) the milk is pasteurised and then concentrated in the vacuum pan, strongly cooled and led on to the drum, the dried milk as it is scraped off being carried by an endless band to the sieves, while a strong current of air passes over it and also over the drying drum.

The principle of the spraying process is essentially different. The milk is pumped through fine orifices into a chamber through which a rapid current of warm air passes and arrangements are made to catch the powder which is so produced. The process seems to have originated with Stauf who proposed to spray whole milk and to employ an air current of about 80°. The object of all these spray processes is to obtain a more soluble product without the coagulation of the albumen or destruction of the enzymes. In the Bevenot process a much finer spray and consequently much higher pressure is employed, and the temperature of the air current is only a little below 100°. The best-known form, however, is the Merrell-Soulé process in which concentrated milk is sprayed into a chamber through which an air current at 150–200° is passing. This process is now being used by the Trufood Co. and in spite of the high temperature employed, the evaporation is so rapid that albumin is not coagulated and enzymes are not destroyed. The product is quite soluble in cold water, the taste of the resulting milk being but slightly different to raw milk, and the fat emulsion is perfect. The process is also applied to the production of separated milk powder, whey powder, etc. The yield is practically theoretical, 8 litres of milk giving about 1 kilo of dried product.

In America 25% of the total dry solids are to consist of fat in the case of dried whole milk, yet it would seem fairer if 20% were taken as a minimum limit.

The ash of whey powder (Trufood process) is very alkaline. Two samples required per 100 grm. of ash, 85 c.c. and 122 c.c. of N sulphuric acid respectively.

If a solution of milk powder is alkaline to litmus it is certain that alkali has been employed in its preparation, but this test will only apply to fresh preparations or those which have been carefully protected from contact with the air, as otherwise a considerable rise in acidity takes place.

This is illustrated in the following table:

	Cubic centimeters of N/1.NaOH per 100 grm. powder to phenolphthaleïn		Ash from 5 grm.	Reaction of ash to litmus
	Fresh	After 5 months		
Cream powder (1).....	10.5	30.0	0.204	Alk.
Cream powder (2).....	11.0	11.5	0.238	Alk.
Whole milk (1).....	10.5	42.0	0.289	Alk.
Whole milk (2).....	12.5	12.5	0.291	Alk.
Skim milk (1).....	8.0	11.5	0.389	Alk.
Skim milk (2).....	16.0	16.5	0.399	Alk.

The ash from 5 grm. of dried milk powder as a rule requires about 0.5 c.c. of $N/10$ sulphuric acid to render it neutral to litmus.

There are undoubtedly changes which take place in dried milk on keeping. These affect its solubility sometimes profoundly. There is also the development of rancidity from which few dried milks, other than skim milk, are at present free. These changes are sometimes attributed to bacteria, but much doubt attaches to such an explanation. With whatever care drying may be done, there is necessarily a change in the state if not in the chemical composition of the milk, and rancidity especially would seem to be due to an oxidation started during the drying process and continued in a catalytic manner during storage of the dried product.

The following are analyses of dried milk powders:

THIN CREAM.

Water	Total solids, %	Fat, %	Protein, %	Ash, %	$\frac{\text{Fat}}{\text{Protein}}$	Source
2.11	97.89	51.55	15.24	.....	3.38	.....
3.23	96.77	40.35	19.84	.....	2.03	.....
3.56	96.44	49.00	14.70	.....	3.30	.....
5.28	94.72	50.69	16.30	.....	3.11	.....
1.85	98.15	46.77	.....	.....	.....	Germany.
2.95	97.05	38.98	21.69	4.76	1.80	Germany.
2.26	97.74	47.82	17.84	4.04	1.71	Germany.
3.33	96.67	44.90	18.89	4.04	2.37	N. America.

WHOLE MILKS.

Water	Total solids, %	Fat, %	Protein, %	Ash, %	$\frac{\text{Fat}}{\text{Protein}}$	Source
2.50	97.50	28.30	32.10	5.34	0.78	England
1.40	98.60	29.20	26.92	6.00	1.08	France.
6.39	93.61	27.35	27.48	6.00	0.99	France.
3.30	96.70	23.97	26.38	6.19	0.91	France.
6.03	93.97	25.60	23.84	6.44	1.07	France.
5.51	94.49	23.75	24.71	6.49	0.96	Austria.
5.65	94.35	23.42	25.48	6.46	0.92	Holland.
5.29	94.71	26.55	25.17	5.57	1.05	Algäu.
4.23	95.77	29.50	26.57	5.80	1.11	N. America.
4.43	95.57	25.97	.....	.....	.....	Germany.
6.73	93.27	30.23	22.75	5.50	1.33	Holland.

PARTIALLY SKIMMED MILK.

Water	Total solids, %	Fat, %	Protein, %	Ash, %	Fat / Protein	Source
5.01	94.99	15.26	38.39	6.67	0.40	Belgium.
5.00	95.00	15.10	33.30	6.90	0.45	France.
4.65	95.35	17.06	29.43	6.78	0.58	.....

SKIM MILKS.

Water	Total solids, %	Fat, %	Protein, %	Ash, %	Lactose / Protein	Source
3.00	97.00	0.75	34.00	7.00	.....	America.
6.29	93.71	1.02	35.45	8.17	1.21	America.
13.19	86.81	1.40	28.89	7.78	1.68	Germany.
13.88	86.12	3.60	30.30	7.75	1.15	Holland.

SWEET WHEY.

Water	Total solids, %	Fat, %	Protein, %	Ash, %	Lactose / Protein	Source
2.20	97.80	1.66	14.16	8.20	5.22	America.
2.10	97.90	1.60	13.72	8.80	5.38	America.

EGG POWDER.

Water	Total solids, %	Fat, %	Protein, %	Ash, %	Lactose / Protein	Source
5.74	94.26	35.90	48.09	3.20	.....	America.
5.00	95.00	43.08	49.22	3.45	.....	America.

The above values are all selected from tables given by Burr (*Milch. Zent.*, 1911, **7**, 118).

The following analysis of a dried milk was made by one of us (1905). The sample was described as containing added fat: Protein, 20.30; lactose, 41.46; moisture, 6.65; ash, 6.52; fat, 19.99; of which last 97% was coconut oil.

The following analyses are due to Veith (*Jahresb. Milch. Instituts zu Hameln*, 1909, 27):

	I	II	III
Water, %	6.44	5.80	5.29
Fat (Gottlieb), %	23.35	23.90	26.55
Protein, %	24.35	23.97	25.17
Lactose (by diff.), %	39.36	39.95	37.42
Ash, %	6.50	6.38	5.57

Also the following "Trufood" preparations:

	Full cream	Skim cream	Whey
Water, %	2.47	3.69	7.81
Fat (Gottlieb), %	25.26	2.30	0.60
Protein, %	26.49	34.98	11.83
Lactose (by diff.),	39.86	51.30	70.80 (est)
Ash, %	5.92	7.73	8.27

In connection with these he draws attention to the great inadequacy of extraction in a Soxhlet extractor for fat estimation in such products.

(I) Fat	23.40 23.30	Gottlieb	17.82 17.73	Extraction
(II) Fat	26.50 26.60	Gottlieb	22.70 22.70	Extraction

Analysis of Dried Milk.—The sample should be carefully mixed.

Moisture.—This is estimated by drying 1 to 2 grm. in a platinum dish to constant weight in a water oven.

Ash.—The dry sample from the moisture estimation is burnt (preferably in a muffle) at a very low red heat. CaO, and P_2O_5 may often require to be estimated in order to detect the addition of sodium phosphate and calcium sucrate.

Fat.—This is best estimated by the Gottlieb process. About 0.5 grm. are weighed into the special apparatus and 4.5 c.c. of hot water added and the whole mixed well with a rod and 0.5 c.c. of ammonia (sp. gr. 0.925) added, mixed, and 5 c.c. of strong alcohol run in and the apparatus floated in hot water until complete solution takes place, which occurs rapidly. The estimation is then finished as in the case of cream. With very old samples of dried milk in which the fat may have become appreciably hydrolysed, the Bondyzynski method, as described under *Cheese*, may be used (page 255).

Sugar.—10 grm. are worked up in a mortar with warm water and a little ammonia added (both to assist the solution and to destroy birotation). The paste is washed into a 100 c.c. flask, cooled, and the solution treated exactly as under condensed milk, the necessary corrections being obtained from the fat and protein estimations.

As calcium saccharate sometimes appears in dried milks, cane sugar may also be estimated.

Protein.—This is estimated on 0.5 to 0.7 grm. by the usual Kjeldahl-Gunning method, using the factor 6.38.

Richmond has pointed out (*Analyst*, 1906, **31**, 219) that analyses

of dried milk seldom add up to 100% and his explanation that it is due to a part of the sugar being present as monohydrate is undoubtedly correct to a certain extent, but the same observer further points out (*Analyst*, 1910, **35**, 516) that when milk is cleared with ordinary acid mercuric nitrate, the precipitation of albumin is not complete, and in the case of dried milks has shown that the milk sugar may be underestimated by 1% polarimetrically. If therefore very accurate estimations are required, it is advisable to employ the method recommended by him of adding to every 100 c.c. of the filtrate from the acid mercuric nitrate, 5 c.c. of a 4% solution of phosphotungstic acid and 5 c.c. of 50% sulphuric acid. The solution is filtered before polarisation, and allowance made for the extra dilution.

H. CHEESE.

This most important milk product is formed from the casein of the milk usually by the action of rennet, the curd or paracasein so obtained carrying down with it nearly all the fat present in the milk. By variations in the after treatment of the curd mass, arise an extraordinary multiplicity of products, all included under the title of cheese. The finished substance is of a highly complex nature on account of the profound protein degradation that usually takes place during ripening. The nitrogenous substances range from the original paracasein down to ammoniacal salts, and together with these are milk fat, some lactic acid, mineral constituents, added salt and colouring matter.

Cheeses may be roughly classed under two headings:

(1) *Hard cheeses*, such as Cheddar, Cheshire, Dutch, Stilton, etc., and

(2) *Soft cheeses*, such as Camembert, Pont l'Evêque, Roquefort, Brie, etc.

The two classes are roughly distinguished by the fact that in hard cheeses, the whey has been separated as completely as possible; while in the soft cheeses, it has been much retained. As a result, hard cheeses ripen slowly and keep for long periods, while soft cheeses ripen rapidly and must be consumed as soon as they have matured sufficiently. Generally speaking, the ripening process is "enzymic" in the case of hard cheeses, and "bacterial" in the case of soft cheeses. On account of the superior keeping quality of the hard varieties they naturally form the bulk of the cheeses of commerce.

The large majority of cheeses are produced from cow's milk, but in

certain parts of the world the milk of sheep and goats is also employed, and there is no reason why any milk should not be used. It is interesting to note that a cheese-like substance may be made from purely vegetable materials. For instance, among the inhabitants of the Soudan, a cheese is made from the seeds of the "*Parkii Africana.*" The seed kernels are used and the product is free from starch. The fresh white cheese is called "*Afiti*" and the ripened product "*Dana-Dana*" and has then the consistency of a hard cheese, possessing an aromatic smell and bitter taste. Bacteria evidently take part in the ripening, and the course seems to be much the same as in soft cheeses.

Busse (*Cent. für. Bakt.*, Abt. ii, 1905, **14**, 480) has examined a cheese made from the seeds of "*Treculia Africana*" and called "*Pembe.*" Cayenne pepper is added at the time of manufacture. The "cheese" becomes strongly acid by the development of lactic acid organisms and so decomposition is prevented.

The large quantity of fat present in these seeds is an important factor in the cheese-like consistency and flavour obtained. A similar type of cheese is also made in Japan from Soya beans.

The fat present in cheese is usually the fat of the milk used, but cheeses are often made from skimmed milk with the addition of margarine fats and sometimes of coconut oil (in certain Swiss cheeses), the fats being emulsified with the milk before manufacture. Such cheeses are known as "filled" or "margarine" cheeses and are a legitimate product, if their nature is clearly disclosed. In cheeses made from unskimmed cow's milk the fat should always be in excess of the protein, but this rule does not hold good in the case of cheeses made from the milk of animals other than the cow, and it must be borne in mind that each locality has its own methods of manufacture and the composition of the cheese must be judged accordingly, and not by any arbitrary standard.

The following paragraphs give a rough outline of the method of manufacture of the two main varieties of cheeses.

Manufacture of Hard Cheese.—Of these Cheddar may be taken as a type.

Perfectly clean milk is first ripened by means of a lactic acid culture. The proper development of acidity is perhaps one of the most important steps in the process. When the acidity is about 0.20% the temperature of the milk is brought to 84–86° F. and the rennet added, sufficient being added to produce coagulation in half an hour; the contents of the vat being gently stirred till coagulation sets in. After this the milk is allowed to stand undisturbed until the curd has

hardened sufficiently for "cutting." The curd is then "cut" with a wire knife lengthwise and crosswise, so that the whole is divided into small cubes. The cutting allows of the escape of whey and the contraction of the curd cubes, this result being known as "firming," and the curd mass is gently stirred in order to prevent the cubes sticking together. It is then gently heated to about 100° F., the rate of heating being regulated by the acidity of the whey found on "cutting." When the acidity of the whey has increased to say 0.25%, it is run off from the curd, gentle stirring being again employed during the process. As soon as the bulk of the whey has run off the curd is "cheddared" by being piled along each side of the vat, so that free drainage is possible, and as the whole gradually sinks into a more or less solid mass, it is cut into blocks and repiled. When this process is complete the curd is "milled" or broken up into small uniform pieces preparatory to "salting." After the salt is completely dissolved by the curd it is cooled and "pressed" in cotton cloths placed in the "hoops," the object of pressing being to weld the curd into a compact and solid mass, and to prepare the cheese for the final "ripening" process.

(For perhaps the best treatise on this subject see the *Science and Practice of Cheesemaking* by Van Slyke and Publow.) Also *The Production of Volatile Fatty Acids and Esters in Cheddar Cheese and their Relation to the Development of Flavour*, by Suzuke, Hastings and Hart, Research Bulletin No. 11, 1910, Wisconsin Agricultural Experiment Station.

Manufacture of Soft Cheese.—The procedure is essentially different to that described above, and on account of the great diversity of this type of cheese, no two processes are similar. The differences lie principally in the fact that the rennet is added to perfectly fresh sweet milk, the coagulum is carefully ladled into the shapes or hoops, and drainage is natural and without pressure, and without heat. The resulting cheese is consequently soft and on account of the quantity of whey left in, ripening is very rapid and far more complex than in the case of hard cheese and the flavour depends almost entirely on the particular moulds and bacteria employed.

Manufacture of Cream Cheese.—Cream cheeses are usually styled "Double cream" or "Single cream." The former is made from a thick cream of 45–50% of fat, and without the use of rennet. The cream is cooled to 60° F. and if desired a little lactic acid starter added. After standing 12 hours the cream is allowed to drain in dry cloths under slight pressure. Single cream cheese is made from cream of

25% fat content, and a little rennet is added with the starter. If sweet cream cheese is desired the starter is omitted.

(For an excellent treatise on soft cheese-making see "*Soft Cheese-making*," by Walker Tisdale.)

The Ripening of Cheese.—The analytical methods employed for cheese will not furnish much information without some knowledge of the changes taking place during ripening. As stated above this process is essentially different in hard and soft cheese.

Ripening of Hard Cheese.—The process in this case is mainly chemical and enzymic. The part played by bacteria is practically limited to the production of lactic acid from the milk sugar, and as 99% of the bacterial flora are of the lactic acid type, the rest play probably an insignificant part, except perhaps in the production of flavour. For a comparison of the bacterial content and ripening process, see Harrison and Cornell (*Rev. gen. du Lait*, **3**, 80, *et seq.*). The lactic acid as it is formed removes calcium from the curd and produces calcium lactate and acid calcium phosphate. The removal of the calcium together with the action of the ferments of the rennet produce during "cheddaring" a marked change in the nature of the protein which, from being almost insoluble in a 5% solution of sodium chloride, becomes almost completely soluble. This soluble form then gradually passes into another insoluble form, and from this last arise, during the true ripening process, the variety of protein bodies found in the finished cheese. From the start of the true ripening process the amount of insoluble protein diminishes and the amount of water-soluble protein and its derivatives increases, so that as Van Slyke has so ably demonstrated, "the amount of water-soluble protein and protein derived substances is a measure of the extent of the ripening process."

The production of these water-soluble and derived proteins is in the light of our present knowledge due to the rennet (perhaps the peptic constituent) in presence of lactic acid and its salts, the protein being gradually broken down into caseoses and peptones. Ripened cheese however contains large quantities of amino-acids and some ammonia and according to Boekhourt and de Vries these are produced by peptonising bacteria which gain entrance to the milk but which do not develop on account of the acid environment. They remain, however, distributed throughout the cheese and form foci for the secretion of proteoclastic enzymes. It has also been suggested that the lactic acid bacteria may eventually disintegrate in the cheese, and liberate such enzymes, but they do not produce any such effect by their ordinary activity. We find then, as a final result, that the nitrogen is present

in very various forms, and the following table due to Van Slyke and Publow illustrates this:

Age of cheese, months	Brine soluble protein	Nitrogen expressed as percentage of nitrogen in cheese in form of:					
		Water, soluble proteins and derivatives	Para-nuclein	Caseoses	Pep-tones	Amino-acids	Ammonia
1½	20.18	21.44	2.06	3.15	3.84	9.88	1.56
3	27.26	30.98	4.45	4.56	4.65	14.36	2.45
6	27.55	36.15	3.57	4.92	4.22	19.96	3.52
9	24.14	43.45	4.02	4.59	3.56	26.53	4.74
12	19.04	44.75	3.52	4.16	3.95	28.38	5.41
18	12.65	47.25	3.40	3.88	2.57	30.46	6.62

The most noteworthy point in the above table is that, while the amount of amino-acids and ammonia steadily increases, the amount of intermediate products remains nearly stationary. This, however, has been fully explained by Van Dam (*Cent. für Bakt.*, Abt. ii, 1910, **26**, 189) as an outcome of experiments on the estimation of the acidity of cheese.

In certain cheeses it is of great importance to know the acidity of the cheese mass as it comes from the press, as on this acidity depends the course of the ripening. The extraction of the mass with either ether or acetone as suggested by Boekhourt and de Vries gives much too high results, as phosphoric acid and soluble phosphates appear in the extract, together with the lactic acid. Van Dam has therefore worked out a simple and easy adaptation of the conductivity method, which gives the true hydrogen ion-concentration, using not an extract of the cheese, but the actual cheese mass itself. In a similar way the action of the rennet has also been studied and the results seem to show clearly the course of events during ripening and explain the above apparent anomaly. He shows that (in the case of Edam cheese, though the facts are also true for other similar cheeses) the H-ion concentration varies from $0.72–1.10 \times 10^5$ and that in the press the development of lactic acid quickly reaches a maximum, while enzyme action which, at first was slight, then suddenly increases. He also shows that the digestion of the paracasein is then influenced by and is proportionate to the H-ion concentration, and that it only takes place through the agency of the chymosin of the rennet. Further the solution of the cheese mass does not proceed immediately to the complete change of the paracasein but a state of equilibrium is brought about between it and its degradation products (peptones and caseoses). This equilibrium is then destroyed by the attack of bacteria or bacterial enzymes on the degradation products with the result that the chymosin again dissolves true paracasein and so on. These findings

completely explain the observed facts, viz.: that the formation of water-soluble nitrogenous substances is always rapid at first and then decreases and that while amino-acids and ammonia steadily increase, the amount of intermediate products remains fairly constant during the ripening process. These results are quite in accord with the researches of Van Slyke and with our knowledge of the action of enzymes in general.

The writers are aware that the above description is open to some objections, but they trust that the presentation is, on the whole, in accordance with ascertained facts.[1]

Whether good cheese can be made from milk which has been heated remains still a rather vexed question, but it would seem that if a proper "lactic" starter is used a fair quality cheese may result.

Ripening of Soft Cheeses.—In soft cheeses the ripening process is rapid and almost completely due to bacteria and moulds. A rapid production of lactic acid is essential (1) to protect the protein from objectionable putrefactive processes, and (2) to produce a proper condition for the rapid development of moulds. A true ripening in soft cheese takes place from the periphery to the centre and the moulds as they push their mycelia into the substance of the cheese produce enzymes which break down the protein, while at the same time they use up the lactic acid already formed. Low temperatures are usually employed to favour the mould development and "spearing" is resorted to if the growth is not sufficiently rapid. In such a cheese as Camembert the mould growth passes through the stages of a white felted mass till on fructification the well-known bluish-green colour appears, the mould finally breaking down and leaving the well-known reddish-brown coating.

For a full investigation into the action of fungi in the ripening of Camembert and Roquefort cheeses see Thorn, *Bulletin* 82 *Bureau of Animal Industry*, U. S. Dept. of Agriculture, Feb. 6, 1906.

Flavour.—The cause of "flavour" in cheese is still somewhat in dispute. In the case of hard cheeses of the "cheddar" type, the consensus of modern opinion seems in favour of the attribution of "flavour" to the amino-acids formed, but in soft cheeses the distinctive flavours are largely due to the various putrefactive bacteria, which produce small quantities of aromatic substances similar to those produced by them in game, etc., and also to the presence of large quantities of moulds which leave in the cheese their own distinctive flavour.

[1] A valuable contribution to this question has been recently published by E. G. Hastings, A. C. Evans and E. B. Hart, in *Research Bulletin* 25, Wisconsin Agric. Expt. Station, July, 1912.

Before passing to the methods of analysis of cheese, the nature of some of the substances present must be considered.

Fat.—The fat of cheese made wholly from milk is ordinary butter fat. In hard cheeses it appears to undergo little change. There is little real evidence, if any, that fat arises from protein decomposition, but free fatty acids certainly occur in cheeses and the acid value of the fat obtained depends very much on the method of extraction.[1] This is well illustrated by some work of Koestler (*Milch. Zent.*, 1908, **4**, 111).

He shows that the character of the fat extracted by different methods varies slightly and solution of the curd in hot hydrochloric acid is not recommended by him. Extraction with ether, either with or without previous drying, is to be preferred, and he gives the following results with extraction methods using a margarine cheese. The ground-up cheese was extracted in one case with methylated ether and in the other with petroleum ether, by shaking out 6 times in a separating funnel.

	Solvent	R. M. value	Sapon. value	Acidity c.c. *N*/10 KOH per 100 grm. of fat
I. Extraction of fresh cheese.....	Meth. ether.	3.02	198.9	161.0
	Pet. ether.	2.12	199.0	110.9
II. Extraction after 24 hours drying at 50°.	Meth. ether.	2.70	201.6	142.3
	Pet. ether.	2.80	199.7	119.0
III. Extraction after 10 days in vacuum desiccator.	Meth ether. .	3.51	199.3	197.3
	Pet. ether.	2.64	198.2	95.4

It is easily seen that methylated ether extracts volatile acids which appear to be formed as the drying proceeds, or the extraction of which are facilitated by drying.

All the fat is not extracted in either way.

In Case I, 35% is not extracted by methylated ether and 71% not by petroleum ether.

In Case II, 36% is not extracted by methylated ether and 29% not by petroleum ether.

Jensen, however, states that besides fatty acids derived from the milk fat, formic, acetic and propionic acids appear which are derived from the protein and milk sugar and that in certain cases butyric acid may be formed from milk sugar only.

Troili-Petersson and Gerda (abs. *Milch. Zent.*, 1911, **7**, 38) account for the fact that, while free fatty acids appear in cheese, no glycerol is

[1] In a recent paper Nierenstein (*Proc. Roy. Soc.*, 1911, **B**, 83, 301) states that old cheddar cheese was found to contain free cholesterol, cadaverine, putrescine and aminovaleric acid, the amounts of these substances being sufficient to account for the increased weight of the ether extract frequently observed. The assumption that fat is produced from proteins during ripening does not seem to be justified.

ever detected, by showing that in certain cheeses (particularly Swedish *Herrensgutkäse*), there are present such organisms as will ferment glycerol and they describe three strains of *Bact. glycerini* a, b and c which they have isolated from such cheese, and they also show that cheeses made from sterilised milk and inoculated with these organisms together with lactic acid organisms and liquefying cocci, ripen normally and with a proper flavour.

Van Slyke also has drawn attention to the presence of small white specks in cheddar cheese cured at a low temperature. These specks appear to be calcium salts of a fatty acid, decomposition of the fat having probably taken place and so furnished the fatty acid.

The analytical figures obtained for the fat extracted from cheese must be taken with a little caution as the effect of ripening is to alter the nature of the fat somewhat, but there is of course no difficulty about distinguishing a "filled" cheese from one made with true milk fat.

With regard to the percentage of fat in a cheese, the nature of the cheese must be borne in mind as not all cheeses are made from whole milk.

The United States Department of Agriculture defines cheese made from whole milk as containing in the water-free substance not less than 50% of butter fat. With regard to cream cheese there is necessarily a certain amount of variation according to the recipe on which the cheese is made. As cream cheese is seldom made with cream of less than 10% butter fat, analysis of the cheese will show whether a cream of lower fat percentage has been used in its manufacture.

Mineral Salts.—Apart from sodium chloride added during manufacture the chief mineral constituents of cheese are calcium salts of phosphoric acid. The precise state of combination of the calcium in milk is not fully understood; it may exist as dicalcium phosphate, or as calcium caseinate, or as both. Lactic acid as it develops removes the calcium from the curd and renders soluble any insoluble calcium phosphate producing calcium lactate and a soluble calcium phosphate. It has been shown by one of us that lactic acid removes calcium from casein in equivalent quantities, and also that casein can combine with free lactic acid. (*J. Hygiene*, 1907, **7**, 216.)

There should not be in a normal cheese any free lactic acid, it should all be taken up by the cheese substances themselves. Failure in this results in a condition of cheese which is termed "short." This condition has been carefully investigated by Boekhout and de Vries (*Cent. für Bakt.*, Abt, ii, 1909, **24**, 122). The fault consists in the production of a granular hard white substance, either in specks or masses, in the

cheese, and according to them is due to the formation of paracasein bilactate. They point out that in cheese there are three substances which can combine with the lactic acid, viz.: calcium phosphate, calcium paracaseinate, and the paracasein. From the first are formed calcium lactate and monocalcium phosphate, from the second calcium lactate and free paracasein, while addition compounds are formed between the acid and the paracasein and which are supposed to be either monolactate or bilactate. It follows then that if either the cheese contains less than the usual quantity of lime salts, or there is an overproduction of acid, there will be more acid to combine with the paracasein and consequently in places the bilactate will be formed, and as a result the fault appears. Where, however, sufficient lime salts are already present, the monolactate results and a normal consistency is obtained. Their experiments show that, on extracting normal and "short" cheeses with 5% salt solution in which the bilactate is said to be insoluble, the content of bilactate in normal cheese varied from 1.3–2.4 c.c. 1/10 N. nitrogen, while in "short" cheese this figure rose to 15.8–18.4 c.c. They also show that the content of CaO in "short" cheese is less than in normal cheese. It follows then that milk exceptionally rich in lactose or the curd of which is poor in insoluble calcium salts is likely to produce this fault in cheese. Crystals of calcium phosphate are sometimes to be seen distributed in the cheese and may be recognised by heating the cheese with diluted alkali till the curd is slimy and then after diluting with water allowing to stand. The crystal conglomerates settle out and may be washed with water and examined under the microscope.

With regard to the amount of sodium chloride present, this will naturally vary with the type of cheese and may be in any percentage from 0.5–5.0%. It is interesting to note that in Edam cheese which is packed in loose salt or pickled in brine, the salt distributes itself through the cheese during the ripening process. The following figures (Boekhourt and de Vries) show this very well:

CHEESE (Edam) DIRECT OUT OF BRINE.

	Sodium chloride
Outer layers	13.3%
Middle layers	4.0%
Inner layers	0.4%
Same Cheese 4 weeks later.	
Outer layers	5.0%
Middle layers	5.2%
Inner layers	4.4%

They state that the lowering of the salt percentage in the outer layer may be due to washing which is done in order to remove excessive yeast and mould growth.

Preservatives, Ripeners and Coatings.—There have been numerous mineral substances found in cheese at various times, though they can scarcely be looked on as usual. *Lead chromate* appears to find entrance either from binding cloths impregnated with the chemical or else is used by mistake for colouring. *Lead dust* has also been found, but its mode of entrance is obscure. *Copper* certainly appears in certain cheeses, especially in the case of some Italian cheese, when copper vessels are used during manufacture. *Zinc sulphate* is said to be added as a "spice." Manfeld (*Ref. Zeits. Unters. Nahr. Genussm.*, 1911, **21**, 428) describes a cheese preservative of the following composition—sodium formate 62%, sodium benzoate 10%, sodium chloride 28%; and also a "ripener" consisting of sodium bicarbonate 70% and ground old cheese 30%.

Various materials have been suggested for hastening the ripening of cheese, such as ammonium carbonate and sodium or potassium carbonate. The following table gives details of three commercial preparations for the hastening of the ripening process. (Reiz, *Milch. Zentralblatt.*, 1905, **1**, 203):

Name	Maturin	Firmitas	Käsepräparat
Price	10 lb., 7.85 marks.	10 lb., 7 marks.	10 litres, 7 marks.
Accelerating power	Ripening in half the time.	Marketable cheese in 10–12 days.	
Quantity employed	1/4–1/2 lb. per 100 lb. of curd.	1–2 lb. per 100 lb. curd.	
Appearance	White powder.	Yellowish powder.	Reddish liquid.
Smell	None.	Like old cheese.	None.
Sp. gr. at 15°	.....	.....	1.076
Moisture	1.05%	11.85%	
$NaHCO_3$	48.72%	57.7%	8.40 grm. per 100 c.c.
Na_2CO_3	Trace	None.	1.91 grm. per 100 c.c.
NaCl	50.25%	3.55%	Trace.

Coatings.—Quite recently a question has arisen as to the coating of cheeses with heavy artificial rinds, particularly in connection with Gorgonzola cheese, in which case a thin coating containing a large proportion of barytes has been sometimes employed. As this adds very materially to the weight it has been looked on as a fraudulent proceeding and legal action has been taken to put a stop to it. (See Hinks, *Analyst*, 1911, **36**, 61.)

The report of a commission held in 1909 in Italy on the question is against the use of baryta in the coating and their recommendations are:

(1) That cheeses which are exported in the cooler months of the year scarcely need a coating.

(2) That for cheeses which will not stand the heat of the summer without losing their shape and taste, a light coating should be employed which should not be of an objectionable nature and should be free from barium sulphate.

It has become an established practice to coat such cheeses as Cheddar intended for export with a thin layer of paraffin wax. This procedure greatly improves the keeping quality of the cheese and is quite legitimate.

Alkaloids.—Adametz and Chrzaszcr (*Oesterreich Molkerei Zeit.*, 1905, No. 3–5) have shown that an alkaloid called by them *tyrothrixin* occurs in Emmentaler cheese in which *B. nobilis* has been used in the ripening process. This alkaloid has given the following chemical properties:

A snow-white crystalline substance of very characteristic pungent odour, easily soluble in alcohol, ether and dilute acids, soluble with difficulty in water at ordinary temperatures and insoluble in sodium or potassium hydroxide.

Phosphomolybdic acid gives a voluminous canary-yellow precipitate.

Phosphotungstic acid a white precipitate soluble in excess.

Potassium mercury iodide solution produces a sulphur-yellow precipitate in long needles.

Gold chloride gives a citron-yellow precipitate which deposits metallic gold in about 15 minutes.

Platinum chloride and tannin produce no precipitate.

The best collection of analyses and descriptions of manufacture of the world's cheeses will be found in Bulletin 146 of the Bureau of Animal Industry, U. S. Dept. of Agriculture, 1911.

The following are analyses of American Cheddar cheese made by Van Slyke:

		Water	Fat	Protein	Ash[1]
Green cheeses	Average.	36.84	33.83	23.72	5.61[1]
	Maximum.	43.89	36.79	26.11	7.02[1]
	Minimum.	32.69	30.00	20.80	3.12[1]
Seven weeks old	Average.	36.06	34.43	24.45	3.61
	Maximum.	41.15	45.36	28.72	5.29
	Minimum.	32.23	23.27	18.45	1.81
Five months old	Average.	34.01	36.81	25.69	3.50[1]
	Maximum.	38.10	44.33	30.09	4.59[1]
	Minimum.	29.85	27.22	21.53	2.72[1]

[1] Includes traces of milk sugar.

and the following of English Cheddar by Voelcker:

	Water	Fat	Protein	Lactose and lactic acid	Ash
Average	35.16	30.45	27.80	3.16	3.42
Maximum	39.43	41.58	32.37	6.80	4.31
Minimum	30.32	23.21	23.28	0.22	2.06

The following analyses of Gorgonzola cheese are by Musso:

	Water	Fat	Protein	Lactose lactic acid, etc.	Ash
Average	37.30	34.67	25.16	1.62	3.82
Maximum	47.10	39.32	28.51	2.00	4.63
Minimum	29.82	29.00	20.33	0.91	3.13

As a result of a number of analyses of Emmentaler cheese made in 1909 and 1910, Koestler (*Milch. Zentralblatt*, 1910, **6**, 289) gives the following mean values:

No. of analyses	Water	Total solids	Fat		Protein		Fat / Protein
			in fresh cheese	in dry solids	in fresh cheese	in dry solids	
37	33.63	66.37	32.20	48.53	27.49	41.42	1.1.7
10	33.09	66.91	31.44	47.00	27.84	41.64	1.1.3

He is of the opinion that the fat should not fall below 45% in the dry solids. The paper also contains a very interesting series of analyses made from sections of a piece of cheese cut right through a complete cheese. The results show that great uniformity of composition exists in such a boring.

The following analyses of Dutch cheese have been made by Paraschtschuk, 1909:

Water	Ash	Fat	Fat in dry solids	Lactic acid	Protein
33.57	5.45	22.79	34.31	1.29	36.90
35.13	6.02	27.78	42.82	1.17	29.90
32.57	5.70	32.29	47.89	1.31	28.17
32.22	5.65	34.04	50.22	0.95	27.14

The R. M. value of the fat varied from 26.74–29.80 and the acid value from 1.9–9.7.

The following analyses of Portuguese cheeses are given by Cordoso and Mastbaum (*Chem. Zeit.*, 1904, **28**, 989).

For curdling the milk, in many cases, the heads of thistles are ground with water in mortars and the strained liquid added to the milk.

Milk of	Name of district	Water	Protein	Fat	Sugar	Ash	NaCl	Acidity as lactic acid	Refraction number	Sap. No.
Sheep.....	Forminhao.	45.79	24.13	21.59	3.70	4.79	1.92	1.18	51.5	.....
Sheep.....	Alcanis.	43.96	19.87	22.30	5.43	8.44	4.63	0.79	51.0	219.0
Sheep.....	Azeitao.	42.54	21.97	25.27	3.82	6.40	3.20	1.33	50.5	.....
Sheep and goat.	Castello branco.	42.83	21.21	28.47	0.58	6.91	4.94	.....	54.5	225.0
Sheep and goat.	Aldeias.	38.26	32.10	19.30	4.43	5.91	2.19	0.73	54.0	239.0
Goat.....	Tinalhas.....	45.55	20.19	23.10	6.78	4.38	1.88	0.96	54.5	242.0
Cow.....	Gouvein.	49.51	17.83	26.10	2.81	3.23	0.94	0.34	50.5	239.0
Cow.....	Lissabou.	54.37	13.63	25.84	2.96	3.20	2.04	0.57	55.0	250.0
Sheep, goat and cow.	Cardiga.	28.39	27.32	34.37	5.00	4.92	2.05	1.83	52.5	238.0

The following are analyses of Camembert cheeses given by Buttenberg and Guth (*Zeits. f. Unters. Nahr. Genuss.*, 1907, **14**, 677 and 1908, **15**, 416):

Kind of cheese	Description	Water (1)	Fat free dry matter (2)	Fat (3)	Ratio (3) : (2)
Magerkäse.....	Extra fine, German.	56.36	35.16	8.48	1 : 4.15
Halbfetterkäse.....	Baby.....	47.22	39.56	13.22	1 : 2.99
Halbfetterkäse.....	Petit.....	54.75	30.06	15.19	1 : 1.98
Fetterkäse.....	Gourmand.....	44.36	36.79	21.85	1 : 1.69
Fetterkäse.....	Demilunaise.....	57.15	25.26	17.59	1 : 1.43
Fetterkäse.....	Petit, G. U.....	53.11	27.57	19.32	1 : 1.43
Vollfetterkäse.....	Vrai Lisieux.....	52.47	25.17	22.36	1 : 1.13
Vollfetterkäse.....	Le Laurier.....	57.35	21.48	21.17	1 : 1.01
Vollfetterkäse.....	Edelweiss.....	51.37	21.44	27.19	1 : 0.79

The following estimations of fat and total solids in various cheeses are due to Vieth (*Jahresb. d. Milch. Instituts zu Hameln*, 1909, 32):

Cheese	Total solids, %	Fat, %	Fat in total solids, %
Tilsiter.....	48.58	10.38	21
	56.22	14.21	25
	57.20	27.90	49
	58.20	28.50	49
	61.20	25.20	41
Chester.....	50.90	14.30	28
Brie.....	38.50	18.76	49
	47.05	23.89	51
Ramadour.....	46.85	20.86	45
	48.90	13.32	27
	43.00	9.50	22
Limburger.....	48.45	21.39	44
	38.77	7.83	20
	43.12	7.40	17
Kaiserkäse.....	40.20	20.85	52
Frühstückkäse.....	39.00	11.65	30
Backsteinkäse.....	45.16	6.22	14

The following analyses of the principal cheeses used in France are by Liudet, Ammaun and Brugière:

Cheese	Water	Fat	Protein	Ash		Soluble nitrogen in % of total nitrogen
				Insoluble	Soluble	
Troyes	58.7	18.6	14.6	1.1	3.7	70.8
Mont D'or	58.7	9.7	25.3	2.4	1.9	39.8
Coulommiers Double Cream	57.8	25.0	13.0	0.5	3.6	44.4
Petit Suisse	54.6	35.0	7.3	0.5	0.1	3.2
Bondon	54.3	23.0	16.1	0.7	4.3	32.9
Camembert	53.8	22.0	17.1	1.2	3.2	86.1
Brie	53.5	22.5	18.0	0.8	3.2	58.1
Rehlochon	53.2	20.5	19.3	1.9	1.8	27.9
Livarot	52.2	15.0	25.9	1.5	2.9	55.9
Pont l'Eveque	51.0	23.1	17.8	2.1	1.9	43.9
Demi-sel	49.6	34.0	11.8	0.6	2.4	12.2
Hollande	42.6	20.0	23.9	2.3	3.2	22.3
Gorgonzola	41.5	29.0	19.7	2.2	2.6	27.2
Cantal	40.9	29.3	20.5	2.2	2.6	46.0
Marolles	40.3	33.5	20.2	1.2	3.3	59.4
Port Salut	38.1	24.5	24.8	3.1	2.2	20.2
Roquefort	36.9	29.5	20.5	1.9	5.1	47.5
Gruyère	35.7	28.0	28.9	3.1	0.4	22.9
Parmesan	34.0	23.0	35.0	3.5	1.7	21.7

The following analyses of Norwegian cheeses are given by Hals (*Zeits. Unters. Nahr. Genussm.*, 1909, **17**, 673):

They are average results.

Cheese	Milk	Water, %	Ash, %	Fat, %	Protein, % $N_2 \times 6.25$	Lactose, %
"Halbfetter"	Goat	23.25	6.31	14.65	5.05	50.74
"Fetter"	Goat and cow.	12.74	4.52	30.82	10.21	41.71
"Magerer"	Sour cow's milk.	22.74	8.74	0.73	11.47	52.47

The cheese is actually made by boiling down whey in copper or iron vessels to the required consistency and then running into wooden tubs. It is practically not ripened at all.

Kräuter Käse is usually made from butter milk mixed with skim milk and with the addition of *Mellilotus caerulea.* It is often in the form of a powder. The following analyses have been published by Buttenberg and Koenig (*Zeits. Untes. Nahr. Genussm.*, 1909, **18**, 413):

	Total solids, %	Fat, %
Powder form from skim milk	76.53	5.10
Shaped cheese from skim milk	36.20–47.59	3.4–7.8
Shaped cheese from richer milk	38.88–55.34	4.68–12.20

The cheese is also made with an addition of coconut oil when the total fat content rises as high as 38–39% and the saponification number of the fat varies from 241.91–256.99 and the R. M. value from 8.36–14.25.

The following are analyses of Margarine cheeses (Koestler, 1908):

No.	Water, %	Total solids, %	Fnt in fresh cheese, %	Fat in dry solids, %	R. M. No.	Sap. No.	Acidity in c.c.s *N*/10.KOH per 100 grm. fat
1	.....	.....	.....	.....	3.7	194.1	219
2	.....	.....	.....	.....	8.1	194.2	.....
3	.....	.....	.....	.....	6.7	194.3	.....
4	36.13	62.87	9.89	15.73	6.6	190.9	.....
5	42.78	57.22	11.66	20.28	3.9	200.7	197
6	44.88	55.12	12.03	21.82	3.7	191.1	361
7	38.44	61.56	11.12	18.06	4.3	195.9	204
8	38.08	61.92	8.06	13.02	1.9	201.6	166
9	36.64	63.36	9.28	14.64	2.7	198.9	136

The fat was extracted from the fresh cheese with methylated ether. Schaeffer gives the following variations in the fat content of cheeses:

Camembert	28.03–51.17	(17 samples)
Brie	38.15–50.85	(6 samples)
Neufchâtel	43.28–52.54	(7 samples)
Gervais	64.07–64.38	(2 samples)
Tilsiter	23.07–48.97	(9 samples)
Russian Steppenkäse	44.90–48.18	(2 samples)
Limburger	12.14–47.68	(28 samples)
Romadour	22.56–45.83	(8 samples)

The Analysis of Cheese.—A representative sample must be obtained. In the case of large cheeses of the hard variety 2 or 3 borings with a large cork borer should be made. The part containing the rind and at least half an inch within it is cut off and the remainder cut into fine shavings with a grating machine or bread grater.

Water.—2 to 5 grm. are weighed out into a large flat weighing bottle or platinum dish and dried in a water bath to constant weight.

More accurate results are obtained by drying for 2 days in a vacuum desiccator and then for 8–10 hours in the water bath.

Ash.—This may be estimated by burning off a few grams of the cheese in a platinum dish, first over a burner till the fat is destroyed and finally at a very low red heat in a muffle.

It is, however, preferable to extract the bulk of the fat with warm ether once or twice, pouring the ether off through a small filter paper which is afterward added to the cheese in the dish and burnt. When

an estimation of phosphates and calcium is required, incineration is best carried out in a covered crucible after the addition of an equal amount of a mixture of 1 part of potassium nitrate and 2 parts of sodium carbonate, otherwise a considerable loss in phosphoric acid will occur. An alternative method is to boil the cheese with 30 c.c. of strong nitric acid and 5 c.c. of strong hydrochloric acid, in a flask (fitted with a cooling tube in the neck) until clear. It is then evaporated to dryness, taken up with water, neutralised with strong potassium hydroxide solution, evaporated and incinerated.

Fat.—This may be roughly estimated by the Gerber or Babcock methods. In the former case 0.5 to 1.0 grm. of the cheese are rubbed down in a small beaker with a little warm water, and a drop or two of ammonia, and washed into the Gerber bottle with so much water as will bring the total quantity used up to 10 c.c. The test is then finished as with cream.

For the Babcock method, Lythgoe directs that about 6 grm. should be placed in a beaker and 10 c.c. of boiling water added, an emulsion being made by rubbing with a rod after the addition of a few drops of strong ammonia. About half the usual 17.6 c.c. of sulphuric acid is added to the beaker, and after stirring, the contents are poured into a Bakcock cream bottle, the beaker being washed out with the remainder of the acid. The test is carried out in the usual manner, the reading multiplied by 18 and divided by the weight of cheese taken.

For accurate estimations, the Schmid-Bondyzynski method is the best. This is carried out as follows: 1–2 grm. of the cheese are weighed into a small flask with 5 c.c. of hydrochloric acid (sp. gr. 1.125, or in the case of moist cheeses 1.19), a little powdered sulphur added and the flask boiled gently. The contents are then cooled and washed into the Gottlieb flask with two quantities of 2.5 c.c. of alcohol and then with small quantities of methylated ether till 12.5 c.c. have been used. The contents are mixed by inversion and then 12.5 c.c. of petroleum ether added, and the estimation finished as described under the Gottlieb method for cream.

The Gottlieb method is quite applicable to cheese, but will give only the neutral fat present. As a certain amount of free fatty acids is usually present, the acid method is preferable.

If the fat is to be extracted for further examination, a large quantity of the cheese is dissolved in boiling hydrochloric acid as above described and the fat either extracted with ether or separated by rotation and clarified by passing through dry filter paper.

In any case, as noted above, the nature of the fat extracted will vary somewhat according to the method employed.

Schaffer and Fellenberg (1910) have estimated the constants for the fat extracted from various cheeses by the hydrochloric acid method and with petroleum and methylated ether, and also with these ethers after close admixture of the cheese with calcium chloride. The highest Reichert-Meissl values were obtained by this last method.

The following are some figures given by them for whole and margarine cheeses:

Cheese	Refractometer No. 40°	R. M. No.	Amount of fat employed for R. M. No.	Sap. No.	Iod. value
Margarine	47.12	8.94	2.0	203.6	43.5
Whole	46.8	26.0	2.0	223.4	42.0
Margarine	46.0	5.0	2.5	201.1	45.0
Whole	42.2	27.2	5.0	222.6	41.1
Margarine	41.2	11.25	2.0	215.3	38.6

In all cases the fat was extracted after mixing the cheese with calcium chloride.

The fat is examined by the methods described under butter-fat (Vol. 2, p. 279).

Lactic Acid.—This is usually estimated by rubbing up 10 grm. of the cheese with warm water at 40–50° and making up the volume to 105 c.c. This is filtered clear and 25 c.c. of the filtrate titrated with $N/10$ sodium hydroxide to phenolphthalein.

As stated above, however, accurate estimations of the lactic acid can only be carried out by the conductivity method and the methods of extraction which have been employed are also open to objection.

Total Protein.—From 1–2 grm. are treated by the Kjeldahl-Gunning method in the usual way. The result, however, is not accurate as some of the protein is in the form of amino-acids and ammonia; the usual factor will, therefore, overestimate the protein.

Examination of the Nitrogenous Compounds.—This will not fall under the heading of ordinary analyses, but as the examination may have to be made in certain cases, the following methods are appended. The most complete method is that of Van Slyke:

Twenty-five grams of the sample are ground in a mortar with sufficient quartz sand. The mixture is washed into a flask with about 100 c.c. of water at 50° and is maintained at this temperature for 30 minutes with frequent shaking. The liquid is then decanted through a cotton wool filter into a 500 c.c. flask and the residue exhausted in a

similar way with further quantities of 100 c.c. of water till nearly 500 c.c. are obtained. The flask is cooled to room temperature and made up to the mark, neglecting the layer of fat on the surface.

This stock solution is used for the following estimations:

Water Soluble Nitrogen.—50 c.c. (=2.5 grm. of cheese) of the solution are treated by the Kjeldahl-Gunning method.

Nitrogen as Paranuclein.—To 100 c.c. of the stock solution are added 5 c.c. of 1% hydrochloric acid and the mixture kept at 50–55° till a clear supernatant liquid appears. The precipitate is filtered off, washed with water, and the nitrogen estimated in it.

Nitrogen as Coagulable Protein.—The filtrate from the last is neutralized with dilute potassium hydroxide and heated in boiling water, till any coagulation which may occur is complete. The precipitate is filtered off, washed, and the nitrogen estimated.

Nitrogen as Caseoses.—The filtrate from the last is treated with 1 c.c. of 50% sulphuric acid, saturated with C. P. zinc sulphate and warmed to 70° till precipitation is complete. The mixture is cooled and filtered, the precipitate being washed with the saturated acid solution of zinc sulphate and the nitrogen estimated in it.

Nitrogen as Amines and Peptones.—100 c.c. of the stock solution are placed in a 250 c.c. flask. To this are added 1 grm. of sodium chloride and a 12% solution of tannin until no further precipitation takes place. The flask is filled to the mark, the contents mixed and poured through a dry filter, and the nitrogen estimated in 50 c.c. of the filtrate. This less the nitrogen due to ammonia in a similar quantity, gives the amino nitrogen. Total nitrogen in stock solution—(nitrogen as paranuclein, coagulable protein, caseoses, amines and ammonia) = nitrogen as peptones.

Nitrogen as Ammonia.—100 c.c. of the filtrate from the tannin precipitation are distilled into standard acid and the ammonia passing over estimated.

Nitrogen as Paracasein Lactate.—The residue of the original cheese insoluble in water is treated with several quantities of 5% solution of sodium chloride until 500 c.c. are obtained. The nitrogen is estimated in 50 c.c.

A rather less complicated method of controlling ripening is given by Sanfelici (*Annuario R. Stazione Sperimentale di Caseificio di Lodi*, 1907, 65).

Twenty grams of finely ground cheese are rubbed up with sufficient warm water and placed in a 500 c.c. flask making up to the mark and allowing to stand for a short time. Three layers form, an upper fat

layer, a middle watery layer, and a lower layer consisting of the undissolved substances. The watery layer is drawn off and filtered several times. In 50 c.c. the total quantity of soluble nitrogen is estimated; in another 50 c.c. the albumoses and peptones are precipitated with phosphotungstic acid and the filtrate made up to 100 c.c. In this the amino-acid nitrogen is estimated; the precipitate itself is washed with dilute sulphuric acid, filtered, the residue dried, and the nitrogen estimated. Another 50 c.c. of the original filtrate are diluted with water and distilled with magnesium oxide and the ammonia estimated. The nitrogen in this last is subtracted from the nitrogen obtained in the phosphotungstic acid filtrate.

The volatile fatty acids of cheese are best estimated by mixing 100 grm. of the finely divided cheese with 200 c.c. of water and 1.5–2.0 c.c. of conc. sulphuric acid and steam distilling till 1,000 c.c. have passed over. The filtrate is then titrated with $N/10$ barium hydroxide, and if desired the mean molecular weight can then be found.

Examination of Cheese for the Presence of Iron and Copper.—Cheese often contains traces of both iron and copper. Iron particularly has a prejudicial effect as, if present in as much as 0.0005% (calc. as Fe_2O_3), the marketable condition of the cheese is prejudiced. Copper appears much more seldom and not till 0.001% (calc. as CuO) is reached, is a prejudicial effect on colour noted. The following method of estimating iron in such traces as occur in cheese has been worked out by Schaeffer:

Twenty grams of cheese are kneaded in a porcelain dish with as much ammonia as is necessary to dissolve the whole mass. From 1–2 c.c. are usually sufficient but the cheese must be reduced to a transparent mass and the smell of ammonia must be apparent. Then 5 drops of yellow ammonium sulphide are added, the whole kneaded again, the mass placed on a piece of opal glass and after 10 minutes the colour compared with standards made by adding known amounts (calc. as 0–0.002% F_2O_3) of ferric chloride solution to iron-free cheese.

The detection of small quantities of copper is not easy, but the following method is satisfactory:

About 20 grm. of the cheese are grated up and heated gently with occasional stirring in a porcelain basin with 5 c.c. of strong sulphuric acid (free from copper) for 1 to 2 hours till all spirting has ceased. The dish is then heated strongly for 1 to 3 hours till the mass is thoroughly charred. The char is broken up and again heated till all carbon is burnt off, and the residue is dissolved by repeated extraction with hot dilute

hydrochloric acid. This solution is made slightly alkaline with ammonia and then faintly acidified with dilute hydrochloric acid. Hydrogen sulphide is passed for 1 hour, the liquid then being allowed to stand covered for 12 hours and the gas again passed for 1/2 to 1 hour. The copper sulphide is filtered off and dissolved in hot dilute nitric acid, and ammonia is then added in excess and the solution evaporated or diluted to 15 c.c., filtered if necessary, and made up to 20 c.c. To this are added 1 c.c. of strong acetic acid and 1 c.c. freshly made solution of potassium ferro-cyanide, and the colour compared with a standard solution of copper sulphate, portions of which are made up to 20 c.c. with 10% ammonium nitrate.

Microscopical Examination of Cheese.—For the examination of the bacteria *in situ* in cheese the following simple method of Rodella (*Cent. für Bakt.*, Abt. ii, 1906, **15**, 143) is excellent. The cheese is cut into small cubes and each cube is laid between 2 slightly warmed cover slips or slides and gentle pressure exerted. An impression specimen of the surface of the cube is thus obtained and after washing with chloroform and alcohol to remove fat, the film is stained with very dilute carbol-thionin or methylene blue. (This method is probably due to Johan-Olsen.)

MEAT AND MEAT PRODUCTS.

By W. D. Richardson.

Meat is defined by the Committee on Food Standards of the Association of Official Agricultural Chemists as follows:

1. Meat, flesh, is any clean, sound, dressed, and properly prepared edible parts of animals in good health at the time of slaughter, and if it bears a name descriptive of its kind, composition, or origin, it corresponds thereto. The term "animals," as herein used, includes not only mammals, but fish, fowl, crustaceans, molluscs and all other animals used as food.

2. Fresh meat is meat from animals recently slaughtered and properly cooled until delivered to the consumer.

3. Cold storage meat is meat from animals recently slaughtered and preserved by refrigeration until delivered to the consumer.

4. Salted, pickled, and smoked meats are unmixed meats preserved by salt, sugar, vinegar, spices, or smoke, singly or in combination, whether in bulk or in suitable containers.

5. Manufactured meats are meats not included in paragraphs 2, 3, and 4, whether simple or mixed, whole or comminuted, in bulk or in suitable containers, with or without the addition of salt, sugar, vinegar, spices, smoke, oils or rendered fat. If they bear names descriptive of kind, composition, or origin, they correspond thereto and when bearing such descriptive names, if force or flavouring meats are used, the kind and quantity thereof are made known.

These definitions are in the main, adequate, although No. 1 is very comprehensive and might well be subdivided; No. 3 is less satisfactory, since it embraces practically all fresh meat whatsoever as well as frozen meat. The everyday distinction and scientific distinction also should be made between chilled meat and frozen meat, the former being held at temperatures of a few degrees above the freezing-point until delivered to the consumer, and the latter in the solid frozen condition. Both classes of meat, provided they are delivered to the consumer in good condition, are to be considered fresh meat.

The principal large animals from which civilised countries derive their meat supply are oxen, swine, sheep, goats and horses. Of these

the first 3 are much the most important. Wild game is consumed to a relatively small extent only. Of the other animal foods, those from domestic fowl and eggs take front rank, while fish and other sea foods complete the list.

Of the large domestic animals, goats are used for food purposes to a slight extent only, and horses are slaughtered for food in comparatively few localities. In the United States of America horse meat is an unknown commodity. The use of meat derived from oxen, swine and sheep is practically universal.

In ascertaining the gross composition of the animals used for human food data may be grouped under the following captions: Live weight and dressed weight; blood, viscera, offal, lean meat, fatty tissue and bone. In each of the last three mentioned, moisture, proteins, fat and ash are estimated according to the methods of analysis farther on.

Composition of Meat.—The gross composition of the principal food animals has been treated at length by Lawes and Gilbert (*Phil. Trans.* 1859, **2**, 494), by Wiley (*Div. Chem. U. S. Dept. Agr. Bull.* **53**), and by Atwater and Bryant (*U. S. Dept. Agr. Off. Exp. Sta. Bull.* **28**, Revised).

The following is the percentage composition of the lean of some of the principal kinds of flesh used for food (Munk's *Physiologie*):

	Ox	Calf	Pig	Horse	Fowl
Water	76.7	75.6	72.6	74.3	70.8
Proteins and gelatin	20.0	19.4	19.9	21.6	22.7
Fat	1.5	2.9	6.2	2.5	4.1
Carbobydrates	0.6	0.8	0.6	0.6	1.3
Salts	1.2	1.3	1.1	1.0	1.1

Hence, the lean of meat contains 4 times the proportion of proteins present in milk, and about the same proportion as is contained in the white of egg (page 432).

The following analyses, showing the average composition of fresh meat, are by König:

Description of meat	No. of samples contributing to average	Water	Nitrogenous matters	Fat	Ash
Very fat ox-flesh	7	55.42	17.19	26.38	1.08
Moderately fat ox-flesh	21	73.25	20.78	5.33	1.33
Lean ox-flesh	9	76.71	20.78	1.50	1.18
Fat cow-flesh	9	70.98	19.86	7.70	1.07
Lean cow-flesh	6	76.35	20.54	1.78	1.32
Very fat mutton	3	47.91	14.80	36.39	0.85
Moderately fat mutton	8	75.99	17.11	5.77	1.33
Horse-flesh	12	74.27	21.71	2.55	1.01

A. H. Church[1] gives the following as the composition of a *mutton-chop*, exclusive of the bone, when quite fresh: Water, 44.1; albumin, 1.7; fibrin (true muscle), 5.9; ossein-like substances, 1.2; fat, 42.0; organic extractives, 1.8; mineral matters, 1.0; and other substances, 2.3%.

The following analyses of *animal foods* are also by Church:

	Water	Nitrogenous matters	Fat	Mineral matter	Remarks
Tripe	79.5	10.0	10.0	0.5	The sample was cleansed, boiled, and freed from excess of fat.
Fowl	.....	.....	.....	.....	The nitrogenous matters included ossein-like substances.
Streaky bacon	22.3	8.1	65.2	4.4	The ash includes 3.8% of common salt.
Mackerel	68.7	13.5	12.5	5.3	The ash includes 2.2% of common salt.

J. König gives the following analyses of the flesh of *wild animals* and of *birds:*

	Water	Nitrogenous matters	Fat	Other nitrogen-free substances	Ash
Hare	74.16	23.34	1.13	0.19	1.18
Rabbit	66.85	21.47	9.76	0.75	1.17
Deer	75.76	19.77	1.92	1.42	1.13
Domestic hen	76.22	19.72	1.42	1.27	1.37
Wild duck	70.82	22.65	3.11	2.33	1.09
Partridge	71.96	25.26	1.43	.....	1.39
Pigeon	75.10	22.14	1.00	0.76	1.00

The following analyses of *animal foods* are due to Payen:

Food	Water	Nitrogenous matters	Carbohydrates, etc.	Fat	Ash
Calves' liver	72.33	20.10	0.45	5.58	1.54
Sheep's kidneys	78.20	17.25	1.32	2.12	1.10
Foie gras	22.70	13.75	6.40	54.57	2.58
Lobster (fresh)	76.62	19.17	1.22	1.17	1.17
Oysters	80.38	14.01	1.40	1.51	2.69
Mussels	75.74	11.72	7.39	2.42	2.73

Lawes and Gilbert, in their elaborate essay, "On the Composition of some of the Animals Fed and Slaughtered as Human Food" (*Phil.*

[1] "Food; Some account of its Sources, Constituents and Uses."

Trans., 1859, 2, pages 493 to 680), give a large number of analyses showing the composition of the entire animals and of various parts thereof. Their results show that the total edible parts of the ten animals analysed contained 3.5 parts of fat for 1 of dry nitrogenous matter.

A. H. Church cites the following figures illustrating the composition of cooked *mutton chops*. The 2 analyses are evidently quite independent, and do *not* represent the composition of the same chop, with and without the gravy and dripping.

	Water	Nitrogenous matters	Fat	Mineral matter	Other substances
Cooked chop, including gravy and dripping.	54.0	27.6	15.4	3.0[1]	.....
Cooked chop, exclusive of gravy and dripping.	51.6	36.6	9.4	1.2	1.2

[1] This figure is evidently an estimation by difference, and is in excess of the truth.

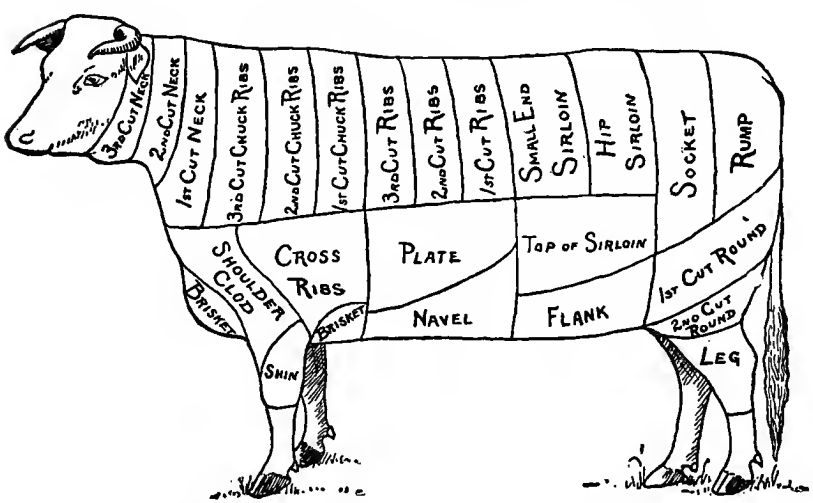


FIG. 11.—Cuts of beef.—(*Nutrition Bulletins, Office of Experiment Stations.*)

The following results were obtained in Allen's laboratory by A. R. Tankard. They represent the composition of various kinds of meat cut from the cold roast joint, and wholly edible. They include such a proportion of fat as would be commonly served and eaten with the lean, but are exclusive of skin, gravy, and dripping.

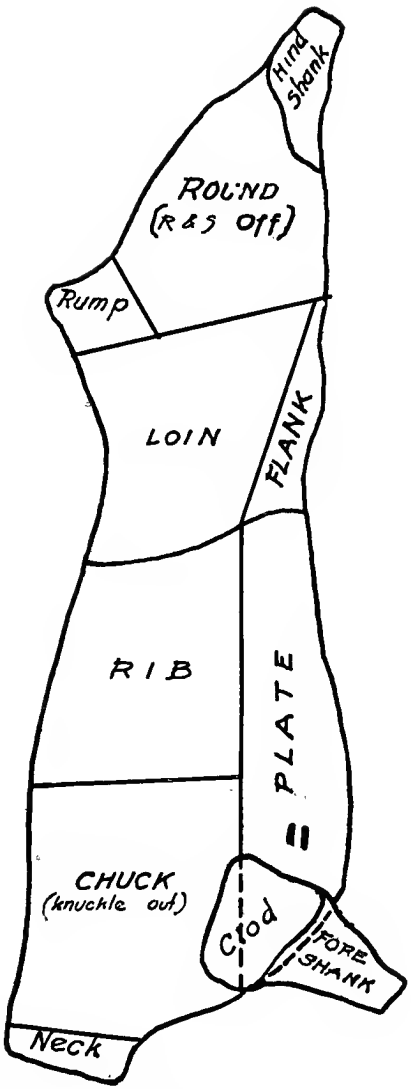


FIG. 12.—Cuts of beef.—(*Bull.* 158, *Illinois Exp. Sta.*)

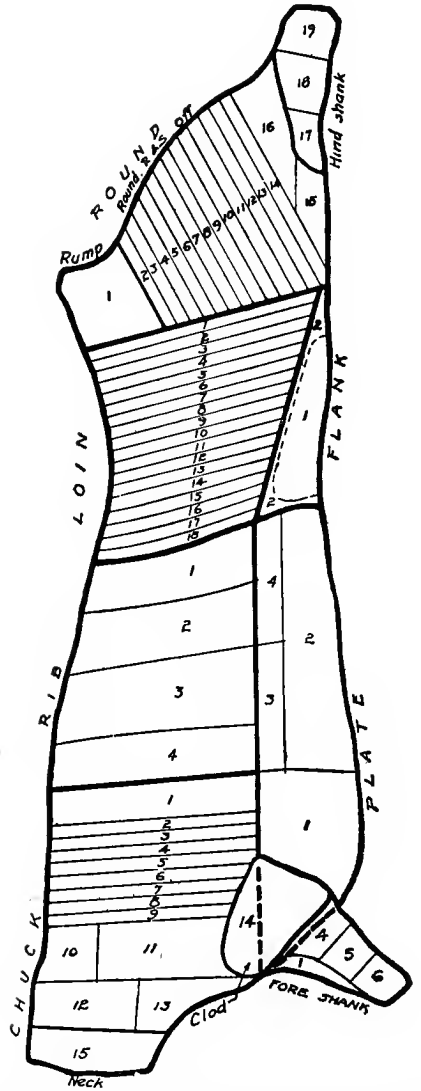


FIG. 13.—Retail cuts of beef.—(*Bull.* 158, *Illinois Exp. Sta.*)

	Mutton	Lamb	Beef	Veal	Pork	Duck	Fowl
Water (loss at 100°)	39.76	59.89	45.63	51.88	44.90	64.13	67.40
Fat (ether extract)	26.80	11.95	24.21	11.39	19.67	6.06	6.68
Proteins (N X 6.3)	29.04	24.69	26.50	32.19	32.63	27.12	24.26
Ash (sulphated)	1.93	1.63	1.21	1.57	1.86	2.04	1.37
	97.53	98.16	97.55	97.03	99.06	99.35	99.71
Cold water extract	3.74	2.81	3.60	6.55	.....	3.70	4.00
Containing ash	0.97	0.92	1.10	1.30	.....	1.20	0.60

Grindley and his coworkers have published many tables of analyses showing the composition of cooked meats (see page 305).

Cuts of Meat.—The methods of cutting up the carcasses of animals vary considerably in different localities and countries and are determined by convenience, custom and subsequent handling. Naturally they are different for different kinds of animals and for different ages and weights of the same kind of animal. The cuts into which the ordinary

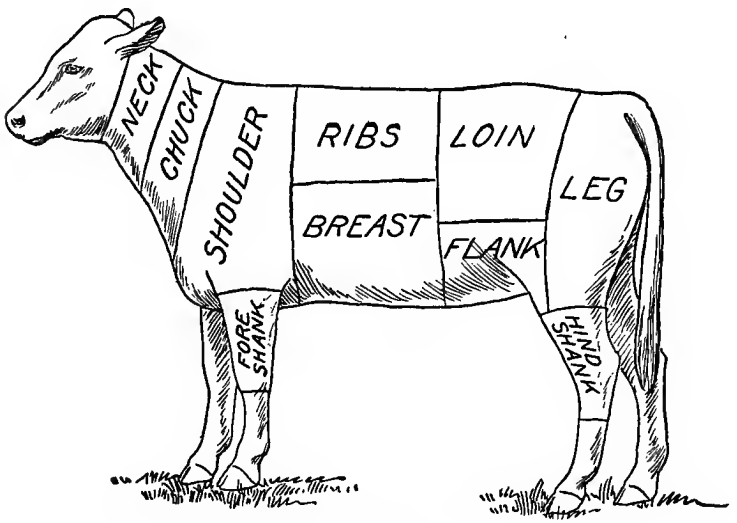


FIG. 14.—Diagram of cuts of veal.—(*Nutrition Bulletins, Office of Experiment Stations.*)

food animals are commonly divided are shown in the following illustrations. For more extended treatment of the subject the reader is referred to the various nutrition bulletins of the Office of Experiment Stations, U. S. Dept. of Agriculture; to Farmer's Bulletin No. 34;

Illinois Agricultural Experiment Station, *Bulletin* 158 (excellent illustrations and tables).

In Fig. 11 is shown a side of beef with the various cuts indicated as used for commercial designation.

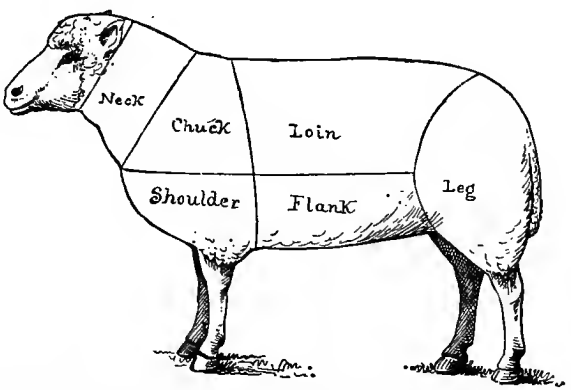


FIG. 15.—Diagram of cuts of lamb and mutton.—(*Nutrition Bulletins, Office of Experiment Stations.*)

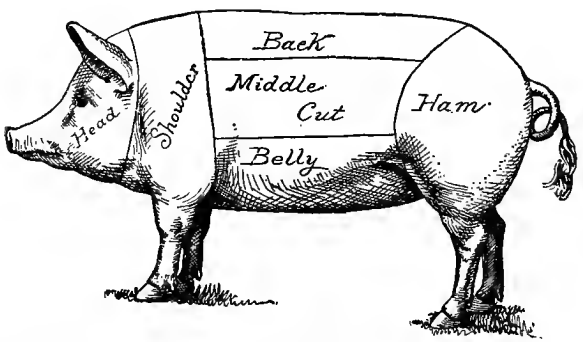


FIG. 16.—Diagram of cuts of pork.—(*Nutrition Bulletins, Office of Experiment Stations.*)

The following tables were compiled by A. E. Leach (*Food Inspection and Analysis*, pages 213–16) from Atwater and Bryant's figures.

COMPOSITION OF BEEF.

Cut	Number of analyses	Refuse	Water	Protein		Fat	Ash	Fuel value per pound cals.
				N × 6.25	By difference			
Chuck: Lean—Edible portion.	2	.....	71.3	20.2	19.5	8.2	1.0	720
As purchased.....	2	19.5	57.4	16.3	15.7	6.6	0.8	580
Medium—Edible portion.	4	.....	68.3	19.6	18.9	11.9	•.9	865
As purchased.....	4	15.2	57.9	16.6	16.0	10.1	0.8	735
Fat—Edible portion.....	4	.....	62.3	18.5	18.0	18.8	0.9	1135
As purchased.....	3	14.7	53.3	15.9	15.4	15.9	0.7	965
Ribs: Lean—Edible portion...	6	.....	66.0	16.5	16.9	9.8	0.8	790
As purchased.....	6	22.6	52.6	15.2	14.8	9.3	0.7	675
Medium—Edible portion.	15	.....	55.5	17.5	17.0	26.6	0.9	1450
As purchased.....	15	20.8	43.8	13.9	13.5	21.2	0.7	1155
Fat—Edible portion.....	9	.....	48.5	15.0	15.2	35.6	0.7	1780
As purchased.....	8	16.8	39.6	12.7	12.4	30.6	0.6	1525
Loin: Lean—Edible portion...	12	.....	67.0	19.7	19.3	12.7	1.0	900
As purchased.....	11	13.1	58.2	17.1	16.7	11.1	0.9	785
Medium—Edible portion.	32	.....	60.6	18.5	18.2	20.2	1.0	1190
As purchased.....	32	13.3	52.5	16.1	15.8	17.5	0.9	1040
Fat—Edible portion.....	6	.....	54.7	17.5	16.8	27.6	0.9	1490
As purchased.....	6	10.2	49.2	15.7	15.0	24.8	0.8	1305
Rump: Lean—Edible portion.	4	.....	65.7	20.9	19.6	13.7	1.0	965
As purchased.....	3	14.0	56.6	19.1	17.5	11.0	0.9	820
Medium—Edible portion.	10	.....	56.7	17.4	16.9	25.5	0.9	1400
As purchased.....	10	20.7	45.0	13.8	13.4	20.2	0.7	1110
Fat—Edible portion.....	5	.....	47.1	16.8	16.4	35.7	0.8	1820
As purchased	5	23.0	36.2	12.9	12.6	27.6	0.6	1405
Round: Lean—Edible portion.	31	.....	70.0	21.3	21.0	7.9	1.1	730
As purchased.....	29	8.1	64.4	19.5	19.2	7.3	1.0	670
Medium—Edible portion.....	18	.....	65.5	20.3	19.8	13.6	1.1	950
As purchased.....	14	7.2	60.7	19.0	18.3	12.8	1.0	895
Fat—Edible portion.....	5	.....	60.4	19.5	19.1	19.5	1.0	1185
As purchased.....	3	12.0	54.0	17.5	17.1	16.1	0.8	1005

COMPOSITION OF VEAL.

Cut	Number of analyses	Refuse	Water	Protein		Fat	Ash	Fuel value per pound, cals.
				N×6.25	By difference			
Chuck: Lean—Edible portion.	1	.....	76.3	.....	20.6	1.9	1.2	465
As purchased.....	1	19.0	61.8	.....	16.7	1.6	0.9	380
Medium—Edible portion..	6	.....	73.3	19.7	19.2	6.5	1.0	640
As purchased.....	6	18.9	59.5	16.0	15.6	5.2	0.8	515
Ribs: Medium—Edible portion.	9	.....	72.7	20.7	20.1	6.1	1.1	640
As purchased.....	9	25.3	54.3	15.5	15.0	4.6	0.8	480
Fat—Edible portion.....	3	.....	60.9	18.7	18.8	19.3	1.0	1160
As purchased.....	3	24.3	46.2	14.2	14.2	14.5	0.8	875
Loin: Lean—Edible portion...	5	.....	73.3	20.4	19.9	5.6	1.2	615
As purchased.....	5	22.0	57.1	15.9	15.6	4.4	0.9	480
Medium—Edible portion..	6	.....	69.0	19.9	19.2	10.8	1.0	825
As purchased.....	6	16.5	57.6	16.6	16.0	9.0	0.9	690
Fat—Edible portion.....	2	.....	61.6	18.7	18.5	18.9	1.0	1145
As purchased.....	2	18.3	50.4	15.3	15.1	15.4	0.8	935
Leg: Lean—Edible portion....	9	.....	73.5	21.3	21.2	4.1	1.2	570
As purchased.....	9	9.1	66.8	19.4	19.3	3.7	1.1	520
Medium—Edible portion..	10	.....	70.0	20.2	19.8	9.0	1.2	755
As purchased.....	9	14.2	60.1	15.5	16.9	7.9	0.9	620

COMPOSITION OF MUTTON AND LAMB.

Cut	Number of analyses	Refuse	Water	Protein		Fat	Ash	Fuel value per pound cals.
				N×6.25	By difference			
Mutton.								
Chuck: Lean—Edible portion.	1	.....	64.7	17.8	18.1	16.3	0.9	1020
As purchased.....	1	19.5	52.1	14.3	14.5	13.1	0.8	820
Medium—Edible portion..	6	.....	50.9	15.1	14.6	33.6	0.9	1700
As purchased.....	6	21.3	39.9	11.9	11.5	26.7	0.6	1350
Fat—Edible portion.....	2	.....	40.6	13.9	13.7	44.9	0.8	2155
As purchased.....	2	16.5	33.8	11.6	11.5	37.5	0.7	1800
Loin: Medium—Edible portion.	13	.....	50.2	16.0	15.9	33.1	0.8	1695
As purchased.....	12	16.0	42.0	13.5	13.0	28.3	0.7	1445
Fat—Edible portion.....	3	.....	43.3	14.7	14.2	41.7	0.8	2035
As purchased.....	3	11.7	38.3	13.0	12.5	36.8	0.7	1705
Flank: Medium—Edible portion.	8	.....	46.2	15.2	14.8	38.3	0.7	1900
As purchased.....	2	9.9	39.0	13.8	13.6	36.9	0.6	1815
Leg: Lean—Edible portion....	3	.....	67.4	19.8	19.1	12.4	1.1	890
As purchased.....	3	16.8	56.1	16.5	15.9	10.3	0.9	740
Medium—Edible portion..	11	.....	62.8	18.5	18.2	18.0	1.0	1105
As purchased.....	11	18.4	51.2	15.1	14.9	14.7	0.8	900
Lamb.								
Chuck: Edible portion.....	1	.....	56.2	19.1	19.2	23.6	1.0	1350
As purchased.....	1	19.1	45.5	15.4	15.5	19.1	0.8	1090
Leg: Medium—Edible portion.	2	.....	63.9	19.2	18.5	16.5	1.1	1055
As purchased.....	2	17.4	52.9	15.9	15.2	13.6	0.9	870
Fat—Edible portion.....	1	.....	54.6	18.3	17.1	27.4	0.9	1495
As purchased.....	1	13.4	47.3	15.8	14.8	23.7	0.8	1295
Loin: Edible portion.....	4	.....	53.1	18.7	17.6	28.3	1.0	1540
As purchased.....	4	14.8	45.3	16.0	15.0	24.1	0.8	1315

COMPOSITION OF PORK, POULTRY AND GAME.

Cut	Number of analyses	Refuse	Water	Protein		Fat	Ash	Fuel value per pound cals.
				N×6.25	By difference			
Pork.								
Shoulder: Edible portion.....	19	.....	51.2	13.3	13.8	34.2	0.8	1690
As purchased.....	19	12.4	44.9	12.0	12.2	29.8	0.7	1480
Loin: Lean—Edible portion...	1	.....	60.3	20.3	19.7	19.0	1.0	1180
As purchased.....	1	23.5	46.1	15.5	15.1	14.5	0.8	900
Fat—Edible portion.....	4	.....	41.8	14.5	13.1	44.4	0.7	2145
As purchased.....	4	16.5	34.8	11.9	10.9	37.2	0.6	1790
Ham: Lean—Edible portion...	2	.....	60.0	25.0	24.3	14.4	1.3	1075
As purchased.....	2	0.9	59.4	24.8	24.2	14.2	1.3	1060
Fat—Edible portion.....	5	.....	38.7	12.4	10.6	50.0	0.1	2345
As purchased.....	5	13.2	33.6	10.7	9.2	43.5	0.5	2035
Poultry and Game.								
Chicken: Edible portion.....	3	.....	74.8	21.5	21.6	2.5	1.1	505
As purchased.....	3	41.6	43.7	12.8	12.6	1.4	0.7	295
Fowl: Edible portion.....	26	.....	63.7	19.3	19.0	16.3	1.0	1045
As purchased.....	26	25.9	47.1	13.7	14.0	12.3	0.7	775
Goose: Edible portion.....	1	.....	46.7	16.3	16.3	36.2	0.8	1830
As purchased.....	1	17.6	38.5	13.4	13.4	29.8	0.7	1505
Turkey: Edible portion.....	3	.....	55.5	21.1	20.6	22.9	1.0	1360
As purchased.....	3	22.7	42.4	16.1	15.7	18.4	0.8	1075
Quail: As purchased.....	1	.....	66.9	21.8	.....	8.0	1.7	775

Identification of Species.—An experienced person can distinguish the meats of the principal food animals, fish, crustaceans, shell-fish, etc., by their appearance, taste, and smell. It would be difficult, however, to describe these differences accurately. The works on meat inspection treat of these matters in some detail. The percentage of glycogen, in many instances, affords means for the detection of horse-flesh. The constants of the fats associated with meats, such as iodine number, saponification number, index of refraction, titer, etc., also afford chemical means of identification in many cases, where the meat is unmixed. For the constants of fats refer to Vol. 2. The analyst of meat food products should be familar with all kinds of meats and meat food products, from the physical and edible standpoint, as well as from the analytical standpoint.

The Precipitin Method.—The fact that more or less specific precipitins can be developed in the blood of one animal by the repeated

FIG. 17.—Nuclei of smooth muscle fibres from the artery of a dog, (*Stöhr.*)

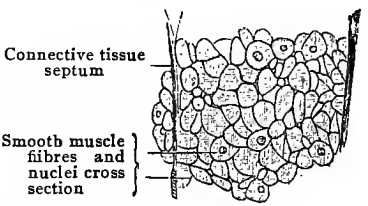


FIG. 18.—Section of the circular muscle coat of the human intestine. × 560. (*Stöhr.*)

or periodic injection of the blood serum from another species of animal, has been proposed as the basis of a method for distinguishing different kinds of meat and for detecting the kinds of meat in a mixture of meats such as sausage. It has been proposed especially as a means for detecting horse meat in sausage, although it can be used for the detection of meat from other species equally well. In brief the method consists in injecting periodically into rabbits, blood or serum from the species for which an antiserum is sought, and after a time preparing the antiserum from the treated rabbits. An extract is them made of the sample and tested *in vitro* with the specific antiserum. Naturally a separate antiserum is generally necessary for each species or variety; however in some cases the same antiserum acts on more than one of several closely related species or varieties.

General Technique.—In order to prepare the antisera a completely equipped bacteriological laboratory with large experimental animals

and a skilled operator are necessary, and this limits the usefulness of the method. Until the specific antisera can be readily purchased the method is necessarily greatly restricted in its application and could be used only in important cases and possibly only in certain legal cases. With reliable antisera available the method could be applied by the ordinary chemist, otherwise he would be precluded from using it. Only the large serum establishments are in a position to prepare the antisera, and without special demand for the sera they would naturally not be prepared except for scientific experiments.

Preparation of the Antiserum.—The special technique for the serum preparation can be only briefly described here. It must be learned under a competent instructor at a properly equipped establishment and requires skill derived from much experience. Rabbits are the experimental animals commonly used. They are injected with defibrinated blood or blood serum from the species for which an antiserum is desired, at 4 or 5 days' intervals, 8 to 15 c.c. being used intraperitoneally or 3 to 8 c.c. by the intravenous method. It is best to work with 5 or 6 rabbits at one time, but the serum from each rabbit is kept separate. When the intraperitoneal method is used there is usually no serious reaction but with the intravenous method the animals sometimes become sick or die. At intervals the blood is tested for its activity by withdrawing 5 c.c. from the large vein of the ear, centrifuging it and working with the clear serum. When sufficiently active and usually 7 to 10 days after the last injection the animal is bled and the clear serum separated after standing 24 hours. A good antiserum should be perfectly clear (not opalescent) and should be highly active. If opalescent it may be clarified by filtration through unglazed porcelain or a layer of kieselguhr on an ordinary filter but it is best to prepare the serum in such a way that it is clear at the start. It is best to preserve the serum by low temperatures (8° to 10°) rather than by antiseptics such as phenol or chloroform. The activity of antisera is measured by determining the dilution of blood in which a precipitate is produced. Thus dilutions are made in 0.85% sodium chloride solutions of 1:500, 1:1000, 1:10,000, 1:20,000, etc., and to 1 c.c. of the diluted blood 0.1 c.c. antiserum is added. The cloud usually appears in 1 to 5 minutes. Very strong and very weak antisera are both likely to cause

FIG. 19.—Muscle fibre of a frog. × 240. *f*, Fibrillæ; *k*, nucleus. (*Stöhr.*)

errors, the strong by producing precipitates in the case of related or remotely related species, and the weak through failure or delay in producing the precipitate. The strong antisera may be weakened with salt solution before use.

Testing for Horseflesh.—The method of testing for horseflesh is typical and the description with slight or no modification will apply to other meats as well. Large pieces of meat can usually be identified by inspection, hence the precipitin method finds its chief application, in food analysis, in the case of comminuted meats such as sausage in which, on the continent of Europe, horseflesh is sometimes used as an adulterant. The method is ordinarily not applicable to any but raw meats; however, reactions have been obtained with rare cooked meat, uncooked cured ham, and old, dry, smoked but uncooked sausage (Nötel, *Z. Hyg.*, 1902, **39**, 373–8; Uhlenhuth, *Deutsche med. Wochenschr.*, **27**, 499–501). Schmidt (*Z. Immunität*, **13**, 166–85) obtained an antiserum by means of blood serum coagulated at 70° then dissolved in dilute sodium hydroxide which reacted with similar but not with normal serum, but no success attended experiments with sodium hydroxide solutions of coagulated muscle proteins. Therefore, for the present at least the method is for the most part, applicable only to raw meats. The following methods are compiled from Nuttal, *Blood Immunity and Relationship;* König, *Nahrungs und Genussmittel*, Vol. 3; and various sources in recent literature to which references are given and to which the reader is referred for more detailed information.

FIG. 20.—Fibrils from the wing muscles of a wasp. (*Schäfer.*)
A, Contracted; *B*, stretched; *C*. Uncontracted. The dark bands are bisected by the light stripes, but they do not show the median membranes.

Method for Whole Pieces of Meat.—While large pieces of meat can generally be identified by inspection, occasion may arise when inspec-

tion may leave a doubt as to the true identity. By the use of sterile knives 30 to 40 grm. of lean meat are removed from the interior of the piece to a sterilized Erlenmeyer flask containing 100 c.c. of sterile 0.85% sodium chloride solution. The extraction proceeds with fresh meat 1 to 3 hours at room temperature or over night in the ice-box. With smoked, pickled or decomposed meat the extraction is longer—even to 24 hours. Very salt meat can be freshened by immersion in sterilized distilled water before extraction. Agitation is to be avoided during the extraction period. The extract is generally usable when it foams on shaking in a test-tube. The extract is clarified by filtration through ignited kieselguhr stirred up in a sterile salt solution and poured over a hard filter on a Büchner funnel or through unglazed porcelain. The test is carried out by means of sterile glassware throughout. The various test-tubes contain:

No. 1, 1 c.c. extract to be tested.
No. 2, 1 c.c. extract to be tested.
No. 3, 1 c.c. known extract of horse meat.
No. 4, 1 c.c. known extract of beef.
No. 5, 1 c.c. known extract of pork.
No. 6, 1 c.c. sterile 0.85% NaCl solution.

All the extracts are prepared as directed for horse meat. To each tube with the exception of No. 2 is added 0.1 c.c. clear, active horse-antiserum in such a way that it runs down the sides of the tubes to the bottom without mixing. Tube No. 2 is treated in the same way with 0.1 c.c. normal rabbit-serum. A cloudiness should be obtained in 1–2 minutes in tube 3 and also in 1 if the meat is in fact horse meat. In 5 minutes this cloudiness increases and in 10 minutes longer settles to the bottom as a precipitate. No indications after 20 minutes are to be considered.

Method for Comminuted Meat, Sausage.—The sample is taken from the centre of the piece or mass and extracted as above. Or it is rubbed in a mortar and then extracted. Sometimes it is advisable to extract first with ether. Uhlenhuth in some cases recommends squeezing through cheesecloth or similar material and adding the press-juice to the extract and finally filtering as before. The extraction of sausage and ground meats is much more difficult than a single lean piece and the operator must be guided accordingly. The test proceeds as stated above except that on account of the smaller amount of horse meat present a very active serum is necessary (1 : 20,000 is active enough to detect 5% addition of horse meat). In addition to the test-tubes

enumerated above two are added containing respectively extracts of beef and horse sausage.

The specificity of antisera is not absolute in all cases. Uhlenhuth obtained a positive reaction with antisera for pig, sheep, horse, donkey and cat blood, when these were tested on the corresponding meats. However, the antisheep serum gave almost as great a reaction with goat's meat as with sheep's and less with beef extract. A similar fact is that the blood of various species of monkeys reacts with antihuman serum but to a lesser extent than human blood. Very active antisera are less specific than average, and tests in dilute solution are more specific than in concentrated, although there is a fairly definite limit to the dilution which is practicable. Closely related species such as

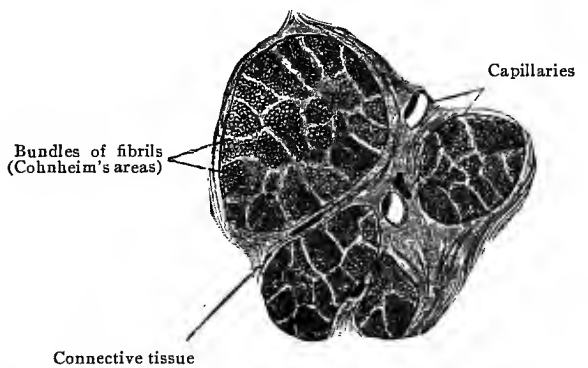


FIG. 21.—Cross-section of four muscle fibres of the human vocal muscle. ×590. (*Stöhr.*)

horse and ass, and dog and fox are difficult or impossible to distinguish by the method.

The precipitate, formed between precipitin and antigen, is derived principally from the antiserum. Thus Welch and Chapman (*Z. Immunität*, **9**, 517–29; *J. Hyg.*, **10**, 177–83) consider it incorrect to speak of coagulation of antigen or to look upon the antigen as the precipitable substance. When the precipitation is complete the weight of the precipitate is independent of the amount of antigen but with partial precipitation the weight of the precipitate is determined by the amount of antigen.

For further discussion of the precipitin method in food analysis and legal cases the chemist may consult Saint-Sernin, Biological Methods

for Distinguishing the Various Kinds of Meat, *Ann. fals.*, **4**, 334–8; Schmidt, *Cairo Sci. J.*, **5**, 271–89.

Structure of Muscle.—The lean of meat, or flesh in the restricted sense of muscular contractile tissue, consists anatomically of numerous small thread-like fibres, the *muscular fibres* lying side by side and united and supported by the *connective tissue.* The connective tissue carries the blood-vessels and nerves and contains among its constituents the gelatin-forming substance, collagen, other proteins such as

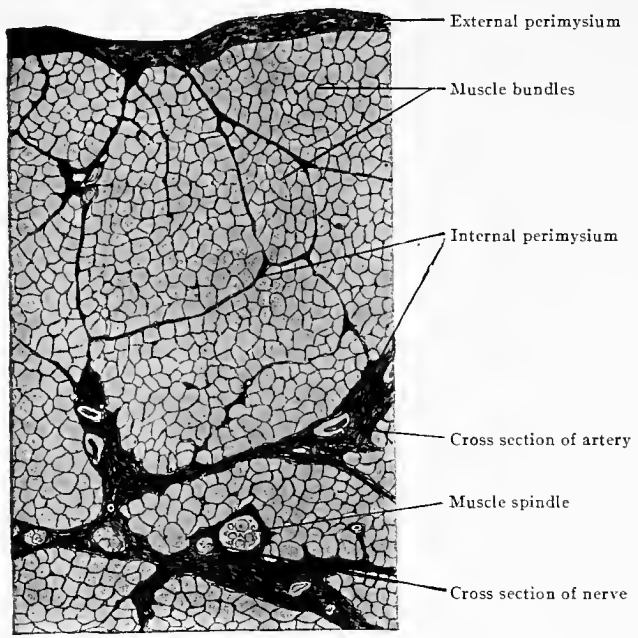


FIG. 22.—From a cross section of the Omohyoid muscle of man. ×60. (*Stöhr.*)

glycoprotein, albumin, and fat. The muscle fibres themselves consist of a delicate structureless covering membrane corresponding to a cell wall, the *sarcolemma,* composed of a protein similar to elastin, and the enclosed sarcoplasm or muscle plasma.

Microscopically, three sorts of muscle fibres are distinguished, corresponding to voluntary or striated, smooth and heart muscle. Voluntary, striated or skeletal muscle is by far the most important and

abundant in the animal body, composing the muscles attached to the skeleton, by which the principal movements of the body are accomplished. Since it forms the major part of meat food products, it is the one of chief interest to the food chemist and analyst. Under the microscope it appears to be formed of numerous parallel cylindrical fibres about 0.05 mm. in diameter and about 18 to 36 mm. long. The characteristic cross striations, consisting of alternate dark and light bands and under certain conditions, fine longitudinal markings, are visible under moderately high powers. The striated muscle fibres are polynuclear and each contains, just below the sarcolemma, numerous oval nuclei. These various structures are shown more prominently by proper staining as by eosin and hæmatoxylin. Still finer structures are observable by more minute microscopic examination, aided by appropriate chemical treatment. Each muscle fibre is seen to consist of minute fibrillæ (sarcostyles) which can be split transversely into numerous discs (sarcous elements). (Compare Figs. 17 to 23.)

Macroscopically the striated muscular fibres are bound together by connective tissue successively into fasciculi, bundles and muscles.

Smooth or non-striated muscle is found principally in the gastrointestinal tract, the blood vessels, genito-urinary tract, and skin. It consists of spindle-shaped cells, about 1/10 by 1/200 mm., each of which contains a central rod-shaped nucleus. Fine longitudinal markings can sometimes be observed.

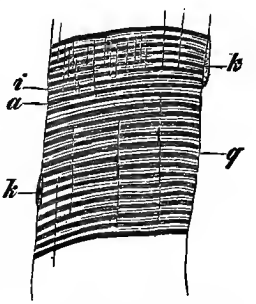


FIG. 23.—Part of a longitudinal view of a human striated muscle fiber. (*Stöhr.*)

a, Anisotropic; *i*, isotropic band; *k*, nucleus; *g*, ground membrane. ×560.

Heart muscle occurs only in the heart. It consists of short, cylindrical, branching cells, attached end to end. Each cell contains a single oval nucleus. The cells show finer cross striations than striated muscle and also fine longitudinal markings.

Composition of Meat.—"Lean meat," that is, muscular tissue, with all the prominent connective tissue, including fat, removed, contains approximately 75% water and 25% solids, in all vertebrate animals. Of the solids approximately 80% are protein and the remaining 20% consist of various water-soluble organic substances conveniently called "extractives," and inorganic salts. This gross composition can be represented in tabular fashion as follows:

Muscular tissue { Water 75% ; Solids 25% { Proteins 20% ; Extractives 4% ; Inorganic salts 1%

During the life of an animal the contractile substance of the muscles has a semi-fluid consistency and contains proteins which are somewhat different from those found after death. By pressing the ice-cold purified muscle of the frog immediately after death under suitable conditions, Kühne obtained the muscle plasma as a syrupy liquid of faintly alkaline reaction which at ordinary temperature soon clotted after the manner of blood plasma. The clot consists of *myosin*, which bears the same relation to the proteins of living muscle that blood fibrin does to fibrinogen.

Human muscle is stated by W. D. Halliburton to contain 73.5% of water and 26.5% of solids, these latter consisting of:

Proteins; including sarcolemma, proteins of connective tissue, vessels and pigments	18.02
Gelatin, Fat } from the connective tissue of muscle	1.99, 3.27
Extractives; creatine, lactic acid, glycogen, etc	0.22
Inorganic salts	3.12
	26.62

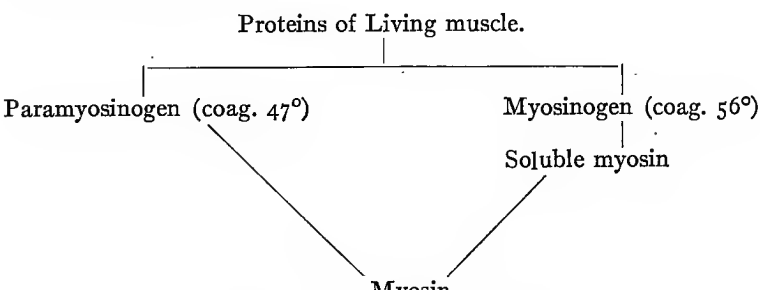
According to J. König, the following represents the general composition of *pure muscle* freed from adherent fat, etc.

Water		75.0 to 77.0
Nitrogenised compounds,	Sarcolemma (muscle fibre)	13.0 to 18.0
		2.0 to 5.0
	Albumin	0.6 to 4.0
	Creatine	0.07 to 0.34
	Hypoxanthine	0.01 to 0.03
	Creatinine, Xanthine, Inosinic acid, Uric acid	Undetermined
	Urea	0.01 to 0.03
Fat		0.5 to 3.5
Other Nitrogen-free compounds,	Lactic acid	0.05 to 0.07
	Butyric acid, Acetic acid, Formic acid, Inositol	Undetermined
	Glycogen	(0.3 to 0.5)
Salts		0.8 to 1.8
Composed of:		
	Potash	0.40 to 0.50
	Soda	0.02 to 0.08
	Lime	0.01 to 0.07
	Magnesia	0.02 to 0.05
	Oxide of iron	0.003 to 0.01
	Phosphoric acid	0.40 to 0.50
	Sulphuric acid	0.003 to 0.04
	Chlorine	0.01 to 0.07

The following table by Hofmann (*Lehrbuch der Zoochemie*) shows the average composition, in parts per 1,000, of the *muscles of vertebrate animals:*

	Mammals	Birds	Cold-blooded vertebrates
Water	745 to 783	717 to 773	800
Organic matters.			
Coagulated albumin, sarcolemma, nuclei, vessels.	145 to 167	150 to 177	?
Alkaline albuminate	28.5 to 30.1	.....	.....
Creatine	2.0	3.4	2.3
Xanthine and hypoxanthine	0.2	.....	.....
Taurine	0.7 (horse)	0.0	1.1
Inositol	0.03	.....	.....
Glycogen	4.1 to 5.0	.....	30 to 50
Lactic acid	0.4 to 0.7	.....	.....
Salts.			
Potash	3.0 to 3.9	.....	.....
Soda	0.40 to 0.43	.....	.....
Lime	0.16 to 0.18	.....	.....
Magnesia	0.40 to 0.41	.....	.....
Oxide of iron	0.03 to 0.10	.....	.....
Phosphoric acid	3.4 to 4.8	.....	.....
Sodium chloride	0.04 to 0.10	.....	.....

According to W. D. Halliburton the proteins of the living muscle in mammals are two in number: *paramyosinogen*, which coagulates at about 47° and *myosinogen*, which coagulates at about 56°. The relationships between the proteins of living and dead muscle are shown in the following diagram:



(The protein of the muscle clot.)

That this diagram is only approximate and represents but a part of the truth is indicated by the various results obtained by extracting dead muscle with pure water and with various concentrations of neutral salts. These results indicate that the term myosin covers a mixture of several proteins and not an individual. Przibram[1] has found that paramyosinogen and myosinogen are present in vertebrates generally, but that paramyosinogen only, is present in invertebrates. In the muscle plasma of fishes is found, in addition to the two principal proteins, a peculiar one, myoprotein, which precipitates on dialysis and with acetic acid, but does not coagulate when heated. Myosinogen in

[1] *Beiträge chem. Phys. u. Path.*, 1902, 2, 143.

coagulating to myosin passes through an intermediate stage known as soluble myosin whereas paramyosinogen passes directly to myosin. The coagulation of the proteins of living muscle upon the death of an animal is coincident with the setting in of the rigid condition known as *rigor mortis*.

The voluntary muscles of mammals are commonly classified into two varieties which are known respectively as red muscle and pale muscle, depending on the depth of red colour which they possess. Prominent examples of pale muscles are the white meat of domestic fowl and of rabbit. The red colour of muscles has been shown to be due to oxyhæmoglobin dissolved in the muscle plasma. The presence of other colouring matters in muscle, possibly derivatives of hæmoglobin, has been claimed by some observers and disputed by others, as having been produced by the reagents used in the separation.

The term nucleo-protein or nuclein is applied to a large group of related substances found in cell nuclei. They are not quantitatively of great importance in muscular tissue. They contain considerable phosphorus (chiefly as lecithin) and some sulphur. By hydrolysis with alkalies they yield proteins and nucleic acids. With boiling acids the true nucleins yield phosphoric acid and the xanthine bases. They are insoluble in water, alcohol, and ether, but usually dissolve in alkalies. (See p. 73.)

The heterogenous group of organic substances known collectively as "extractives" of muscle are comprised in the following list. Some of them, although present in flesh foods in small quantity, are of considerable importance to the analyst.

Muscle Extractives.

Non-nitrogenous Extractives.	Nitrogenous Extractives.
Glycogen	Creatine
Dextrin and sugars	Creatinine
Lactic acids	Xanthine
Inositol	Hypoxanthine
	Uric acid
	Urea
	Carnine
	Inosinic acid
	Taurine

Fat is sometimes included under non-nitrogenous extractives but can best be treated separately. However, it is always found inti-

mately associated with muscular tissue. Many of these substances are brought into solution when meat is extracted with boiling water and hence occur in the various meat extracts which are found on the market. (See Meat Extracts—Creatinine and Creatine, page 410.) The nitrogenous extractives are intimately associated with protein metabolism and are of greater importance to the physiologist than to the analyst. Only those extractives which are relatively important from the analytic standpoint will be considered specifically.

Non-nitrogenous Extractives.—Glycogen $(C_6H_{10}O_5)_n$, together with its hydrolytic products, dextrin, maltose, and dextrose, is constantly present in muscular tissue in small quantity. As the sugars are constantly consumed in metabolic processes, it follows that glycogen is constantly forming. It is not uniformly distributed through the musculature and is present in certain organs—notably the liver—in greater quantity than in others. Neither is it present in the same proportion in different animals, and since among the large food-animals it is present in horse meat to a greater extent than in other meats, its estimation has been used as the basis of a method for the detection of horse-flesh. Glycogen is a white, amorphous, odourless and tasteless substance, as prepared from animal tissues, of which the liver of the dog is one of the most suitable (Abderhalden, *Biochemische Arbeitsmethoden* **2**, 162). It gives an opalescent solution with water, can be boiled with strong potassium hydroxide solution without decomposition and is hydrolysed by mineral acids and diastase into dextrose.

Glycogen is strongly dextrorotatory, the value of $[\alpha]_D$ being $+196.6$.

Glycogen does not reduce Fehling's solution. It is precipitated by barium hydroxide as $BaO(C_6H_{10}O_6)_3$, and by basic lead acetate as $PbO(C_6H_{10}O_6)_2$.

When boiled with dilute nitric acid, glycogen yields oxalic acid. Boiled with dilute sulphuric or hydrochloric acid, it is converted into dextrose. It does not ferment with yeast, but diastase and saliva convert it into maltose and erythro-dextrin, a little dextrose being also formed. On the other hand, in the hydrolysis of glycogen in the liver, dextrose and not maltose is the chief product. (Compare p. 376.)

Quantity of Glycogen in Flesh.—During life the amount of glycogen in muscular tissue represents the balance between the quantity formed and that hydrolysed and is dependent upon a number of circumstances among which are diet and work. The amount found varies with the kind of animal and the kind of tissue; and since the hydrolysis of glycogen through the agency of enzymes goes on after death, it is also dependent on the age of the meat.

A. Bujard (*Forsch. Ber.*, 1897, **4**, 47; abst. *Analyst*, 1897, **22**, page 160) has published the following determinations of glycogen made by the method of Niebel and Salkowsky (*Zeit. Fleisch und Milkhyg.*, 1891, 185).

	%		
	Water	Glycogen; direct	Glycogen on dried substance
Horse flesh	61.83	0.846	2.24
Horse flesh	72.90	0.174	0.64
Horse flesh	70.47	1.366	4.62
Horse flesh	71.84	0.59	2.09
Horse flesh smoked	43.00	0.108	0.19
Beef (ox)	73.62	0.206	0.74
Beef	75.55	0.018	0.073
Veal	76.12	0.346	1.44
Veal	74.47	0.066	0.25
Pork	54.05	trace	trace
Pork	66.29	.....	.....

Bujard also reports the following figures which were obtained by later methods. These methods, however, have now been superseded by Pflüger's. (See below and compare p. 378.)

	Water	Glycogen direct		Glycogen in dried substance	
		Niebel method	Mayrhofer method	Niebel	Mayrhofer
Horse flesh	78.44	0.440	0.445	1.721	1.741
Horse flesh	74.87	0.600	0.520	2.388	2.069
Horse flesh	76.17	1.827	1.727	7.667	7.247
Horse flesh	76.00	0.592	0.610	2.466	2.542
Veal	74.6	.....	0.086	.....	0.342
Pork	75.6	.....	0.186	.....	0.744

W. Niebel (*Fleisch. Milch Hyg.*, 1891) gives the following figures showing the amounts of glycogen found in the flesh of various animals. The analyses were made by the older methods.

Kind	Age	Glycogen
Horse meat	3 hours	0.700%
Horse meat	3 hours	1.026%
Horse meat	1 day	0.373%
Horse meat	2 days	0.603%
Horse meat	3 days	0.523%
Horse meat	4 days	0.524%
Horse meat	5 days	1.072%
Horse meat	5 days	0.460%
Beef	4 hours	0.204%
Beef	1 day	0
Beef	2 days	0
Beef	½ hour	trace
Beef	5 days	0.076%
Pork	4 hours	0
Pork	2 days	0
Mutton	2 days	0

These figures show, in general, the decrease of glycogen in stored meat, and at the same time the varying amounts present in the same kind of meat and the irregularity of the decrease.

Niebel also reported the following figures showing the amounts of glycogen and dextrose in various kinds of meat of different ages.

Kind of meat	Age, days	On the original basis				On the dry and fat-free basis		
		Water, %	Fat, %	Dextrose, %	Glycogen, %	Glycogen, %	Dextrose, %	Total carbohydrates as sugar, %
Horse....	8	75.2	3.5	0.417	0.812	3.810	1.957	6.190
Horse....	.....	75.2	2.6	0.263	0.532	2.396	1.139	3.801
Horse....	.....	75.3	2.6	0.142	0.744	3.397	0.648	4.421
Horse....	.....	71.7	6.6	0.180	0.940	4.782	0.828	6.151
Horse....	8	71.9	7.1	0.222	0.606	2.886	1.057	4.387
Beef.....	.....	75.3	3.6	0.066	trace	trace	0.314	0.314
Beef.....	1	75.3	3.6	0.190	trace	trace	0.900	0.900
Beef.....	.....	75.3	3.6	0.036	0.164	0.777	0.177	1.033
Beef.....	.....	75.3	3.6	0.071	0	0	0.336	0.336
Beef.....	.....	75.3	3.6	0.070	0	0	0.331	0.331
Veal.....	1	78.8	0.9	0.210	0	0	0.336	0.336
Veal.....	1	78.8	0.9	0.250	0	0	0.331	0.331
Pork.....	.....	73.8	5.1	0.156	0	0	0.739	0.739
Pork.....	2	73.8	5.1	0.100	0	0	0.479	0.479
Pork.....	.....	73.8	5.1	0.100	0	0	0.985	0.985
Mutton...	.....	66.4	14.4	0.005	0	0	0.052	0.052
Mutton...	.....	70.0	8.0	0.171	trace	trace	0.777	0.777

Niebel also gives figures for various sausages with and without horse flesh.

Because of the relatively large amount of glycogen in horse flesh, its determination has been used for the purpose of detecting horse meat in mixtures of meats with some success. W. Niebel considers the presence of more than 1% dextrose, after conversion of the other carbohydrates, on the basis of the fat-free meat, to be proof of the presence of horse flesh, in the absence of starch. However, because of the continued decomposition of glycogen after death, the failure to find more than this equivalent does not necessarily prove the absence of horse meat and the very general practice of using sugar in the curing of meats introduces another uncertain factor.

Detection of Glycogen.—Microscopically glycogen can be detected in tissue preparations by means of Lugol's solution (iodine 1, potassium iodide 2, distilled water 200); but it should be noted that previous contact with water and also contact with the water of the test solution will dissolve the glycogen. Hence, tissue should be hardened in alcohol, not in water solutions. The iodine stains the glycogen reddish-brown.

Chemically, use is made of the iodine reaction also for the detection

of glycogen. Depending on the concentration of the glycogen solution in water, the colour produced by iodine varies from yellowish-brown, through reddish-brown to deep red. 50 to 100 grm. of the finely ground sample are boiled with an equal weight of water for 20 minutes and the extract strained through fine cheese cloth free from starch. To the filtrate a few drops of iodine solution (iodine 2, potassium iodide 4, water 100) are added. The reddish-brown colour produced disappears on heating and reappears on cooling. (Compare p. 377.)

Pflüger makes use of 2 test-tubes of equal diameter, placing respectively an equal volume of water and the glycogen solution in them. Both are then heated to the same temperature for the same period, whereupon the colour disappears from the one containing glycogen and, on cooling, it reappears. In the presence of certain impurities the colour disappears slowly in the cold, rapidly on heating and does not return. Evidently the iodine, under these circumstances, enters into chemical combination with the impurity. To avoid this result 10 c.c. of the impure solution are brought to a concentration of 3% potassium hydroxide and 10% potassium iodide, 50 c.c. of 96% alcohol added, and filtered. The filter is washed first with a mixture of 1 volume of 3% potassium hydroxide and 10% potassium iodide in water, and 1/2 volume of 96% alcohol, then with 60% alcohol, finally with nearly absolute alcohol (97.8%). The precipitate is dissolved in water, the alcohol removed by evaporation on the water-bath, and the solution neutralised with acetic acid. Thus freed from the interfering impurity the solution is used for the glycogen test previously described.

If starch is known to be present or is suspected, it can be precipitated by treatment with twice the volume of acetic acid (glacial), filtered off, and the test for glycogen applied to the filtrate.

Estimation of Glycogen.—Pflüger's (E. Pflüger, *Das Glycogen*, 1905; *Pflüger's Archiv.*, 1906, **114**, 242) methods for the separation and estimation of glycogen have superseded the older ones and these have now only a historic interest.

His methods will be followed here.

Separation of the Glycogen.—50 grm. of the finely hashed sample are treated in a flask with 50 c.c. of 60% potassium hydroxide and digested in a boiling water-bath for 3 hours with occasional thorough agitation. The solution is cooled and transferred to a beaker, 100 c.c. of water being added, a part of which is used to rinse the digestion flask thoroughly. The glycogen is precipitated with 2 volumes of 95% alcohol (200 c.c.). The precipitate is allowed to settle (not more than 24 hours) and the supernatant liquid is poured through a

filter. The precipitated glycogen is now agitated three times successively with considerable 60% alcohol containing a few drops of saturated sodium chloride solution (equal to 7 mg. NaCl per litre), settled, and the wash-alcohol decanted through the filter. This treatment is followed by two treatments with 96% alcohol, one with absolute alcohol, three with ether and one with absolute alcohol. The glycogen on the filter is washed into the beaker containing the rest of the glycogen, with hot water, and the whole brought into solution by stirring with water.

Estimation of Glycogen.

Three methods are available: (*a*) direct, (*b*) as dextrose after hydrolysis, (*c*) by means of polarisation.

(*a*) **Direct.**—2 mg. sodium chloride per 100 c.c. water are added and the glycogen is reprecipitated with 2 volumes of 95% alcohol. The precipitate is brought on a filter, washed with 95% alcohol containing 7 mg. sodium chloride per litre, then with absolute alcohol and finally with ether. It is dried to constant weight.

(*b*) **By Hydrolysis and Estimation as Dextrose.**—The glycogen solution is neutralised with hydrochloric acid of sp. gr. 1.19, drop by drop, and washed with hot water into a 500 c.c. flask, 25 c.c. of hydrochloric acid of sp. gr. 1.19 are added and the volume made up nearly to the mark, leaving sufficient space for neutralising after the inversion. The solution will contain approximately 2.2% hydrochloric acid. Hydrolysis is accomplished by digestion in a boiling water-bath for 3 hours, after which the solution is made slightly alkaline by adding 60% potassium hydroxide solution drop by drop from a burette. The volume is made up to the mark and reducing sugar (dextrose) determined by Allihn's method or better Pflüger's improvement of this method (Abderhalden, *Biochemische Arbeitsmethoden*, **2**, 174). (See Vol. I.)

To obtain the amount of glycogen, the dextrose result by the gravimetric method is multiplied by 0.927.

(*c*) **By Polarisation.**—The faintly alkaline glycogen solution is made weakly acid, and disregarding the slight precipitate which forms, is washed into a volumetric flask of 100 or 200 c.c. capacity. An aliquot is filtered and used for the polarisation. The specific rotation for glycogen is 196.6°. It is best to take the mean of several readings and also to take readings at 1/2 and 1/4 the original concentration. Pflüger believes the rotation depends not only upon the concentration but also the size of the colloidal particles.

Dextrin and Sugars.—These, as mentioned, are hydrolytic products

of glycogen. They do not remain long in the tissues but soon enter into metabolic processes, the sugar (dextrose) being destroyed by a glycolytic enzyme.

Estimation of Reducing Sugar (Dextrose).—100 grm. of the finely hashed meat are extracted successively with 200, 100 and 100 c.c. of water by boiling in an evaporating dish. Each time the solution is poured through fine linen or cheese cloth and the meat wrung out by hand in the same medium (suitable precautions being taken) into a 500 c.c. flask. The solution is clarified with lead subacetate, the lead removed by sodium sulphate in an aliquot and the reducing sugar determined as dextrose by Allihn's or other standard method.

Lactic Acid, Hydroxypropionic Acid, Ethylidene Lactic Acid, $CH_3.CHOH.COOH$.—The 3 forms, dextro-, lævo-, and inactive ethylidene lactic acids are of biological interest, but only the dextro modification (para- or sarco-lactic acid) appears to be present in animal tissues and secretions. To it the acidity of meat (following *rigor mortis*) is due. It is of minor importance to the analyst, who, for methods of preparation and estimation can consult Vol. 7, pp. 437–440. The total quantity in flesh varies from 0.1 to 1.0%.

Inositol, $C_6H_{12}O_6$ or $C_6H_6(OH)_6$, is an aromatic compound formerly classified with the hexoses and found in many plant tissues as well as in animal tissue. It is the optically inactive modification which is found in the latter. The dextro and lævo modifications are known. Inositol is non-fermentable and does not reduce Fehling's solution, although it changes its colour. Horse flesh contains 0.003% inosite (Jacobsen).

Nitrogenous Extractives.—E. Fischer (*Ber.*, 1897, **40**, 549, 559, 1839, 2226, 2604, 3089) classified many of the nitrogenous extractives of meat under the term "purine bases" considering them as derivatives of purine $C_5N_4H_4$. The relationships will not be considered in detail here, but can be ascertained by reference to the literature.

They are formed not only as products of protein metabolism, but some of them also as hydrolytic products of proteins (especially nucleins) and as the result of bacterial decomposition of the proteins. At one time some of the purine bases were confused with the more active poisonous products of bacteria (the toxines), and the terms "ptomaïnes" and "leucomaines" were introduced, the former referring to poisonous basic substances produced by bacteria and the latter to basic substances produced by metabolism in the animal body. The word ptomaïne has persisted and is still currently used especially in the expression "ptomaïne poisoning" which is popularly applied by physicians and

laymen to various sorts of sickness resulting from eating spoiled animal and vegetable foods.

Caffeine, theobromine and other bases derived from plants are closely related to the meat extractives of the purine group.

Creatine.—Creatine, methylglycocyamine, methylguanidine acetic acid, $C_4H_9O_2N_3$, $NH{:}C\langle^{NH_2}_{N(CH_3)CH_2COOH}$, is constantly present in flesh, in which it is found to the extent of 0.1–0.35% and with creatinine is an especially characteristic constituent of meat extracts (*q.v.*). It was discovered by Chevreul in 1834 in meat extract and was investigated by Liebig in 1847. It crystallises with 1 molecule of water in colourless rhombic prisms, m. p. 100°, with loss of water. It has a neutral reaction in water, in which it dissolves readily when the water is heated, and a slightly bitter flavour. It dissolves in alcohol less easily than in water and in ether is nearly insoluble. When its aqueous solution is acidified and digested, creatine is completely converted into its anhydride creatinine. With barium hydroxide solution it takes up the elements of water, forming urea and sarcosine.

The amounts of creatine reported in flesh of various kinds are as follows (König, *Nahrungs und Genussmittel*, **2**, 422): horse, 0.07–0.22%; rabbits, 0.21–0.34%; swine, 0.12%; cattle, 0.19–0.28%; duck, 0.20%; chickens, 0.21–0.33%.

Creatine can be prepared from muscular tissue (or better from meat extract) by extracting with boiling water, filtering, clarifying with lead acetate, filtering, removing the excess of lead with hydrogen sulphide, filtering, evaporating at low temperature and purifying the crystals which separate by recrystallising.

Methods for the estimation of creatine are given under meat extract (*q.v.*). (Compare Vol. 7, p. 310.)

Creatinine, methylglycocyamidine, $C_4H_7ON_3$, $NH{:}C\langle^{NH{-}CO}_{N(CH_3)CH_2}$, is invariably present in flesh along with creatine. It is, therefore, present in beef extract and in larger proportion than creatine. It also occurs constantly in urine (0.25%). It can be prepared from creatine by digestion of the aqueous solution with acids. Creatinine crystallises in colourless rhombic prisms and dissolves easily in cold water and alcohol. It is a much stronger base than creatine, can expel ammonia from ammonium salts and forms crystalline salts with acids. Bases cause creatinine to take up water with formation of creatine. Boiled with barium hydroxide it is decomposed into methylhydantoin and ammonia. Sodium phosphomolybdate precipitates it from acid solu-

tion. Zinc chloride forms a characteristic compound with it which is precipitated from solutions as a crystalline powder scarcely soluble in water and insoluble in alcohol. It is also precipitated by picric acid from concentrated solutions and with the same reagent in alkaline solutions gives a reddish-yellow colour which is made the basis of Folin's method for its estimation. (See under meat extracts, page 389.)

A method for the estimation of creatinine is given under meat extracts (compare Vol. 7, p. 316).

Xanthine, 2:6-*Dioxypurine*, $C_5H_4O_2N_4$, is widely distributed in animals and plants, being found in muscle, liver, spleen, pancreas, and in potatoes, beets and tea. It was discovered by Marcet in urinary calculi in 1823. As prepared from flesh it is white and amorphous or forms granular masses of crystalline leaves or, with 1 molecule of water, it crystallises in rhombic plates. It is insoluble in alcohol and ether but dissolves in about 14,000 parts of water at 16° and 1,400 at 100°. It is but slightly soluble in dilute acids, easily in alkalies. It is decomposed at 156° with formation of ammonium cyanide, carbon dioxide, formic acid and glycocoll. With nascent hydrogen it forms sarcine (hypoxanthine). It can be synthesised by heating hydrocyanic acid with water and an excess of acetic acid in a sealed tube to 145°. Its hydrochloride crystallises in needles and hexagonal prisms. The platini-chloride compound crystallises in yellow prisms.

When xanthine is evaporated to dryness with nitric acid in a porcelain dish on the water-bath, a yellow residue is left which when treated with sodium or potassium hydroxide becomes first red and then purple. This is known as the *murexide test*. *Weidel's reaction* is applied by treating a xanthine solution in a test-tube with chlorine water (or with hydrochloric acid and potassium chlorate), heating, and evaporating carefully to dryness in a porcelain dish. If the dish and contents are placed in contact with ammonia fumes (as under a bell-jar) first a red, then a purple colour are developed (compare Vol. 7, p. 327).

The amount of xanthine in muscular tissue is small. According to Kossel, the flesh of pigeons and hens contains from 0.01% to 0.1%.

Hypoxanthine, *Sarcine*, 6-*Oxypurine*, $C_5H_4ON_4$, is constantly present in muscle and various glands, accompanying xanthine. It also occurs in plants. Sarcine forms white crystalline needles, which dissolve with difficulty in cold, but easily in hot water (70–80 parts). It is nearly insoluble in alcohol, soluble in weak alkalies and ammonia and in acids. It does not give the murexide test (compare Vol. 7, p. 334).

The following amounts are reported by Hofmann in the flesh of animals (*Lehrbuch der Zoochemie*, 1879, **83**): cattle, 0.016–0.022%; horse, 0.013–0.014%; rabbits, 0.026%; dog, 0.025%.

Uric acid, $C_5H_4O_3N_4$, occurs in muscle, blood, urine (especially in carnivorous animals, hippuric acid being characteristic of herbivorous), and in the excrements of birds, reptiles and insects. It was discovered by Scheele in 1776 in urinary calculi. It forms a white, crystalline, granular powder, is odourless and tasteless, insoluble in alcohol and ether and difficultly soluble in water. (In 15,000 parts at 20°, 1,800 parts at 100°.) On evaporating to dryness with nitric acid a yellow residue is left which turns purple-red if moistened with ammonia and violet with sodium or potassium hydroxide (murexide test). Uric acid is a weak dibasic acid. Uric acid and urea form the chief nitrogenous end-products of protein metabolism and are of immense importance to the physiologist and physiological chemist (see Vol. 7, p. 357).

Urea, Carbamide, $CO(NH_2)_2$, the most important nitrogenous end-product of protein metabolism, occurs in the urine of mammals and especially in that of carnivorous animals. In the urine of man it is present in the proportion 2–3% and a full-grown man excretes about 30 grm. daily. Urea occurs also in all animal fluids and tissues, including muscular tissue. It was discovered by v. Rouelle in 1773, in urine, and was synthesised by Wöhler in 1828 from ammonium isocyanate—a fundamental work in the history of organic chemistry. It can be prepared from urine by evaporation and treatment of the concentrated urine with strong nitric acid. The precipitate is dissolved in boiling water, decomposed with barium carbonate, the mixture evaporated to dryness and the urea dissolved out with alcohol. For methods of synthesis, properties, etc., see Vol. 7, p. 288.

Urea crystallises in colourless rhombic prisms or needles, which taste somewhat like saltpetre. It is readily soluble in water (1 part), in alcohol (15 parts) and is practically insoluble in ether. It melts at 132° and above this temperature decomposes. When heated above 100° with water or when boiled with acids or alkalis it decomposes into carbon dioxide and ammonia. The same reaction is brought about by certain bacteria.

Carnine, $C_5H_2(NH_3)_2O_3N_4$, was discovered by Weidel in 1871 in meat-extract. It occurs also in the muscle of frogs and flesh of fish. It is insoluble in alcohol and ether, difficulty soluble in cold, easily soluble in hot, water. It gives Weidel's reaction.

Inosinic acid, $C_{10}H_{13}O_8N_4P$, occurs in small quantity in the muscles of rabbits, birds, (turkey 0.21%) and fish.

Taurine, aminoethylsulphonic acid, $SO_2\begin{matrix}/C_2H_4NH_2,\\ \backslash OH\end{matrix}$ occurs in very small quantities in the muscular tissue of mammals and in mollusks, also in taurocholic acid in the bile of cattle. It was discovered by Gmelin in 1824. It crystallises in monoclinic prisms which are soluble in hot water, insoluble in alcohol (see Vol. 7, p. 246).

Amino Acids.—These are found in various flesh foods, animal fluids and tissues, and also occur as hydrolytic products of the proteins when these are digested with mineral acids, and as products of bacterial decompositions. The following belong to this class: Glycocoll, amino-acetic acid $NH_2.CH_2.COOH$; Sarcosine, methylglycocoll, $CH_2(NH.CH_3).COOH$; Leucine, amino-caproic acid, $C_5H_{10}\cdot NH_2.COOH$; Butalanine, amino-valeric acid; Tyrosine, *p*-hydroxyphenyl-α-aminopropionic acid, $C_6H_4(OH)C_2H_3(NH_2).COOH$.

Enzymes of Meat.

Various enzymes have been identified in meat, not so much by methods of isolation but principally by the reactions of muscular tissue under definite conditions *in vitro*, or by inference from reactions known to occur in the living tissue. Only the more important ones are considered here, since many have been reported on insufficient authority or evidence. Of them all the most important from the practical and analytical standpoint is the proteoclastic enzyme and next to this the fat-splitting enzyme.

Proteoclastic Enzyme—Protase.—The presence of this enzyme is evidenced by the fact that after the onset of *rigor mortis*, which results in a stiffening of all the muscular tissues, when meat is held, as is customary, in the chill-room or cooler at temperatures varying usually from 1 to 3° (34 to 38° F.), a progressive softening of the muscular tissue occurs which makes it more tender and hence more palatable. Practical use is made of this enzyme by packing houses and markets in the ordinary method of holding meats as outlined above, to ripen them. This ripening is entirely distinct from bacterial action and as commonly practised is not accompanied by the latter. It is more fully described elsewhere.

The enzyme can be prepared in an impure condition by extracting fresh, finely ground, lean beef several times with chloroform water, saturating with sufficient alcohol to make a 60% solution, shaking,

allowing to stand overnight and decanting the supernatant liquid from the precipitate. The latter is dissolved in water and precipitated with sodium phosphate and calcium chloride in molecular proportions. After separating the liquid, the residue is taken up with chloroform water and filtered, the filtrate dialysed, and precipitated with alcohol (to 80%). The precipitate is settled, filtered, washed with 95% alcohol, and dried at a low temperature.

Thus prepared the enzyme appears to be most active in a weak alkaline solution (such as occurs in living muscle); but in the case of meat (after *rigor mortis*) it is obvious that it acts in an acid medium.

Fat-splitting Enzyme—Lipase.—This enzyme is associated with all the fatty tissues of the animal body, and it can be demonstrated in them by the simple process of holding the finely ground tissue (best emulsified with an equal weight of water) under aseptic conditions at a temperature of 40°, and observing the increase in fatty acids. Whether it is intimately associated with muscular tissue, free of fat, is a question; but connective tissue containing fat is so closely bound up with muscular tissue, that it would be practically impossible to prepare muscular tissue which would contain no fat-splitting enzyme. The lipase of animal fats appears to be active not only at the optimum temperature (near 40°) but also at much lower temperatures, even below the freezing-point. But as the temperature is reduced below 40° the activity is progressively lessened, until just above the freezing-point it is slight indeed, and at —10° the formation of fatty acids cannot be detected in ordinary animal fats even after the lapse of 2 years. However, if finely ground pancreas which contains much fat-splitting enzyme is mixed with ground fat and the mixture emulsified, an increase of fatty acid can be demonstrated, at —10°. The activity of lipase at low temperatures may depend on its insolubility in water. These points are of importance to the food analyst as is indicated in another place.

The more quickly fatty tissue is rendered, after death, and the lower the temperature used in rendering, the less will be the amount of fatty acids found by analysis, in the rendered fat. In carefully rendered animal fats the percentage of free acid (as oleic) is usually from 0.2 to 0.35%. It is assumed therefore that fats as they occur in the body during life are neutral or practically so, that is they contain no appreciable amount of fatty acid. This would indicate that lipase is formed in fatty tissue upon the death of animals, or that during life there is some counterbalancing tendency to offset the formation of fatty acids. It should be noted that it has been shown that some fat-splitting cata-

lytic agents (Twitchell reagent, lipase) are also synthesising agents under appropriate conditions.[1] Fat splitting occurs in the presence of considerable water with low glycerol concentration, fat synthesis with high glycerol concentration, fatty acid being present (Twitchell[2]). Dietz[3] showed the synthetic action of lipase in a mixture of a little butyric acid and much isoamyl alcohol. The amount of fatty acid found in the fats associated with meats may be as much as 10% (as oleic), for example, in old cured hams and summer sausage.

Amyloclastic Enzyme—Diastase.—Diastatic enzymes occur in the saliva, pancreatic juice, blood, lymph, and liver, and to a lesser extent in most tissues. Dextrin, maltose and dextrose have been demonstrated in muscular tissue.

Maltase, oxydase, catalase, a glycoclastic enzyme, and myosin enzyme have all been claimed in muscular tissue. The last, if it exists, is not identical with fibrin enzyme (Halliburton).

Nitrate-reducing enzyme has been claimed to exist in muscular tissue by several observers. The writer has failed to demonstrate its presence in fresh meat, although nitrate reduction occurs in meat as soon as bacteria begin to grow therein, and in this fact is probably to be found the reason for the reports of its presence. There are excellent reasons also, in connection with the curing of meats, when saltpetre is used, to indicate that such an enzyme (or reducing substance) does not exist in fresh meat. If it were generally present all saltpetre-cured meats would invariably be red, which is not the case.

Mineral Constituents of Flesh.—The ash of muscle ranges from 0.8 to 1.8% in the flesh in its natural condition, or from 3.2 to 7.5% in the water-free flesh. The inorganic salts consist chiefly of calcium and potassium phosphates and sodium chloride.

J. König gives the following figures as representing the percentage composition of the *ash* (free from carbon dioxide) of the flesh of terrestrial animals:

	Minimum	Maximum	Mean
	%	%	%
K_2O	25.0	48.9	37.04
Na_2O	0.0	25.6	10.14
CaO	0.9	7.5	2.42
MgO	1.4	4.6	3.23
Fe_2O_3	0.3	1.1	0.44
P_2O_5	36.1	48.1	41.20
SO_3	0.3	3.8	0.98
Cl	9.6	8.4	4.66
SiO_2	0.0	2.5	0.69

[1] Dunlap and Gilbert, *J. Am. Chem. Soc.*, 1911, 33, 1787.
[2] *J. Am. Chem. Soc.*, 1907, 29, 566.
[3] *Z. Physiol. Chem.*, 1907, 52, 279.

From the analysis of the flesh of a large number of animals, J. Katz[1] finds the ash-constituents to vary between the following limits. The figures are parts per 1,000 of the fresh flesh: Potassium, 2.4 to 2.6; sodium, 0.3 to 1.5; calcium, 0.02 to 0.39; magnesium, 0.18 to 0.37; iron, 0.04 to 0.25; and chlorine, 0.32 to 0.8. The phosphorus from phosphates ranged from 1.22 to 2.04; from lecithin, 0.13 to 0.48; and from nuclein from 0.09 to 0.32 parts per 1,000.

The following table (p. 294) gives a compilation of his results:

Albu and Neuberg[2] discuss in detail the metabolism of the various mineral salts in the animal body, and give tables of composition.

GENERAL METHODS OF SAMPLING AND ANALYSING MEATS.

Methods of Sampling.

As a rule it is better to separate the sample of meat first into its parts such as lean, fat, gristle and bone and to analyse any or all of these separately. In ascertaining the composition of the whole animal or of the larger cuts, these separated portions are weighed, in order to afford a basis for the final calculation. Ordinarily it is only the lean and fat and especially the lean with its small proportion of inseparable fat tissue which is analyzed. In the great majority of cases only the last is analyzed. Large and small butcher knives and a bone saw are required for the dissection. If the sample is in the form of a medium or large-sized piece, a convenient procedure is first to subdivide it into parallel slices about 0.5 to 1 in. thick, later removing from these the fat, gristle and bone, and finally cutting the lean into small cubes. These cubes are then run preferably through an Enterprise hasher once or twice and the ground sample thoroughly mixed. In lieu of the Enterprise hasher an ordinary household chopping bowl and knife may be used to advantage.

In handling samples of meat products, their perishability should always be kept in mind and every effort made to prevent decomposition. Low temperatures afford the best means of preservation and if the sample is to be kept for any length of time, the small cubes as obtained by the method described, should be placed in air-tight jars—such as Mason jars—and held in frozen condition. If juice separates during thawing, it should be well incorporated with the sample after

[1] *Pflüger's Archiv.*, 1896, 68, 1.
[2] *Mineral Stoffwechsel*, Berlin, Julius Springer, 1906.

MINERAL CONSTITUENTS OF FLESH.

Flesh of	Water	Potassium oxide	Sodium oxide	Ferric oxide	Calcium oxide	Magnesium oxide	Phosphoric acid (P_2O_5)				Chlorine	Sulphur
							Total	Soluble in water	Soluble in alcohol	Residue		
Pig	72.89	0.306	0.210	0.008	0.011	0.046	0.487	0.350	0.085	0.053	0.048	0.204
Ox	75.80	0.441	0.088	0.035	0.003	0.040	0.389	0.279	0.065	0.046	0.057	0.187
Calf	75.39	0.458	0.166	0.013	0.020	0.050	0.503	0.334	0.097	0.072	0.067	0.226
Deer	75.27	0.405	0.095	0.015	0.013	0.048	0.569	0.411	0.096	0.062	0.040	0.211
Rabbit	76.83	0.479	0.067	0.008	0.026	0.048	0.579	0.469	0.068	0.043	0.051	0.199
Dog	76.42	0.392	0.127	0.006	0.010	0.039	0.512	0.345	0.110	0.055	0.081	0.227
Cat	75.14	0.456	0.097	0.013	0.012	0.047	0.461	0.351	0.066	0.043	0.057	0.219
Hen	68.38	0.560	0.128	0.013	0.015	0.061	0.580	0.456	0.057	0.067	0.060	0.292
Frog	81.62	0.371	0.074	0.009	0.027	0.039	0.426	0.349	0.047	0.030	0.040	0.163
Shellfish	80.60	0.403	0.133	0.008	0.031	0.044	0.313	0.263	0.029	0.021	0.241	0.223
Eel	63.10	0.290	0.043	0.008	0.055	0.030	0.405	0.336	0.046	0.023	0.034	0.135
Pike	79.83	0.301	0.040	0.006	0.056	0.051	0.485	0.392	0.036	0.058	0.032	0.248

hashing. Antiseptics should be used only in cases where low temperatures are not available for preservation and are never to be recommended. Special methods for sectioning and sampling cured meats are given under this heading (page 366).

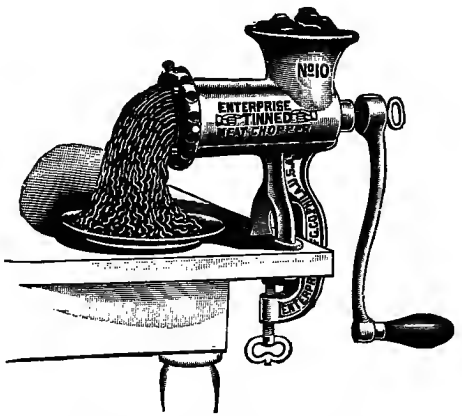


FIG. 24.—Enterprise meat hasher.

Methods of Analysis.

Moisture.—From 5 to 20 grm. of the finely hashed sample are weighed —best by difference from a weighing bottle—into a watch glass or metal moisture dish and the moisture determined by difference after drying under low pressure in a vacuum drying oven heated by electricity. The exact temperature and pressure can be varied according to circumstances, but a pressure of 60 mm. or less and a temperature of 40–50° are desirable. The sample should be taken to constant weight, but a few trials at a given pressure and temperature will suffice to set a minimum limit at which desiccation is complete.

Where a vacuum oven is not available, moisture may be determined by difference after drying in one of the forms of hydrogen ovens in a current of hydrogen.

For ordinary work it is sufficiently accurate to dry the sample in the air-bath (oven) at the temperature of boiling water or better at 100–105°. The bulb of the thermometer should be located near the drying sample.

Moisture may also be estimated by drying 5–10 of the sample in the vacuum desiccator, in the presence of sulphuric acid.[1] This method is slower than that of the vacuum oven and it is important that the sample be spread over the bottom of the dish in a thin layer, in order to prevent decomposition during the drying. The Hempel form of vacuum desiccator is best to use for the purpose.

Ash.—The dried sample or a convenient portion of it or a specially dried sample (weigh out 10 grm.) is charred in a porcelain, silica, or platinum dish. The charred mass is extracted by digestion with hot water, the extract filtered off through an ashless filter paper, the char thoroughly washed with hot water and returned with the filter paper to the original dish and ignited to a white ash. The extract is evaporated to dryness in the same dish, dried at 100–105°, very gently ignited below a dull red heat, cooled and weighed. Direct ignition of the meat to ash is never advisable.

Fat.—It is difficult to extract all the fat from muscular tissue by the ordinary methods and consequently more elaborate metods have been devised, such as alternate extraction with a volatile solvent and digestion of the residue with pepsin. But such a complicated method as this introduces as many errors as it obviates so that for all ordinary work a simpler procedure, such as the following, gives results which are quite satisfactory.

The residue from the moisture determination by one of the vacuum methods, or a sample (2–10 grm.) weighed and dried *in vacuo* for the purpose, is ground in an agate mortar with sand which has been purified in the usual way. The ground mixture is transferred to a Schleicher and Schüll extraction shell, or an alundum[2] extraction thimble, and extracted for 16 hours or longer in a Soxhlet or other suitable form of continuous extraction apparatus, with petroleum ether or absolute ethyl ether. It is sometimes advisable to remove the residue from the shell at the end of the extraction period, regrind it with sand and re-extract it. The ether is evaporated from the extract on the steam bath and the last traces of solvent removed by heat in an oven, *preferably in a vacuum oven to avoid oxidation.* The residue is then cooled as usual in a desiccator and weighed. It is always well to examine the dried fat for solubility in light petroleum ether. Insoluble impurities should be filtered off, well washed and the fat again dried *in vacuo* and weighed.

The ordinary fat solvents dissolve from meat not only fats but also

[1] J. Kōnig, *Nahrungs und Genussmittel*, 3, 25, Berlin, 1910.
[2] Norton Company, Worcester, Mass.

lecithin, and sometimes dextrin and other carbohydrates. In ordinary work these impurities are present in small quantity and do not seriously affect the result.

For other methods of fat estimation in animal tissue consult Abderhalden, *Handbuch der biochemisches Arbeitsmethoden* (Berlin, 1910), 2, 238 (good bibliography).

Separation of Fat for Further Examination.—This can be accomplished in several ways. If the fatty tissue can be separated by means of the knife, it should be hashed and rendered in a porcelain dish at as low a temperature as possible, on the steam bath or on wire gauze over a flame. Or the fat may be extracted by shaking the hashed tissue at intervals in a flask for a long period with ether or petroleum ether. The criticism of this method is that extraction may not be complete and the softer portions of the fat tend to dissolve first. A third method consists in extracting the tissue in the same way as for an analysis but on a larger scale. After separation the fat is examined according to the methods for fat analysis (see Vol. 2). Estimations should never be made on the fatty tissue but only on the separated fat.[1]

Total Nitrogen.—(See also page 406.) 3–4 grm. of the hashed sample are weighed out from the weighing bottle and digested in an 800 c.c. Kjeldahl flask according to the modified Kjeldahl-Gunning method, using potassium sulphate and mercury but no permanganate.[2] 25 c.c. of strong sulphuric acid are added to the flask, then 10 grm. nitrogen-free potassium sulphate and 0.7 grm. mercuric oxide or its equivalent in metallic mercury. The digestion should be continued about 5 hours, but no absolute time can be set as this depends on local conditions. The appearance of the digestion does not afford a positive criterion as to the completion of the process. The time of digestion should be sufficient to yield maximum figures, and should be determined by each chemist for his own conditions. After digestion is complete the acid liquid in the flask is diluted with nitrogen-free water, caustic soda solution added to alkaline reaction and distilled into $N/2$ sulphuric acid as usual. The back titration is made in the presence of cochineal as indicator.

Volatile Acids and Volatile Sulphur Compounds (sulphur dioxide, hydrogen sulphide, hydrosulphides, thio-ethers).—100 grm. of the hashed sample are weighed into an evaporating dish containing

[1] *J. Am. Chem. Soc.*, 1910, **32**, 568.
[2] For general directions see *Bull.* 107 *Revised, Bur. Chem. U. S. Dept. Agric.*; also *Proc. Ass. Off. Agr. Chem.*, 1908, 1909, 1910 and Vol. 1, p. 59.

200 c.c. distilled water and after stirring, the mixture is poured into a 1,000 c.c. distilling flask. The dish is rinsed with 100 c.c. water. This procedure allows a thorough breaking up of the sample and obviates sticking during distillation. 1 c.c. syrupy phosphoric acid is added and distillation is carried on in a current of steam. The distillate is condensed by means of a Hopkins condenser and collected in a 500 c.c. flask containing 10 c.c. sodium hydroxide, by means of a tube running to the bottom of the flask. The excess of sodium hydroxide is titrated back with $N/2$ hydrochloric acid, using phenolphthaleïn as indicator. Bromine is then added to oxidise sulphur compounds, the solution taken to dryness in a porcelain dish and the residue ignited until carbon is burned off. The residue is taken up with hydrochloric acid and water and the sulphates estimated by means of barium chloride in the usual way.

Total Sulphur.—For methods of estimating sulphur see Abderhalden, *Biochemische Arbeitsmethoden*, **3**, 794, 1910. Schreiber, *Circular* 56, *U. S. Dept. Agric. Bur. Chem.*, 1910.

Total Phosphorus.—50 grm. of the sample are weighed into a 250 c.c. beaker and 50 c.c. of a mixture of 6 parts nitric and 1 part hydrochloric acid are added. The resulting solution is taken nearly to dryness (10–15 c.c.) on the hot plate, taken up with hot water and made up to volume after cooling, in a 250 c.c. flask. The solution is filtered and portions of 100 c.c. are taken in duplicate for the estimation. This solution is precipitated with the ordinary acid ammonium molybdate solution, the precipitate dissolved in ammonia and reprecipitated with nitric acid and a little of the molybdate solution. The precipitate is filtered, dissolved in ammonia and precipitated as usual with magnesia mixture and weighed in a Gooch crucible as magnesium pyrophosphate.

Soluble Inorganic Phosphorus.—100 grm. of meat in a porcelain dish are extracted successively with four 250 c.c. portions of boiling distilled water and filtered through a folded filter. The last portion is squeezed through cheese-cloth and the filter and contents washed with boiling water. Two portions of 500 c.c. each are evaporated to about 100 c.c. 30 c.c. of nitric and 5 c.c. of hydrochloric acid are added and the volume reduced on the hot plate to 10–15 c.c. When oxidation is at an end hot water is added and the phosphorus determined as described under total phosphorus.

Cold-water Extract.—For the investigation of the soluble substances present in meat, it is customary to work on a cold-water extract or on the juice of the lean portions. Cold water does not extract

all the soluble matters, since the juice contains a considerably higher portion of them than does a cold-water extract made according to any of the usual methods. The failure of water to extract all the soluble matters is probably due to the indiffusibility or the slow diffusibility of the soluble proteins through the sarcolemma of the muscle fibres. The method was proposed by König,[1] was developed and used extensively by Grindley[2] and modified by Richardson and Scherubel.[3] The following simplified method has been found to be satisfactory, yielding results which agree with those obtained by the more complicated procedure of Grindley.

Duplicates of 100 grm. each of the finely hashed lean meat, are weighed into 8 in. porcelain dishes and 250 c.c. of cold distilled water added. The mass is macerated, with occasional stirring, preferably in a room of low temperature (in summer 2–5°). If a chill room is not available, the temperature should be kept down by means of ice. The extract is then filtered through linen or cheese-cloth of suitable mesh, placed in a funnel, and the meat wrung out by hand in the cloth. The meat is then returned to the dish and the process repeated with lesser amounts of water 5 times or until a litre of extract is obtained; or 4 portions of about 250 c.c. may be used. If the method is carefully followed only negligible amounts of extractive are obtained by a second extraction. The extract obtained is filtered for all determinations.

Technique for obtaining Meat Juice.—The meat, which should be in the form of lean pieces of moderate size (1–10 pound), is first frozen hard. The frozen meat is brought to the laboratory and, without thawing, is sawed and chopped into blocks about 1 inch on an edge. These pieces are then wrapped in linen or muslin, and firmly tied. Pressure is applied to the meat in a colander or other container with perforated bottom, by means of weights or in a screw press. The pressure is continued for a length of time which varies from a few hours to 24 hours. It is advantageous to remove the meat after the juice has ceased to run freely, grind it in an Enterprise hasher and then return it to the press. At least 20% of juice should be obtained. The pressing should of course be done in a cool place, and the juice collected in such a way as to avoid evaporation of water. The freezing of the meat appears to change the sarcolemma to a sieve-like structure which permits the passage of colloidal substances. After thawing the original semi-permeable structure is resumed, so that a

[1] *Chemie der meschlichen Nahrungs und Genussmittel*, 3.
[2] *J. Am. Chem. Soc.*, 1904, 26 1086; 1905, 27, 658; 1906, 28, 25 and 468.
[3] *Ibid.*, 1908, 30, 1515.

cold-water extraction of frozen and thawed meat removes no more solids than an extraction of unfrozen meat.

Methods for the Analysis of the Cold-water Extract (Applicable also to Meat Juice).—All calculations, for the sake of convenience, are made to the basis of the original meat.

Total Solids.—100 c.c. are evaporated to dryness in a platinum, porcelain, or silica dish, on the steam bath, then dried in the oven at 100–105°, for 1 hour, or better in the vacuum oven, cooled and weighed.

Ash.—The residue from the total solids estimation is charred, the char is extracted with hot water, filtered on an ashless filter and washed with hot water. The filter paper and contents are returned to the original dish and ashed, the filtrate is added and evaporated to dryness on the steam bath. The residue is then dried in the oven, cooled and weighed.

Total Nitrogen.—The nitrogen in 50 c.c. is determined by the modified Kjeldahl-Gunning method, as given above, using potassium sulphate and mercury but no permanganate. If nitrates are present —as in cured meats—these are first removed by adding 10 c.c. of saturated ferrous chloride solution and 5 c.c. strong hydrochloric acid and boiling a sufficient length of time in the Kjeldahl flask. (See Methods for the Analysis of Cured Meats, page 368.) Nitric nitrogen is determined in a separate portion of 50 or 100 c.c. by the Schloesing-Wagner[1] method (page 369).

Nitrogen in Coagulable Proteins.—A 50 c.c. portion of the extract is transferred to a 100 c.c. beaker and concentrated on the steam bath to one-half the volume. The precipitate is filtered off and washed with hot water. The filtrate is made neutral to litmus paper with $N/10$ sodium hydroxide. If any precipitate forms upon further heating it is filtered off and washed with hot water. This second precipitate is often separately estimated as "syntonin." The two precipitates and filter papers are transferred to a Kjeldahl flask and the nitrogen determined as given above under "Total Nitrogen," the distillate being received in $N/10$ sulphuric acid. The calculation is usually made to nitrogen but if desired the factor 6.25 may be used to estimate the coagulable proteins.

Nitrogen in Albumoses and Proteoses.—To the filtrate from the coagulable proteins determination, 1 c.c. of 50% sulphuric acid is added. (The filtrate should be concentrated so as to measure about 30 c.c.) Finely powdered zinc sulphate is next added (about 35 grm.

[1] Richardson, *J. Am. Chem. Soc.*, 1908, 30, 421.

per 50 c.c.) until the solution is saturated, when it is placed on the steam bath for a few moments and finally set aside to stand overnight. The next morning the precipitate is filtered off and washed with saturated zinc sulphate solution acidified with sulphuric acid. The filter and precipitate are then transferred to a Kjeldahl flask and the nitrogen estimated as usual, distilling into $N/10$ sulphuric acid.

Nitrogen in Meat Bases.—When nitrates are absent, the filtrate from a second coagulable estimation is taken and the total nitrogen estimated therein by the usual method, as given above. When nitrates are present they are removed, after separating the coagulable proteins, by adding 10 c.c. of saturated ferrous chloride (freshly made) and 5 c.c. strong hydrochloric acid, and boiling a sufficient length of time. The usual total nitrogen estimation is then made. The "meat-base" nitrogen is obtained by subtracting from this amount (albumose+meat−base nitrogen) the albumose nitrogen. If it is desired to calculate to the meat-bases, the factor 3.12 may be used.[1]

Nitrogen in Proteins Precipitated by Tannic Acid.—This method known in its present form as the "tannin-salt" method was proposed by Schjerning[2] and further developed by Bigelow and Cook[3] for the estimation of peptones in meat extracts (*q.v.* page 409). As applied to the cold-water extract of meat it is conducted as follows: 20 c.c. of the cold-water extract are placed in a 100 c.c. volumetric flask, 50 c.c. of a saturated salt solution added and then 30 c.c. of a 24% tannic acid solution. This tannic acid must be of highest possible purity. The precipitation is made in a cold room (or ice-box) not above 12° and the flask allowed to remain there overnight. The next day the solution is filtered and the nitrogen estimated in 50 c.c. of the filtrate. The figure thus obtained is multiplied by 2. A blank is made on 30 c.c. of the tannic acid solution and the nitrogen found deducted from that found in the filtrate (×2). This figure, subtracted from the total nitrogen in the extract, gives the amount precipitated by the tannic acid. The tannic acid nitrogen minus the coagulable nitrogen gives the albumose and peptone nitrogen. In the writer's experience this method is difficult to handle and obtain concordant results. The personal equation appears to influence results and there is a positive and uncertain source of error in the nitrogen present in the purest tannic acid obtainable. Oftentimes the nitrogen introduced with the tannic acid is much greater than the amount to be estimated. If a nitrogen-free tannic acid were obtainable, the results would doubtless

[1] See under "Meat Extract," page 409.
[2] *Z. Anal. Chem.*, 1900, **39**, 545.
[3] *U. S. Dept. of Agric. Bur. Chem. Bull.*, 73; *J. Am. Chem. Soc.*, 1906, **28**, 1485.

be much more satisfactory. Where the amount of albumoses and peptones to be estimated is small, as in the cold-water extract or juice from fresh meat, only a nitrogen-free tannic acid could make the method a satisfactory one to use. The method is, of course, more satisfactory for use in the analysis of meat extracts and commercial peptones. Some of the creatine is precipitated by the tannin-salt method.

Acidity of the Extract.—50 c.c. are diluted with recently boiled and cooled distilled water and titrated against $N/10$ sodium hydroxide, using phenolphthaleïn as indicator. The acidity is expressed as lactic acid on the basis of the original sample. The CO_2 which may be present in the extract interferes with the accuracy of the method in some cases. A second estimation is sometimes made on another 50 c.c. portion after boiling 1/2 minute to remove CO_2. The fact that acid is produced when proteins coagulate by heat, introduces an error which may approximately equal the one obviated.

Creatinine and Creatine.—For the estimation of these substances 200–500 c.c. of the water extract are evaporated to small bulk, filtered, the precipitate thoroughly washed with hot water and the filtrate treated according to the method given under Meat Extract, page 410.

Hot-water Extract.—The residue from the cold-water extraction is exhausted several times by boiling with water. If 100 grm. are used, 4 extractions of 1 hour each using 250 c.c. of water each time should be sufficient; but the process should be continued until practically no more gelatin is extracted as shown by the biuret test. If the cold-water extraction has been carried out properly, the hot water extract will consist chiefly of gelatin (or gelatone) and smaller quantities of peptones derived from insoluble or coagulable proteins. Each extract is strained through cheese-cloth (placed in a funnel) into a 100 c.c. flask. Some chemists combine the hot water extract with the cold-water extract and analyse the whole at one time, but a better procedure is to analyse them separately. If the latter course be followed, it is usually sufficient to estimate the nitrogen in an aliquot of the hot-water extract, and calculate to collagen (or gelatin) by using the factor 5.55.

Other Methods for the Separation and Estimation of Nitrogenous Substances.

The foregoing methods will be found adequate for most investigations, but certain other methods, especially for the separation of nitrogenous substances are applicable to certain cases.

Solubility of Nitrogenous Substances in Sodium Chloride Solutions.—That the extraction and the solubility of the nitrogenous substances in meat, is not a simple matter, is indicated not only by the fact that water does not extract as much of the soluble substances as are present in expressed meat juice, but by the following figures[1] which show the amounts of solids and organic nitrogen dissolved from hashed beef by solutions of sodium chloride of different strengths. Such solutions might be used to separate nitrogenous substances in meat.

EXTRACTION OF BEEF KNUCKLES WITH WATER AND SOLUTIONS OF SALTS OF DIFFERENT CONCENTRATIONS.

100 grm. meat extracted with the solvent and extract made up to 1 litre. 50 cc. (representing 5 grm. of meat) taken for each estimation. Two experiments (*a*) and (*b*).

Solvent	Grm. solids in 50 c.c. solution	Grm. ash in 50 c.c. solution	% meat solids in solution	% organic extractives	% meat ash	% total nitrogen
Distilled water.	(*a*) 0.2824	(*a*) 0.0597	(*a*) 5.65	(*a*) 4.46	(*a*) 1.19	(*a*) 0.770
	(*b*) 0.2625	(*b*) 0.0540	(*b*) 5.25	(*b*) 4.17	(*b*) 1.08	(*b*) 0.651
0.6% salt.....	(*a*) 0.6097	(*a*) 0.3441	(*a*) 6.50	(*a*) 5.31	(*a*) 1.19	(*a*) 0.931
	(*b*) 0.6412	(*b*) 0.3227	(*b*) 6.45	(*b*) 5.37	(*b*) 1.08	(*b*) 0.858
2.0% salt.....	(*a*) 1.2688	(*a*) 0.9580	(*a*) 7.40	(*a*) 6.21	(*a*) 1.19	(*a*) 1.080
	(*b*) 1.2075	(*b*)	(*b*)	(*b*)	(*b*) 1.08	(*b*) 0.913
5.0% salt.....	(*a*) 2.6200	(*a*) 2.2392	(*a*) 8.81	(*a*) 7.62	(*a*) 1.19	(*a*) 1.232
	(*b*) 2.5252	(*b*) 2.1517	(*b*) 8.55	(*b*) 7.47	(*b*) 1.08	(*b*) 1.158
10.0% salt.....	(*a*) 4.8024	(*a*) 4.4465	(*a*) 8.30	(*a*) 7.11	(*a*) 1.19	(*a*) 1.322
	(*b*) 4.7867	(*b*) 4.4216	(*b*) 8.38	(*b*) 7.30	(*b*) 1.08	(*b*) 1.274
20.0% salt.....	(*a*) 9.1873	(*a*) 8.8395	(*a*) 8.14	(*a*) 6.95	(*a*) 1.19	(*a*) 1.001
	(*b*)	(*b*)	(*b*)	(*b*)	(*b*)	(*b*) .....
1.1% K_2HPO_4	(*a*) 0.8388	(*a*) 0.5145	(*a*) 7.67	(*a*) 6.48	(*a*) 1.19	(*a*) 0.896
	(*b*) 0.8194	(*b*)	(*b*)	(*b*)	(*b*)	(*b*) 0.858

The 10% salt solution appears to extract the largest amount of nitrogenous substances, possibly not without alteration, whereas the 5% solution extracts more organic solids. However, on account of the large quantity of salt present, the ash figures are not to be considered as being absolutely accurate, although the estimations were made as carefully as possible. The nitrogen figures should be considered more accurate. In the case of the 5 and 10% salt solutions, results in soluble meat solids and nitrogen are obtained of the same order as those representing the soluble solids and nitrogen in meat juice itself, calculated to the basis of the meat.

Alcohol Separation of Nitrogenous Substances.—The alcoholic separation of nitrogenous substances (see also page 420) has been applied by Knorr[2] to the extraction of the meat bases from the meat. His method is as follows: A suitable quantity of the fat-free sample is extracted with strong alcohol which dissolves the meat bases, together with small amounts of non-nitrogenous extractives such as

[1] Richardson and Sherubel, *J. Am. Chem. Soc.*, 1908, 30, 1543.
[2] *U. S. Agric. Report*, 1886, 355–357.

lactic acid, glycogen and inositol. The alcohol extract is dried and weighed and the nitrogen determined in the residue, as usual. A cold-water extract is made of another portion—removing certain soluble proteins and the meat bases—and an aliquot of the extract is dried and weighed, and the nitrogen therein determined. In another aliquot of the cold-water extract, the coagulable proteins are determined by heating the solution to boiling, filtering off the precipitate, drying it at 100°, and weighing it.

Knorr's average results from 11 analyses of meat are shown in the following table:

Dried residue from cold-water extract	13.76%
Dried coagulable albumin	2.24%
Total cold-water extract	3.56%
Total nitrogen	3.37%
Nitrogen in residue from alcohol extraction	2.86%
Dried alcohol extract	3.03%

Mallet's phosphotungstic acid method[1] **and the bromine precipitation method** (see page 421) have been applied to the separation of nitrogenous substances but are not so useful as the more recent methods described. In both cases the separations are not sharp and the results do not usually agree as well as could be desired.

The Cooking of Meat.

Meat is tender before the occurrence of *rigor mortis*, but when once that condition has set in it requires to be kept some days before it again acquires this character.

The tangible change in composition undergone by meat in the process of roasting consists chiefly in the loss of water by evaporation and formation of gravy, with the loss of fat in the form of dripping. The proteins become concentrated in the cooked meat, while at the same time certain dark-coloured substances of carameloid nature are formed, which greatly modify and improve the flavour and odour of the meat.

When meat is boiled in water, a considerable quantity of organic and inorganic matters pass into solution, and when the liquid is not intended to be consumed the loss should be reduced to a minimum by immersing the meat in boiling water for a few minutes, and then adding more water, in quantity sufficient to reduce the temperature of the liquid to about 77°, which temperature should not be greatly exceeded during the remainder of the process of cooking. By operating

[1] *U. S. Dept. Agric. Bur. Chem., Bull.* 54.

in this manner an insoluble coating of coagulated proteins is formed on the meat, and loss by solution reduced to a minimum. On the contrary, when it is desired to extract the meat as thoroughly as possible, as in preparing soup, beef-tea, or mutton-broth, the meat should be placed in cold water, and the temperature gradually raised.

Soup contains the extractives of the meat from which it is prepared, a portion of the proteins, and most of the gelatinoids.

Beef-tea contains only insignificant quantities of proteins, gelatinoids or fat, and hence possesses true nutritive properties to but a very limited extent. Its value appears to be due to the stimulating action of the extractives, especially creatine, creatinine, xanthine, lactic acid and salts.

Liebig's extract of meat is practically concentrated beef-tea, and owes its value to the same constituents. The nature of commercial meat-extracts is discussed fully in the sequel.

Composition of Lean Beef.—The tables which follow show the composition of fresh lean beef according to the general methods of analysis given. In making these analyses it was thought advisable to select the leanest and most uniform muscular tissue which could be found. The part selected is commonly known as the beef "knuckle" to butchers, and is the crural triceps of anatomists consisting of the rectus femoris, vastus externus, vastus internus and anterior gracilis muscles.

The effects of cooking on meat have been very fully investigated and discussed by Grindley and his associates.[1] The principal points of interest for the analyst in connection with the cooking of meat are the coagulation and hydrolysis of the proteins and the great shrink in weight, due chiefly to the loss of water coincident with the coagulation of the proteins. Cooked meats usually contain 55–65% water whereas fresh meats contain 70–75%. The shrinkage in weight usually amounts to 30–40% of the original weight. The lower the temperature and the shorter the time of cooking the less the shrinkage. Cooking commences when the proteins of lowest coagulation point begin to coagulate, but the temperatures generally employed vary from 65° to a little above 100°, or to a considerably higher temperature on the surface only. Grindley (*l. c.*) concludes that when beef is cooked in water from 3 to 20% of the total solids is found in the broth, but that beef which has been used for the preparation of beef-tea or broth has lost comparatively little of its nutritive value, though much of the flavouring material has been removed.

[1] *U. S. Dept. Agric. Off. Exp. Sta. Bulls.* 102, 141, 162, and 193, 1901–1907.

PART I.—ANALYSES FRESH BEEF KNUCKLES. ALL SAMPLES HELD AT 2–4° UNTIL ANALYSED. AGE, 0–7 DAYS. ALL FIGURES ON BASIS OF ORIGINAL MEAT.

Source	Lab. No.	Killed	Analysed	Age, days	Moisture %	Ash %	Fat pet. eth. ext. %	Total N. %	Amm. N. Method 1 %	Amm. N. Method 2 %	Cold water extract							
											Total solids %	Ash %	Organic extractives %	Total N. %	Coag. N. %	Albumose N. %	Meat base N. %	Acid as lactic %
Unknown	.....	11/9	11/9															
Choice steer	3813	1907 4/17	1097 4/22	0.0	76.78	1.11	1.93	3.41	0.029	.....	6.24	0.97	5.27	0.807	0.444	0.026	0.332	0.63
Old Bull	3814	1908 4/17	1908 4/22	5.0	74.78	1.26	3.34	3.47	0.030	0.010	6.03	1.24	4.79	0.777	0.397	0.022	0.373	0.67
Heifer	3815	1908 4/21	1908 4/22	5.0	76.20	1.31	1.08	3.65	0.033	0.110	6.09	1.27	4.82	0.849	0.448	0.027	0.385	0.68
Old cow	3816	1908 4/21	1908 4/22	1.0	75.26	1.23	2.30	3.58	0.029	0.010	6.02	1.17	4.85	0.795	0.413	0.021	0.363	0.67
Unknown	459	1908 1/14	1908 1/18	1.0	77.27	1.23	0.95	3.46	0.027	0.011	5.55	1.13	4.42	0.742	0.358	0.022	0.360	0.69
Unknown	460	1908 1/14	1908 1/18	4.0	77.17	1.20	1.05	3.45	0.028	0.010	6.16	1.16	5.00	0.854	0.452	0.030	0.383	0.68
Unknown	.....	1908 1/26	1908 1/29	4.0	76.96	1.27	0.85	3.46	0.030	0.010	6.15	1.15	5.00	0.840	0.445	0.034	0.376	0.66
Unknown	3263	1908 4/2	1908 4/6	3.0	76.28	1.30	1.28	3.43	0.028	0.009	5.78	1.11	4.67	0.778	0.393	0.025	0.367	0.72
Unknown	3264	1908 4/2	1908 4/6	4.0	76.30	1.28	0.78	3.56	0.033	0.011	6.02	1.22	4.90	0.837	0.409	0.024	0.398	0.82
Unknown	.....	1908 10/12	1908 10/15	4.0	76.33	1.27	0.90	3.54	0.032	0.010	6.20	1.23	4.97	0.812	0.394	0.024	0.392	0.78
Unknown	2326	1907 3/2	1907 3/9	3.0	76.73	1.13	1.82	3.34	.....	.....	6.02	0.95	5.07	0.766	0.401	0.014	0.363	0.64
Unknown	.....	1908 10/12	1908 10/19	7.0	77.04	1.28	1.06	3.43	0.022	0.010	5.94	1.25	4.69	0.840	0.423	0.019	0.378	0.65
Unknown	.....	1907	1907	7.0	75.56	1.10	1.25	3.59	.....	.....	5.89	1.06	4.83	0.787	0.393	0.033	0.355	0.58
Maximum	.....	.....	.....	7.0	77.27	1.31	3.34	3.65	0.033	0.011	6.24	1.27	5.27	0.854	0.452	0.034	0.398	0.82
Minimum	.....	.....	.....	0.0	75.26	1.11	0.78	3.34	0.022	0.009	5.55	0.95	4.42	0.742	0.358	0.014	0.360	0.63
Average	.....	.....	.....	3.7	76.35	1.23	1.43	3.49	0.029	0.010	6.01	1.14	4.87	0.806	0.413	0.024	0.371	0.68

PART II.—ANALYSES FRESH BEEF KNUCKLES. ALL SAMPLES HELD AT 2–4° UNTIL ANALYSED. AGE, 0–7 DAYS. FIGURES CALCULATED TO MOISTURE-, ASH-, AND FAT-FREE BASIS.

Source	Lab. No.	Killed	Analysed	Age, days	Total N. %	Amm. N. Method 1 %	Amm. N. Method 2 %	Organic extractives %	Cold water extract				
									Total N. %	Coag. N. %	Albumose N. %	Meat base N. %	Acidas lactic %
Unknown	....	11/9 1907	11/9 1907	0.0	16.90	0.144	.....	26.12	3.99	2.20	0.129	1.65	3.12
Choice steer	3813	4/17 1908	4/22 1908	5.0	16.83	0.145	0.048	23.23	3.77	1.92	0.107	1.81	3.25
Old bull	3814	4/17 1908	4/22 1908	5.0	17.05	0.154	0.051	22.51	3.96	2.09	0.126	1.80	3.17
Heifer	3815	4/21 1908	4/22 1908	1.0	16.88	0.137	0.047	22.87	3.75	1.95	0.099	1.71	3.16
Old cow	3816	4/21 1908	4/22 1908	1.0	16.83	0.131	0.053	21.51	3.61	1.74	0.107	1.75	3.36
Unknown	459	1/14 1908	1/18 1908	4.0	16.76	0.136	0.049	24.30	4.15	2.20	0.146	1.86	3.30
Unknown	460	1/14 1908	1/18 1908	4.0	16.54	0.143	0.048	23.90	4.02	2.13	0.162	1.79	3.15
Unknown	.....	1/26 1908	1/29 1908	3.0	16.22	0.132	0.043	22.09	3.68	1.86	0.118	1.74	3.41
Unknown	3263	4/2 1908	4/6 1908	4.0	16.45	0.152	0.051	22.64	3.87	1.89	0.111	1.84	3.79
Unknown	3264	4/2 1908	4/6 1908	4.0	16.47	0.149	0.047	23.12	3.78	1.83	0.112	1.82	3.63
Unknown	.....	10/12 1907	10/15 1907	3.0	16.44	.....	.....	24.95	3.77	1.97	0.069	1.79	3.15
Unknown	2326	3/2 1908	3/9 1908	7.0	16.63	0.107	0.048	22.75	4.07	2.05	0.092	1.83	3.15
Unknown	.....	10/12 1907	10/19 1907	7.0	16.25	.....	.....	21.87	3.56	1.78	0.149	1.61	2.63
Maximum	.....	.....	.....	7.0	17.05	0.154	0.053	26.12	4.15	2.20	0.162	1.86	3.79
Minimum	.....	.....	.....	0.0	16.22	0.107	0.043	21.51	3.56	1.74	0.069	1.61	2.63
Average	.....	.....	.....	3.7	16.63	0.139	0.049	23.22	3.84	1.97	0.117	1.77	3.25

Changes in Flesh Following Death.

Alteration occurs in flesh foods in 3 ways: (1) physically; (2) chemically; (3) biochemically.

The physical changes are those which affect the appearance, the structure of the tissue, macroscopically and microscopically, and, in general, changes noticeable by the senses which do not affect the composition or nutritive value of the food.

The chemical and biochemical changes are those which affect the composition of the food, hence the nutritive value (advantageously or otherwise) and which may produce alterations in odour and flavour. The chemical agents of deterioration in the case of flesh foods are water and oxygen; in the absence of the former—that is, in desiccated flesh foods—the changes which occur in the lean portions, even after long storage at ordinary temperatures, are small. In the absence of water and oxygen—for instance desiccated meat *in vacuo*—flesh foods will keep indefinitely without change. In desiccated flesh foods, if oxygen is present, rancidity usually occurs after a shorter or longer period of storage; this change is entirely chemical in nature and does not require the presence of micro-organisms. Practically all the spoilage which occurs in normal flesh foods is due to bacteria or moulds; that is, these micro-organisms are the exciting causes of the changes which occur, and in their absence, even though water and oxygen are abundantly present, the changes will be slow.

A tabular view of deterioration in the fat as well as in the lean of meat may be presented in the following form. In this scheme the formation of bacterial toxins is not considered and this topic will be discussed later.

In the living animal, muscular tissue consists chiefly of semifluid muscle plasma, which has a faintly alkaline reaction. After the death of the animal, *rigor mortis* sets in, the meat becomes acid in reaction, due to the formation of lactic acid and the previously soluble proteins coagulate in part to form the muscle clot, the principal protein of which is myosin. Earlier investigators looked to the muscle carbohydrates as the source of lactic acid but later investigations indicate that it may have a protein origin. The onset of *rigor mortis* may be delayed in various ways as by low temperatures or an abundant supply of oxygen; but in any event it gradually passes away and the muscular tissue again relaxes. This relaxation is coincident with the process known as ripening which is in effect an auto-digestion which causes the meat to become more tender. The ripening process is due to proteo-

CHEMICAL AND BIOCHEMICAL DETERIORATION OF FLESH FOODS.

Three Principal Causes of Influences.				
	Fat...	Hydrolysis—active agent water—products fatty acids and glycerol.		
		Oxidation (rancidity) active agents water and oxygen; products lower fatty acids, aldehydes, etc.		
Chemical. (1) Water. (2) Oxygen of Air.	Lean...	Hydrolysis.....	In the absence of micro-organisms this occurs slowly at ordinary temperatures, rapidly at high temperatures, with production of gelatin and albumoses (proteoses). At ordinary temperatures enzymes act as accelerating agents.	
		Oxidation.....	Occurs slowly at ordinary and moderately high temperatures, in presence of oxygen of air, even in absence of micro-organisms.	
Biological. (3) Micro-organisms.		Decomposition by micro-organisms. Hydrolysis chief chemical action. In presence of oxygen oxidation plays a part, the sulphur compounds being affected and much CO_2 being evolved.	Putrefaction (anaerobic).	Putrefaction produces reduction products of foul odour (NH_3, H_2S, amines, skatole (acids). Decay produces products with scarcely any pronounced disagreeable odour (no H_2S). Mouldering produced the typical odour or "mouldiness," possibly due to acid amides.
			Decay (aerobic).	
			Mouldering (aerobic by moulds).	

clastic enzyme (protase) naturally present in meat and is not caused by bacterial decomposition although bacterial decomposition may overtake it and become the dominant effect when meat is held too long or at too high a temperature or in too small pieces. A slight bacterial decomposition, or "gamey" flavour is desired by some persons, and meat of this sort is eaten by them with no apparent ill effects. The ripening process in the case of large pieces (sides or quarters of beef) held at 34 to 38° F. reaches its maximum in from 15 to 21 days when bacterial action has not penetrated much below the surface of the meat (about 1/16 to 1/8 in.), but beef is sometimes held at these temperatures by hotels, etc., for 30 and even 60 days.

The bacteria which cause the decomposition of flesh foods gain access to the meat after the death of the animal; for it is the concensus of scientific opinion that normal muscular tissue from an animal healthy at the time of slaughter is free from bacteria at that time. That bacteria sometimes gain access to the circulation during life is true but the fact that the blood promptly kills them, during health, makes the point an academic one, of no significance for practical purposes.

The Spoilage of Meat.[1]—If adequate precautions are not taken

[1] See *U. S. Dept. Agric. Bur. An. Ind., Bull.* 132.

to ensure its preservation, meat undergoes progressive decomposition, beginning with the death of the animal from which it was derived. This process can best be followed in the case of large pieces of beef held in chill rooms at 1 to 3° (34 to 38° F.) which is the common modern practice. Into these rooms the sides are placed immediately after killing and dressing and they are held there until transferred to the refrigerator car or ship for shipment to the consumer or middleman.

Starting with recently slaughtered meat free from bacteria there are two ways by which micro-organisms enter the flesh and excite decomposition: first, by direct extension of surface colonies into the firm tissue; second, by extension and locomotion along surfaces such as those afforded by blood- and lymph-vessels, and nerve and connective-tissue sheaths.

By the first method, penetration is slow and very regular. The progress of the extension of the innumerable bacteria from the surface toward the interior can be easily observed. If a section be cut across a lean piece of beef which has been stored in the cooler for about 20 days or more, a dark, narrow, sharply defined border can be seen extending all around the cut. The line separating this border from the interior marks the limit of bacterial penetration by the first method. It will readily be seen that motility cannot play a great part in this process; rather the advance occurs by the actual extension of the limits of those confluent colonies which have spread over the surface and later penetrated the meat. It will also be evident that the harder and drier the surface the less readily will penetration occur at the start, although after the surface layer is pierced progress will be easier. Hence the advantage of a certain amount of desiccation in the cooler in the preservation of meats. Again, the connective tissue which surrounds muscles and with which the ordinary large cuts of beef, mutton and pork are more or less covered is especially difficult of penetration by bacteria when it is dry. In this condition it resembles parchment.

The width of the bacterial border shown in section increases with the time of storage. Under favourable conditions at 2 to 4° it will be about 1 cm. in 30 days; under unfavourable conditions (good desiccation) it may be only 2 or 3 mm. in the same length of time. Fresh beef formerly shipped abroad from Chicago reached the ports of England 3 or 4 weeks after the death of the animal. It is needless to say that it arrived in a satisfactory condition of preservation.

It is characteristic of this method of bacterial penetration that the line of demarcation between the decomposing surface and undecomposed interior remains always sharp and distinct.

The second method of penetration wherein bacteria follow various moist surfaces and tubes including arteries, veins, nerve sheaths, lymph-vessels and connective tissue, allows them to reach at a relatively early period the interior of the meat, where they set up foci of decomposition which spread and finally merge with each other and with the surface decomposition until the meat contains bacteria throughout and is in an active state of putrefaction. The extension of the interior foci can be roughly followed by making smear or impression preparation from various places on the surface of the sectioned meat, on cover-glasses or slides. In the earlier stages of decomposition, infected and uninfected areas will be found side by side, the infected spots will be few and small, the untainted areas large. Gradually the former increase in number and size; the latter diminish until with the encroachment of the surface layer of bacteria no uncontaminated spot remains.

So far as the external surface is concerned, if the air be dry, no slime will form. If the air (and hence the surface of the meat) is moist, a thick layer of slime will form and later an abundant growth of mould will appear. The slime consists of quantities of bacteria. The ordinary bacterial decomposition of meat—except on the external surface—is a typical putrefaction.

In this decomposition the tendency is chiefly toward the formation of simpler compounds by hydrolysis, and if the various changes are able to reach the limit, on account of a sufficiently abundant supply of water (which, among other functions, serves to dilute and render ineffective inhibitory substances) and of oxygen, the final products will be principally three: namely, water, ammonia and carbon dioxide. The sulphur compounds will remain as sulphates and the phosphorus compounds as phosphates. While the tendency of bacterial decomposition of flesh is principally in the direction indicated, at the same time the bacterial cells produce certain complex substances synthetically out of simpler materials.

Again the decomposition process does not follow a uniform course from complex to simplest compounds, but even in the beginning ammonia, hydrogen sulphide, and other simple compounds of pronounced odour are split off and afford a basis for the chemical detection of decomposition.

Methods for the Detection of Decomposition in Meat.

By Inspection.—If the sample under investigation has not been specially treated in any way, but is simply chilled meat held at tem-

peratures above the freezing-point, organoleptic tests and especially the odour afford one of the best means of judging unsoundness. Taste is of value in many instances and appearance in some, but the last is at times particularly misleading, although it may be a guide as to the previous handling of the meat. Experience is, of course, the first requisite in forming a judgment. An expert can distinguish slight different characteristic odours in the flesh of different animals but good meat from any source has no pronounced odour and especially no pronounced disagreeable odour, whereas in decomposing meat a strong, "musty," "mouldy," "sour" or "putrefactive" odour can be detected. The last is due largely to ammonia and hydrogen sulphide.

By Microscopic Examination.—It is very doubtful whether histological examinations afford any reliable data for the detection of decomposition in meat especially in the earlier stages. Wiley and Pennington[1] report progressive deterioration in frozen meats on histological examination, but Richardson and Scherubel[2] have shown that the changes reported are probably physical effects due to freezing. Although histological examination is of little value in detecting decomposition, microscopic examination for bacteria will always discover them in large numbers in decomposing meat. The only likely error in this examination is failure to locate the seat of decomposition. Smear preparations should be made at several points, stained with methylene blue and examined with oil immersion objective as usual.

By Testing for Hydrogen Sulphide. *Eber's Sulphide Test.*[3]—In the bacterial decomposition of meat among the first products to be split off from the proteins is hydrogen sulphide, and consequently when proper precautions are observed, a method for the detection of unsound meat can be based upon this fact. Eber applied his test to meat from diseased animals especially tuberculous animals and found that, in general, such meats yielded more hydrogen sulphide than meats from healthy animals. Fresh meat when warmed on the water-bath gives off hydrogen sulphide (and possibly other volatile sulphur compounds), but Eber found the quantities evolved in the case of healthy and diseased meats too nearly alike to distinguish them by this means. Working with the amounts evolved with dilute sulphuric acid, he obtained more characteristic results. His method is as follows: 25 grm. of the finely hashed sample are placed in an Erlen-

[1] Cold Storage Results on Fowls and Eggs. Abs. *Science*, 1908, 27, 295; *Bull.* 115, *Bur. Chem. U. S. Dept. Agr.*, 1908.
[2] *J. Am. Chem. Soc.*, 1908, 30, 1515–64.
[3] *Z. Fleisch. u. Milch. Hyg.*, 1897, 7, 207, 227; 8, 41.

meyer flask of convenient size and 50 grm. of 10% sulphuric acid added. In the neck of the flask a strip of filter paper moistened with 10% lead acetate solution is fixed by means of a plug of cotton wool and the apparatus placed in a well-ventilated chamber in the dark for 24 hours at a temperature of about 15° (20–21° would be more convenient). At the end of the period the filter paper is compared with a standard set of similar strips. The colour will vary from yellowish-brown to black.

The set of standards can be made by treating a solution of pure potassium or sodium sulphide in the same way, using amounts varying from 0.002 mg. to 0.01 mg.

It is doubtful that the details given above are the best which can be devised and more work should be done with the method. As it is entirely empirical and comparative, in the absence of a standard method, each operator can work out details for his own conditions. It is a useful method in many cases. Meats which have been treated or cured with nitrates do not respond to Eber's sulphide test.

By Estimation of Ammoniacal Nitrogen.—The various methods for estimating the nitrogen in ammonium salts together with other substances easily decomposed by means of weak alkalies, probably afford the best available chemical methods at the present time for the detection of decomposition in flesh foods. Various details for making the estimation have been proposed; but they all agree in treating the substance with a very weak alkali, volatilising the ammonia set free, absorbing it in standard acid and titrating the excess. Although putrefactive processes occasionally give rise to acid products in excess of basic ones, as a general rule free ammonia is produced from first to last. Much ammonia is also left combined with various organic acids. Based on the free ammonia evolved from putrefying meat, Eber[1] has proposed a qualitative test for incipient putrefaction as follows: The sides of a glass cylinder are moistened with a mixture of hydrochloric acid 1 part; alcohol 3 parts; and ether 1 part. A piece of the sample on the end of a wire is introduced into the cylinder and if a white cloud of ammonium chloride forms, it is an indication of putrefaction.

The test might be modified to include those cases where acid products of putrefaction are in excess by treating the hashed sample in an Erlenmeyer flask with barium carbonate at ordinary temperature and suspending a strip of filter paper moistened with the test solution above.

[1] *Arch. Wissensch. u. prak. Thierheilk*, 1893, 18, 1894, 19, 81.

In estimating ammoniacal nitrogen quantitatively several difficulties arise. The aim of the estimation is to effect a separation of the simplest basic nitrogenous compounds—resulting from decomposition—from the more complex, but proteins themselves are decomposed by boiling with even the weakest alkalies and water and even by prolonged digestion with water itself. To avoid this result as far as possible three distinct principles are made use of:

(1) Use of the weakest alkalies as magnesium oxide and barium carbonate.

(2) Use of alcohol to reduce hydrolysis.

(3) Use of low temperatures, evolving the ammonia by air-aspiration.

(1) 100 grm. of the well-hashed sample are weighed into a 1 litre round bottom distilling flask and 10 grm. of freshly calcined magnesium oxide and 450 c.c. of water are added and the whole mixed. The mixture is quickly brought to the boiling-point and the ammonia distilled into $N/10$ sulphuric acid, 50–60 minutes being used in the distillation and exactly 200 c.c. being distilled over. Care is necessary in the distillation, especially at the start, to prevent sticking of the material to the bottom of the flask and charring. Cochineal indicator is used in the back titration. Comparable results are obtained in one distillation but if sufficient water is added to replace the volume distilled and the distillation repeated, more ammonia comes over. After 4 or 5 distillations a practical limit is reached. The amount of ammoniacal nitrogen calculated as nitrogen found by this method in the lean meat from beef, pork and chickens, lies between the limits 0.025% and 0.035 %. (See tables below.)

(2) 100 grm. of the sample are extracted with 150 c.c. of 60% (by volume) alcohol 3 times, the extract filtered through fine linen or good cheese cloth and the residue brought upon the filter cloth and wrung out tightly each time. To the combined extracts in a 1 litre round bottom distilling flask are added 10 grm. of recently calcined magnesium oxide, and exactly 200 c.c. are distilled into $N/10$ sulphuric acid, 1/2 hour being taken for the distillation. Cochineal indicator is used in the back titration. A blank is made on reagents and solvent. Results by this method are, for fresh meats, about one-third to one-half those obtained by method 1.

Results by the two methods given above are shown in the following tables:

Ammoniacal nitrogen obtained by successive distillations of hashed fresh meat, the flask being made up to volume, between distillations.

No. of distillation	Method 1		Method 2	
1	0.030	0.029	0.010	0.010
2	0.015	0.014	0.001	0.001
3	0.009	0.009	0.001	0.001
4	0.006	0.007	0.001	0.001
5	0.005	0.005	.....	.....
6	0.006	0.007	.....	.....
7	0.005	0.005	.....	.....
8	0.004	0.004	.....	.....
9	0.004	0.004	.....	.....
10	0.004	0.003	.....	.....
Sum	0.088	0.087	0.013	0.013

Ammoniacal nitrogen obtained by successive distillations of hashed frozen meat, the distillation flask being made up to volume between distillations.

No. of distillation	Sample 1				Sample 2			
	Method 1, %		Method 2, %		Method 1, %		Method 2, %	
1	0.034	0.032	0.009	0.009	0.0034	0.0032	0.008	0.008
2	0.014	0.014	0.001	0.001	0.012	0.012	0.001	0.001
3	0.009	0.010	0.001	0.001	0.009	0.010	0.001	0.001
4	0.009	0.009	0.001	0.001	0.008	0.008	0.001	0.001
5	0.008	0.008	0.001	0.001	0.006	0.008	0.001	0.001
6	0.006	0.006	.....	.....	0.006	0.006	.....	.....
7	0.005	0.005	.....	.....	0.005	0.005	.....	.....
8	0.004	0.004	.....	.....	0.005	0.004	.....	.....
9	0.004	0.003	.....	.....	0.003	0.003	.....	.....
10	0.003	0.002	.....	.....	0.002	0.002	.....	.....
Sum	0.096	0.093	0.013	0.013	0.090	0.090	0.012	0.012

The following figures show the rise in ammoniacal nitrogen during putrefaction, the results being obtained by the methods given above.

Putrefaction of Beef at Room Temperature (Summer) 24–33°.

20 grm. meat digested with 450 c.c. water after addition of 1 c.c. of putrefying infusion of meat.

Hours.....	0	5	21	29	45	93	117	165	309
Ammoniacal nitrogen Method 1, %.....	0.038	0.039	0.069	0.099	0.182	0.621	1.056	1.379	1.648

Experiment on Putrefaction of Beef at Room Temperature 24–33°.

20 grm. meat digested with 100 c.c. of water after addition of 1 c.c. of putrefying meat infusion. Just before distillation enough alcohol was added to make 450 c.c. (total of 60% alcohol Method 2).

Hours.....	0	16	64	92	118	140	188	284
Ammoniacal nitrogen Method 2, %.....	0.012	0.018	0.191	0.353	0.607	0.814	1.015	1.045

Richardson and Scherubel[1] report ammoniacal nitrogen figures obtained by distillation of various amino acids, acid amines and meat

[1] *J. Am. Chem. Soc.*, 1908, 30, 1515.

bases with magnesium oxide which have a bearing upon the methods given above.

Both of the preceding methods can be conducted by distillation under reduced pressure, with the advantage of lower temperatures and consequently less hydrolysis. Distillation at ordinary pressure appears to be satisfactory and hence it is a question to be determined by the operator whether it is worth while to resort to this further refinement of the process with its attendant complications.

(3) Pennington and Greenlee[1] have applied the Folin method for the estimation of preformed ammonia in urine by means of an air current at ordinary temperatures, to the estimation of preformed ammonia in flesh foods. The method cannot be commended for its simplicity, and the results obtained are comparable with the results obtained by method 1. The error most likely to affect method 1 is the production of ammonia by hydrolysis of compounds containing more firmly bound ammonia than in ammonium salts and the method about to be discussed suffers from the difficulty of separating all the ammoniacal nitrogen in an air current. The method can be conducted as follows: 50 grm. of the finely divided meat are introduced into a litre round bottom flask with 300 c.c. water and 10 grm. recently calcined magnesium oxide. The purifying and absorption train is arranged as follows: The air is dried and freed from basic substances by drawing it through sulphuric acid in a flask provided with a Hopkins safety bulb. It then passes through the suspension of meat. The meat flask is followed by an empty 250 c.c. catchall flask and this by the absorption flask containing $N/10$ sulphuric acid. The last member of the train is a small empty guard flask to catch any of the standard acid mechanically sprayed over; and this flask is connected to the air pump. A volume of 8,000–10,000 cu. ft. during a period of 3 to 6 hours is necessary to remove the preformed ammonia. The back titration is made using cochineal as indicator.

Pennington and Greenlee found that the same amount of ammonia was evolved with 0.5–2.0 grm. of sodium carbonate as with magnesium oxide, which furnishes excellent evidence that the ammoniacal nitrogen evolved by their method is well differentiated from that in the other nitrogenous compounds present in meat.

Methods for the Detection of Changes in the Fat.

The fat associated with meat may also undergo characteristic changes, which, under certain conditions and when the necessary precautions

[1] *J. Am. Chem. Soc.*, 1910, 32, 561; *Z. physiol. Chem.*, 37, 161.

are observed, may give information in regard to the condition, age or handling of the product. The examination is made for free fatty acids and for rancidity.

Formation of Free Fatty Acids.—The hydrolysis of fats with formation of fatty acids and glycerol is brought about in fatty tissue after death by the enzyme lipase and later, if no steps are taken to prevent putrefaction, by bacteria. In the rendering of fats too, especially by means of steam and water under pressure, some hydrolysis occurs, so that good grades of fats and oils contain as much as 0.2 to 2.0% free fatty acids.' In the case of edible fatty tissue hydrolysis by bacteria is negligible, and therefore the fatty acids present are due to hydrolysis by the natural enzyme of the fat. This action appears to go on not only at ordinary temperatures but even at low temperatures in tissues which are well supplied with the enzyme. The lipase of hog pancreas is moderately active at a temperature of $-12°$ or even lower but experiments made with beef fat, and leaf fat from swine indicate that the natural lipase is inactive at such low temperatures or is not present in sufficient quantity to produce a measurable effect. The fats were taken within 24 hours after death and held at -9 to $-12°$ and analyses made from time to time up to 2 years. The amount of free acid varied from 0.3 to 0.6%, depending upon the sampling and rendering, at the beginning and throughout the experiment. In some varieties of summer sausage and also in old dried hams ("Italian" style ham, "Virginia" ham, "Westphalian" ham, etc.) the free fatty acids in the fat may rise to 9–10%.

Estimation of Free Fatty Acids.—If possible the fat tissue should be separated from the lean of the sample, hashed and rendered at a low temperature in a porcelain dish in the steam bath or over a low flame. The melted rendered fat is then filtered. This is the best method for freeing the fat from organic impurities. 10–20 grm. of this fat are then titrated in hot neutral alcohol against standard alkali using phenolphthaleïn as indicator. The result is calculated either to acid number (milligrams KOH per grm. of fat) which is preferable, or by conversion, to percentage of oleic acid (mol. wt. 282).

If the fat is distributed throughout the lean and cannot be separated by hand, it may be extracted by means of ether or petroleum ether after drying the sample, but this introduces the po sibili'y of two errors, first the acids formed by oxidation during the drying (supposing this to be done in the air); second the extraction of organic acids from the lean by the solvent. These latter, if they are water soluble, can be removed by washing in a separator or on a wet filter with hot water.

Pennington and Hepburn[1] have proposed making the free acid estimation on the fatty tissue directly without rendering, but this is not to be recommended except in cases where the quantity of the sample is too small to admit of rendering. They hash the fatty tissue, place the weighed sample (±10 grm.) in an Erlenmeyer flask, boil with alcohol neutral to phenolphthaleïn and titrate against standard alkali using the same indicator. The end-colour is fugitive and its persistence for 1/4 minute is taken for the endpoint.

The calculation is made as previously described.

Examination for Rancidity.—Rancidity is a condition of fats resulting from oxidation. It is characterised by the production of volatile substances of disagreeable odour. This action goes on in the air at ordinary temperatures, but it is enormously accelerated by sunlight and to a considerable extent by heat. Inversely it is retarded by low temperatures and storage in the dark. The process is purely a chemical one in which bacteria are not concerned, although under some conditions it may be aided indirectly by bacterial action. This may occur when fats or fatty tissue are emulsified with water containing bacterial nutritive substances.

Moisture (the humidity of the air is sufficient) appears to be essential to rancidity, and it is likely that the oxidation affects principally fatty acids following hydrolysis of the fat. Glycerol is not subject to rancidity, although when fats turn rancid the glycerol in *statu nascendi* appears to be further split. In ordinary examples only a small part of the fat is affected or altered by the process. The products which are formed are largely lower fatty acids and aldehydes, free alcohols and esters and as these are volatile, a fat exposed to air and sunlight continually loses weight, unless the effects of rancidity are more than counterbalanced by the drying process in which oxygen is absorbed from the air.

The characteristic odour, to an experienced chemist, affords the best evidence of rancidity. This odour is characteristic, although not identical in all cases, and unmistakable, but difficult to describe.

A chemical test for rancidity has been proposed by von Raumer,[2] but the test is of limited usefulness and it is doubtful that it affords positive evidence of rancidity, since overheated fats respond to it. Schiff's reagent is used, prepared as follows: 40 c.c. of a 5% solution of fuchsin (magenta) are mixed with 250 c.c. distilled water and the dye is bleached by adding 10 c.c. of sodium hydrogen sulphite solution

[1] *J. Am. Chem. Soc.*, 1910, 32, 569.
[2] *Analyst*, 1897, 265.

(sp. gr. 1.375) and 10 c.c. of sulphuric acid (sp. gr. 1.84). After standing the mixture becomes colourless. 5 c.c. of the rendered melted fat are mixed with 10 c.c. of the reagent, heated to 90–100°, and shaken. Rancid fats give a deep red to violet colour to the reagent which does not fade, while fresh fats give none, or at most a faint pink which fades in 30 minutes in the cold. When rancid fat is distilled with steam, volatile aldehydes, as well as the lower fatty acids, come over and can be tested for according to the usual methods.

According to Kries[1] if the melted rancid fat is shaken with hydrochloric acid (sp. gr. 1.19) and 1% phloroglucin solution in ether, a red colour due to aldehydes and ketones is produced. The ether used must be free from peroxide.

Poisonous Meat, Meat Poisoning, Ptomaïne Poisoning.

In the discussion of unsound meat in these pages the presence and detection of animal parasites are purposely not considered, since with the possible exception of inspection for trichinæ the subject would be out of place in a work of this character. For complete information on the subject consult Ostertag: *Handbuch der Fleischbeschau*, or the English translation: Ostertag-Wilcox, *Handbook of Meat Inspection;* also Edelmann, Mohler and Eichhorn: *Meat Hygiene.*

There are a number of different cases coming under this heading which may be classified as follows:

(1) Flesh rendered poisonous by contamination with mineral or organic poisons.

(2) Flesh made poisonous by the food of the animal.

(3) Flesh made poisonous by metabolic products of the cells of the living animal.

(4) Flesh made poisonous by the presence of pathogenic bacteria in the living animal.

(5) Flesh made poisonous by bacteria after death of the animal (true ptomaïne poisoning).

(1) **Flesh rendered Poisonous by Contamination with Mineral or Organic Poisons.**—The cases of this sort which sometimes occur, come properly within the scope of toxicology. Reference should be had to a standard work on this subject as detailed methods of analysis cannot be given in this place. Methods for estimating metals and their compounds which may be absorbed by contact, especially from metallic containers, are given under "Canned Meats." Tin is

[1] *Chem. Ztg.*, 1904, 28, 956.

the metal most commonly to be looked for; other metals possibly present are zinc, copper, lead, arsenic and antimony.

(2) **Flesh made Poisonous by the Food of the Animal.**—Not only do food animals consume food which makes meat derived from them unpalatable but sometimes also actually poisonous. For vertebrates such food may be colchicum, equisetum, lupines, buckwheat (intermingled with the fodder) or sprouting potatoes, ricinus, mustard cake, etc.[1] Shell-fish and poisonous tropical fish also appear to derive their poisonous nature in certain cases from their food.[2] (For list of poisonous fish see Andrews, *Handbook of Public Health*, 1898, 51.) Flesh foods may also be made poisonous occasionally by the animals' accidental eating of definite mineral or organic poison (lead, phosphorus, arsenic, etc.) or by improper medication (tartar emetic, mercury, strychnine, carbolic acid, etc.) of the animal.[3] The animal may or may not be killed or made sick by the food or poison.

(3) **Flesh made Poisonous by Metabolic Products of the Cells of the Living Animal.**—In the catabolic processes of the animal cell, many protein degradation products are formed, before the end-products urea, carbon dioxide, water, etc., are reached. Many of these substances are basic and nitrogenous and related chemically to the ptomaïnes (see below under *Flesh made Poisonous by Bacteria after Death*); some, indeed, as choline, neurine, betaine, tri-methylamine, neuridine, cadaverine, etc., are identical with the ptomaïnes.[4] These metabolic products of the living cell are known collectively as leucomaines. Most of them are harmless or nearly so, but on the other hand, some are decidedly poisonous (neurine, choline). Normally they are soon eliminated and do not accumulate in the tissues in excess. The predisposing cause for the presence of undue amounts of leucomaines in the flesh of higher animals is over-fatigue, for example in over-hunted game, hard-driven cattle and animals which have struggled violently after having been caught in a trap. An occasional illness caused by eating the flesh of hard-hunted game may be due to leucomaines.[5]

(4) **Flesh made Poisonous by the Presence of Pathogenic Bacteria in the Living Animal.**—Two cases may be distinguished under this head: First, those due to specific bacteria which existed and occasioned disease in the animal before slaughter. Such an organism is the *B. enteritidis* of Gaertner. These cases have in times past

[1] Ostertag-Wilcox, *Handbook of Meat Inspection*, 379. See also Letheby, *On Foods*, 221.
[2] Letheby. Also Guenther, *The Study of Fishes*, 189.
[3] Walley, *Meat Inspection*, 57. Ostertag-Wilcox, *Handbook of Meat Inspection*, 379. Mitchell, *Flesh Foods*, 216.
[4] Mitchell, *Flesh Foods*, 218. Vaughan and Novy, *Cellular Toxins*.
[5] Gautier, *Les Toxines*, 1896, 438, 455, 456

occurred not infrequently in Germany (consult Ostertag) following cases of "emergency" slaughter of diseased and dying animals. It seems almost incredible that such meat should be eaten at all, even by a half starved population, and doubly incredible that it should be eaten without adequate preliminary cooking.

There are also many cases on record wherein the various bacteria of pyæmia and septicæmia, from animals afflicted with these diseases at the time of slaughter, caused sickness among the consumers of the meat.[1]

Second, are those cases of sickness caused by pathogenic bacteria which are present in or on the flesh food in question, but which produced no disease in the living animal from which it was derived. The best-known examples of this class are botulism caused by *B. botulinus* and those cases due to the contamination of shellfish in waters polluted by sewage.[2] Typhoid fever and Asiatic cholera are the diseases most to be feared from this source. The bacteria *B. coli* and *B. enteritidis sporogenes* have been found abundantly present in such food although they are not normally present in the common shellfish, or in sea water uncontaminated by sewage.

Botulism or sausage poisoning, also called *allantiasis* and *ichthyosism*, caused by *B. botulinus* of van Ermengem requires special mention. This disease is the most typical and the best known of those caused by pathogenic bacteria which are also capable of living on meat products as *saprophytes*. It has been of most frequent occurrence in those parts of Germany and Saxony where it is customary to eat meats such as ham and sausage in raw condition. The period of incubation usually varies from 18 to 48 hours. Fatal cases result in death in from 4 to 8 days. In the past as many as 25 to 30% of the cases have proved fatal, but the disease is now rarely observed. *B. botulinus* is an anaerobe and develops especially in meat products whose preparation—pickling, smoking, ageing, drying—favors *anærobiosis*. The bacteria may develop in rather closely circumscribed areas and hence some parts of the infected meat may be quite innocuous; others decidedly injurious. The symptoms of the disease may be summed up as central nervous disturbances, secretory disturbances and motor paralysis. Fever is absent. Van Ermengem's conclusion that the disease is caused by toxins preformed in the meat (not formed in the body) does not harmonise with the fact that an incubation period intervenes before the onset of the characteristic symptoms, and requires further investigation.

[1] Mitchell, *Flesh Foods*, London, Griffin, 1900, 220.
[2] *U. S. Dept. Agri. Bur. Chem. Bull.* 136, 1911. This bulletin contains an excellent bibliography.

As stated, the disease is now rare and thorough cooking appears to obviate the danger.

(5) **Flesh made Poisonous by the Action of Bacteria after Death of the Animal (True Ptomaïne Poisoning).**—The expression "ptomaïne poisoning" is commonly used by the physician and the public to designate almost any sort of sickness caused by the eating of spoiled food. The word ptomaïne was brought into the literature in the years 1874 to 1877 by Selmi before the doctrine of bacterial toxins had been developed, to designate poisonous, basic, sometimes crystalline, bacterial products, to which the injurious action of bacteria was ascribed. In the bacterial degradation of the proteins a long series of simpler basic substances is produced, some harmless and some harmful. Among these relatively simple basic substances are those which Selmi named the ptomaïnes or animal alkaloids, and of these some are very poisonous and cases of meat poisoning from eating putrid or decomposing meat have undoubtedly been due to them. Some of them are not decomposed by boiling water but retain their toxicity even after boiling for some time, thus cooking does not render them harmless, and this property distinguishes them from the bacterial toxins, none of which withstands a boiling temperature. The toxins proper (*ectotoxins*) appear to be elaborated within the bacterial cell and are excreted into the surrounding medium. Thus they are both soluble and diffusible. Contrasted with these are the so-called *endotoxins*, which are elaborated within the cell and remain therein. Thus, three classes of poisons, all of bacterial origin, must be considered in connection with meat poisoning.

The knowledge that a water extract of decomposed animal matter had poisonous properties is very old and older still is the knowledge that spoiled food might prove toxic. Yet the action of spoiled foods is by no means uniform. A food which decomposes spontaneously—that is by uncontrolled bacterial action—may prove harmful at one time and not at another, or harmful to one person and not to another. Meat which is even very slightly tainted will cause severe gastro-intestinal symptoms when eaten by some individuals, whereas meat in which bacterial decomposition is much more advanced may be eaten by preference by others and cause no ill effects whatever. Such so-called "gamey" meat is a regular article of diet by certain epicures.

More striking still are certain nitrogenous foods which are allowed to undergo a more or less controlled putrefaction, or other fermentation, for the purpose of developing strong flavours. These foods are very generally eaten and appear to be entirely harmless. Important

among them are the strong cheeses, such as Roquefort and Limburger. Fish is eaten in a state of incipient decomposition by certain islanders. A preparation of eggs which have undergone a controlled putrefaction is consumed by certain Chinese, while in the Dardanelles, fish-roe cheese is prepared by allowing the roe to undergo a fermentation and pressing and drying the product in the air.

It is plain that the facts in regard to the harmless consumption of decomposed nitrogenous food greatly complicate the subject of ptomaïne poisoning. The conditions which give rise to it are necessarily somewhat obscure. It appears certain that not every putrefactive fermentation of flesh foods gives rise to poisonous ptomaïnes, at least in the earlier stages of decomposition, while the product is still fit for food, and in some cases if they are formed, they appear to be fugitive, disappearing a little later. Thus it has been observed that a flesh food poisonous at one period of its history may become harmless subsequently. Nor is there positive evidence that ptomaïne poisoning is caused by a specific bacterium or by a limited number of specific bacteria.

There can be no doubt that in recent years since the properties of toxins have become established, attention has been directed away from Selmi's ptomaïnes as the active agencies in cases of food poisoning. It is likely that attention will again have to be directed to them in order to account for many of the obscure minor cases of food poisoning, in which the gastrointestinal symptoms follow quickly after the ingestion of the food—too quickly to admit of an incubation period which is characteristic of the best known and most typical kind of bacterial poisoning, botulism, caused by *B. botulinus*.

The following table of the ptomaïnes and basic degradation products of proteins is due to Mitchell (*Flesh Foods*) and follows Gautier's scheme of classification.[1]

Monamines of the Aliphatic Series.

Trimethylamine, $(CH_3)_3N$, herring pickle.
Di-ethylamine, $(C_2H_5)_2NH$, putrid meat extract.
Tri-ethylamine, $(C_2H_5)_3N$, decomposed cod-fish.
Propylamine, $C_3H_7NH_2$, decomposing cod-liver.
Butylamine, $C_4H_9NH_2$, decomposing cod-liver.
Amylamine, $C_5H_{11}NH_2$, cod-liver oil.

Diamines of the Aliphatic Series.

Putrescine, or Tetramethylene-diamine, $C_4H_{12}N_2$. Putrid horse flesh.

[1] See Vol. 7, p. 345 *et seq.*, and Abderhalden, *Biochemische Untersuchungsmethoden*, 1909, 2, 1002–1042.

Cadaverine, or pentamethylene-diamine, $C_5H_{14}N_2$.

Putrid fish and blood.

Neuridine, $C_5H_{14}N_2$, putrid meat, albumin, gelatin.

Saprine, $C_5H_{14}N_2$, decomposed flesh.

Guanidines.

Methylguanidine, $C_2H_7N_3$, putrid horse flesh and beef.

Aromatic Ptomaïnes, free from Oxygen.

β-Phenylethylamine, putrid gelatin.

Parvoline, $C_9H_{13}N$, putrid horse flesh after several months.

Corindine, $C_{10}H_{15}N$. Putrid cuttle-fish.

Di-hydrocollidine, $C_8H_{13}N$, putrid fish and horse flesh.

Oxygenated Ptomaïnes.

Neurine, $C_5H_{13}ON$, putrid meat on fifth or sixth day.

Choline, $C_5H_{15}O_2N$, accompanies neurine.

Muscarine, $C_5H_{15}O_3N$, putrid fish.

Betaine, $C_5H_{11}O_2N$, in mussels (leucomaine).

Homopiperidinic acid, $C_5H_{11}O_2N$, decompositon of meat fibrin.

Mytilotoxine, $C_8H_{15}O_2N$, in poisonous mussels.

Mydatoxine, $C_6H_{13}O_2N$, putrid horse flesh after nine to fifteen months.

Gadinene, $C_7H_{18}O_2N$. (Putrid fish, especially cod.)

Methylgadinene, $C_8H_{20}O_2N$.

Unnamed base of Brieger, $C_7H_{17}O_2N$, accompanies mydatoxine.

Aromatic Oxygenated Bases.

Tyrosamines, C_7H_9NO, $C_8H_{11}ON$, $C_9H_{13}ON$, decomposing cod-liver.

Mydine, $C_8H_{13}ON$, decomposing human flesh.

p-Hydroxyphenylethylamine, cheese, cod-liver, etc.

Examination of Supposed Unsound Meat Products.—Passing judgment upon a sample of meat which is supposed to have been the cause of sickness is one of the most difficult tasks which confronts the chemist. No general rules applicable to all cases can be formulated, but the following statements can be made which will assist the investigator in arriving at a conclusion.

(1) If the meat was consumed in fresh condition—that is, not salted or pickled and if it was well cooked, and if it appears from the examination to be sound and untainted (absence of bacteria in interior, low ammoniacal nitrogen, etc.) the conclusion would be that the meat was harmless unless it could be included under the exceptional cases 1, 2 or 3 given above.

(2) If the meat was consumed in fresh condition but uncooked, suspicion should attach to it in any case. Thorough inspection before

and after slaughter reduces the danger to a minimum; but fresh meat should never be eaten raw.

(3) If the meat was salted or pickled and cooked just before consumption, in nearly all cases it could not be considered harmful. On the other hand, if cooked at the time of manufacture and then stored for a longer or shorter period, further investigation should be made. The results of ammoniacal nitrogen determinations and Eber's sulphide test are not so conclusive in the case of salted and pickled meats as of fresh meats.

(4) If the meat was salted or pickled and consumed in the raw state, it may always be looked upon with suspicion until further examination proves it to be innocuous.

The analyst who examines meat products which are suspected of having caused illness, should be familiar with the appearance, odour and flavour of the various kinds of fresh and cured meats and meat products in sound condition. No rules or descriptions can be laid down which will be in any sense the equivalent of individual experience with the various products.

Sound meat derived from mammals properly slaughtered and properly handled and stored should be reasonably firm to the touch (not flabby) and should scarcely moisten the finger. It often has a marbled appearance, especially in the case of young fat animals, due to the distribution of fatty tissues between the muscle bundles. It should have the characteristic odour of the species but no disagreeable odour. The presence of excessive moisture (above 74%) is often recorded as an indication of spoiled meat, but it is doubtful that this idea is well founded, and if it were, before the increase in moisture could be noticed or determined other criteria would point certainly to decomposition.

As to colour, there are considerable differences to be observed depending upon the species and age of the animal, the location of the cut and the method of slaughter. An animal killed by bleeding yields lighter-coloured meat, than by other methods. Typical beef has a good red colour and is neither pale nor dark purple. Veal and pork are much lighter in colour partly due to the method of slaughter. Some birds possess dark muscular tissue only; others, as the domestic fowl, both dark and light. Fish muscle is generally white but in some species a pink or reddish colour prevails. All of these points must, however, be learned by the analyst from careful inspection.

After the physical examination which should take note of colour, odour, texture, and the other general characteristics and condition of the sample (fresh, salted, smoked) the chemical examination proper is

begun. All or a part of the following determination and tests may be made.

(1) Examination for metals. (See Canned Meats.)
(2) Determination of ammoniacal nitrogen (page 313).
(3) Application of Eber's sulphide test (page 312).
(4) Free acid and rancidity of fat (page 317).
(5) Examination for ptomaïnes.
(6) Bacteriological examination.

These tests have all been considered previously with the exception of the last two. If decomposition has advanced to the point where ptomaïnes are formed, evidence of decomposition will be disclosed by the first three tests. If a specific examination for ptomaïnes is called for the investigator is referred to the methods in Vaughan and Novy's Cellular Toxins, as the methods, which are seldom used at present, demand more discussion than can be given here.

The bacteriological examination requires the expert services of a bacteriologist. The methods presume thorough knowledge of that science and can only be briefly outlined here.

Feeding experiments are important and should be conducted with a number of species of animals, such as guinea pigs, rabbits, mice, rats, dogs and cats. If any of the animals become sick the symptoms should be noticed, and if death ensues the body should be examined for pathogenic bacteria. Negative results do not necessarily indicate absence of pathogenic bacteria. According to Dr. E. C. Novy the further examination should proceed as follows:[1] "Another set of animals should be injected with a cold extract of the meat made with sterile water. If the animals die, they are to be examined for pathogenic bacteria. A third set of animals should receive similar injections, though larger portions, of this aqueous extract which has been previously filtered through sterile porcelain. If the animals die from such injections, the same as with unfiltered solutions, it is evident that a soluble bacterial chemical poison is present.

"The identification of the toxin produced by the germ is wholly out of the question. The most that can be done satisfactorily is to obtain, as above, a germ-free solution of the poison.[2]

"A bacteriological examination proper should be made of the original poisonous meat and of all the animals that died either from eating the meat or from the injections of the aqueous extracts. The organism present in the animal, if any, must be isolable directly from the meat.

[1] *Bull.* 107 (*revised*), *U. S. Dept. Agri. Bur. Chem.*, 1908.
[2] See Abderhalden, Biochemische Untersuchungsmethoden, Vol. 2, page 1002.

If it happens, as it sometimes has, that the dead animals contain no germs, it is proof that they were killed by a toxin elaborated by a germ in the meat previous to the injection. Cultures from the meat will then reveal the germ, and the effects of its pure cultures should correspond to those observed with the poisonous meat.

"To prepare the cultures from the original food, the latter should be cut out with a sterile knife and the material should be taken from the inside, thus avoiding all chances of contamination.

"Several sets of beef-tea tubes and agar plates should be made. One set should be set aside in a Novy anaerobic jar at room temperature; a second similar set should be placed at a temperature of 37°. A third set should be grown in the presence of air at room temperature, and a like set at a temperature of 37°."

The following is a good technique to follow in taking samples of meat for bacteriological examination. The surface of the meat is first seared with a Bunsen burner. If the sample is to be cut across, a complete circle is seared around the sample in this way. The section is then made by means of knives (and saws if necessary, as in the case of pork hams) sterilized by boiling in 3% sodium carbonate or by heating in the naked flame. At the place where a sample is to be taken the cut surface is again seared by a branding iron in the form of a circular disc 1 or 2 in. in diameter, and the sample cut out by means of sterile scalpels. These pieces can be dropped at once into sterile culture media, or can be further cut up with suitable precautions. Preferably all these operations are to be carried out under a glass case entirely closed except for a moderate sized opening on the side facing the operator.

In passing judgment on meat food products for soundness or unsoundness and excluding cases 1, 2, and 3 given above and thus including unsoundness due to bacterial influences only, consideration should be had for the following points. Ordinary bacterial decomposition or putrefaction of meat may or may not render it harmful, but such meat should always be looked upon with suspicion. Incipient decomposition (gaminess, *haut gout*), may be without effect or may cause gastrointestinal disturbances resulting in slight purging or rather violent diarrhœa. These results may occur in from 8 to 24 hours and are usually, but not always, of short duration. On the other hand, advanced decomposition (putrefaction) even after the product has been cooked, will invariably cause violent symptoms and possibly death in the average subject. Besides ordinary saprophytes and their products there may be present representatives of two different classes of

pathogenic bacteria of which *B. enteritidis* and *B. botulinus* are typical, and which may occasion serious specific diseases and death. Thus meat which is slightly decomposed can never be said to be positively harmful; yet it is always to be looked upon with suspicion. This appears to be the unavoidable conclusion, although the harmlessness of such meat in the majority of cases is a matter of everyday record.

The Preservation of Meat.—There are only four general methods used for the preservation of meat, in common with other foods. They are:

Desiccation.
Heat Sterilisation.
Low temperatures.
Antiseptics.

Exclusion of atmospheric air is often given as a method of preservation, but at best it is only a partial means and very imperfect. It acts by creating conditions unfavorable for the development of aerobic micro-organisms, but does not prevent the development of facultative and anaerobic organisms, and as these together outnumber the exclusively aerobic organisms, putrefaction is not prevented and not greatly retarded. In the absence of air (or more accurately, oxygen) rancidity, which is a chemical (not bacterial) oxidation of fats, does not occur, and therefore exclusion of air is a most valuable adjunct to the methods enumerated above, and in combination with these produces the best-known methods for the preservation of flesh foods.

Desiccation.—This is one of the oldest and most effective methods for the preservation of all kinds of foods, including meat, and has been used from a time, long antedating written history. It is the method most commonly used by savage and semi-civilised tribes at the present day and naturally so on account of its simplicity and effectiveness. Ordinary fresh meat as prepared and stored in large establishments possesses a dry surface, and this is an important point for its preservation. The surfaces of cured meats such as hams, bacon, smoked beef and sausage are also maintained in dried condition and the drying is quite as important for their preservation as the smoking, if not more so. Certain cured meats such as so-called "Italian" hams and summer sausage (*Cervelat, Salami,* etc.) are purposely hung to dry for a long period after which they remain in good condition indefinitely. *Jerked meat, biltong, charque, carne secca, carne Tassajo* are names applied to dried meat in different parts of the world. The method of preparation usually consists in cutting the meat in thin strips and drying in the sun or over a slow fire with or without previously coating

the product with salt, sugar or flour. Meat powder and dried blood (Sweden) are also articles of commerce.

Pemmican is a preparation of meat made by the American Indians chiefly from buffalo or deer flesh or fish. The meat was cut into strips, dried and reduced to a powder in a mortar. About an equal quantity of fat was then incorporated with it. The modern product, as used chiefly by explorers in cold regions, usually has a fruit, such as currants, added.

Dried fish are prepared by splitting them down the middle and drying in the air, by civilised and uncivilised peoples. Smoked fish, which are more or less desiccated, are also common articles of commerce.

The chief characteristic of desiccated meats is, of course, the absence of much moisture. Fresh lean muscular tissue contains about 75% water and as water is the first requisite for the growth and reproduction of bacteria, it is natural that a considerable reduction in this quantity makes the product a poorer medium for bacterial growth and thus insures its preservation to a greater or lesser extent. On the other hand, dried meat products are apt to become rancid, on account of the fat they contain, when exposed to the air, and this is especially true of comminuted products where a large surface is exposed.

Heat Sterilisation.—Heat sterilisation has been a matter of knowledge, more or less imperfect, ever since the art of cooking foods was practised, but the contamination of such sterile foods by exposure to sources of bacterial infection as the air was not fully understood until recently. However, in a practical way, the process of preservation by heat sterilisation accompanied by sealing in hermetically closed containers was disclosed by Appert in 1809. His original process provides for heating the product in earthenware vessels and protecting it from subsequent infection by hermetic sealing. It has been recognised that no air should be left above the product for the best results and this has been brought about variously by completely filling the containers, as with jelly, fats or oils, by leaving the space filled with steam which later condensed leaving a vacuum and by filling the space with an inert gas such as nitrogen or carbon dioxide. Appert's process in one or another modification has been used for preserving practically all kinds of food and is used at present in large canning establishments for the production of immense quantities of tinned vegetables, fruits and meats including fish.

Heat sterilisation of meats is based on the following facts. The vegetative forms of micro-organisms in moist condition are all killed below the temperature of boiling water and their spores are killed at

temperatures not higher than 120° corresponding to a gauge steam pressure of 14.7 pounds. In canning factories, heat sterilisation is known as "processing" and is carried out in autoclaves called "retorts." The steam pressures used generally lie between 4 and 10 pounds as shown on the gauge.

The principal kinds of sterilised canned meats are boiled beef, corned beef, tongue, canned chicken and poultry, and various deviled meats and pastes. Among sterilised canned fish, salmon, sardines and other small fat fish are the principal varieties.

The preservation of meat (and other kinds of food) by confining it in hermetically sealed vessels is now practised on an enormous scale. In the case of meat, the material, freed from bone, is packed in the tins, and an addition of jelly or gravy, salted and flavoured, is often made. The tins are then closed, with the exception of a small orifice and processed. The current of steam issuing from the tin through the aperture left for the purpose carries the air with it, and the high temperature effectually destroys any lower organisms. When the air is judged to be thoroughly expelled, the orifice is closed by solder. When the operation is carefully performed, the contents of the tin will keep in a good condition for an indefinite period.

Two typical cases of meat-canning operations will be described as they are practised in the larger meat-canning factories.[1]

The canning of corned beef begins with boiling the beef for about an hour to shrink the meat. The liquid resulting from this preliminary boiling goes into the manufacture of one grade of meat extract (cured meat extract). The meat is then put into cans which are capped with the vent open. Vacuum is then established in one of three ways: (1) sealing in a vacuum machine under a vacuum of 22 in. or more; (2) processing in a retort and sealing soon after removal; (3) sealing, placing in boiling water, removing and puncturing, and re-sealing. Method 2 is the one generally followed. For a 1-pound can the first processing lasts about 1 hour; after sealing the can is returned to the retort and processed at about 8 pounds pressure for 1 1/2 hours. For larger cans longer processing is required.

The canning of unsalted beef (boiled beef, "Roast" beef) is similar to that described. Higher temperatures are necessary, however, to insure sterilisation, because of the fact that bacteria are more easily killed in salt solution than in water.

Potted meats are first boiled for about 1/2 hour, then hashed, spread

[1] See *Bull.* 13, *Part* 10, *U. S. Dept. Agric. Bur. Chem.*; C. N. McBryde, *Annual Report U. S. Bur. Animal Ind.*, 1907, p. 279; Richardson, *Ency. Am. Agric.*, 3, page 261. *Bull.* 151, *Bur. Chem. U. S. Dept. Agric.*, 1912, (General and Historical).

on trays and heated 20 minutes below the boiling-point in retorts. As quickly as possible the cans are stuffed by stuffing machines. The caps are soldered on and the vents closed by hand, and the cans processed at about 8 pounds pressure for 1/2 to 1 1/2 hours depending on the size of the can. In some cases the vents are closed in the vacuum machine before processing.

The following analyses by J. König show the composition of commercial potted foods. The foie-gras paste was obtained from Strassburg, and the remaining samples from Cross & Blackwell, London.

	Water	Nitrogenous matters	Nitrogen-free extract	Fat	Ash	Common salt
Foie-gras paste	46.04	14.59	2.67	33.59	3.11	.22
Potted beef	32.81	17.17	3.36	44.63	2.03	.....
Potted ham	25.57	16.88	.....	50.88	6.78	5.72
Potted tongue	41.52	18.46	0.46	32.85	6.71	5.98
Potted salmon	37.64	18.48	0.70	36.51	6.67	5.65
Potted lobster	51.33	14.87	4.04	24.86	4.90	0.38
Anchovy paste	36.81	12.33	5.18	1.59	44.09	40.10

Tinned meat, preserved in the foregoing manner, is sometimes overcooked, though this fault has been less evident of late years. Occasionally, through imperfect sealing of the tins, the contents undergo change, and when there is any evidence of this they should on no account be eaten. Incomplete sterilisation will result in gradual bacterial fermentation of the meat, with production of gas, and sometimes with formation of poisonous ptomaïnes. Hence any can which is bulged by internal pressure, or from which gas issues on opening, should be rejected.

H. A. Baker (*8th Int. Cong. App. Chem.*, New York, 1912, Section VIIIc, Bromatology) states that "springers" is a trade term given to cans with bulging ends which contain perfectly sound and sterile food products. The gases in the head space of "springers" are never more than three: Carbon dioxide, nitrogen and hydrogen. Very often no hydrogen is found. Oxygen is practically never found. The carbon dioxide is formed during the time of processing, or when the product is not worked through quickly from the beginning of preparation to final sterilisation, or when it is allowed to stand in the container before sterilisation. Nitrogen is simply a residue from unremoved air. Hydrogen when formed is the product of attack of organic acids on the metallic container. "Springers" are usually produced in warm weather, but can be avoided by suitable precautions. They are more apt to occur in the case of vegetable products than in the case of meat products.

Occasionally severe and even fatal ptomaïne poisoning has occurred by the use of decomposed canned foods, but such cases bear such a small proportion to the enormous number in which the meat is found good and wholesome, that, with care in the directions above named, danger from this cause is very remote.

Analysis of Canned Meats.—The chemical examination of canned meats requires in certain cases besides the usual determinations described under "General Methods of Analysis of Meats" an examination of the gases contained in the can and an examination for metals—especially poisonous metals—derived principally from contact with the metal package.

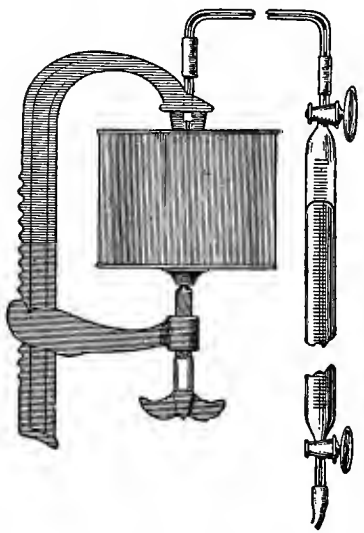


FIG. 25.—Apparatus for collecting the gases of canned foods.

The gases can be collected by means of the apparatus of Doremus.[1] A bevelled, hollow steel needle is attached to the upper arm of an adjustable clamp. The point and lower part of the shaft are covered by a rubber stopper, which serves as a soft pad. The lower arm is moved along the body of the clamp until the can to be pierced is held between the rubber stopper and the head of the screw. The upper part of the needle is connected by means of a capillary tube, filled either with water or mercury, with a receiver also filled with either of these liquids. The receiver may be a stop-cock eudiometer or a nitrometer.

[1] *Am. Chem. J.*, 1897, 19, 733.

The apparatus adjusted, a turn or two of the screw clamps the can tightly between the rubber-pad at the top and the screw-head below. The rubber yields to the pressure, making a tight joint around the needle. When the latter pierces the tin the contained gases of the can escape gently into the eudiometer.

H. A. Baker (*8th Int. Cong. App, Chem.*, New York, 1912, Section VIIIc, Bromatology) employs an apparatus with perforating needles both at the bottom and top of the can. Through the lower needle he introduces a current of water (under about 15 lb. air pressure) which sweeps the gas in the can under examination through the upper perforating needle and into a gas burette. With this apparatus Baker has investigated the disappearance of oxygen in canned food containers in an endeavor to explain the fact that oxygen is practically never found in the "head space" of cans in which food is packed, although slight traces have been found in cans containing foods of less than two months' age. The analyses usually show carbon dioxide 8 to 15% with the balance nitrogen. Hydrogen is also found to be present in some instances, particularly with acid fruits. Baker concludes that oxygen disappears in tinned food containers in at least the three following manners:

(1) By combining with the metals tin and iron, forming oxides.

(2) By oxidizing tin or iron salts.

(3) By combination with nascent hydrogen, when organic acids act on the metallic container.

For the analysis of the separated gases reference should be made to Hempel's Gas Analysis (translated by L. M. Dennis), or to other standard works on the subject. The gases, methane, hydrogen, nitrogen, carbon dioxide, carbon monoxide and oxygen are among those to be expected. Nitrous oxide, ammonia and hydrogen sulphide are also among the possibilities, although they would naturally be present in very small quantity.

General Composition of Canned Meats.—On account of the cooking it has received the meat proper in the can will contain less moisture than uncooked meat, but the can as a whole may contain more water than would correspond to the water content of uncooked meat (see Cooking of Meat, page 304).

Warden and Bose have published (*Chem. News*, 1890, **61**, 304) some unusually complete *analyses of typical samples* of canned beef and mutton. They found the moisture to range from 49 to 57%; the fat from 10 to 22; the proteins (*i.e.*, N×6.25) from 24.5 to 29; the ash from 0.62 to 4.36; the chlorine from 0.11 to 2.65; the phosphoric acid

from 0.31 to 0.40; the hot-water extract from 5.35 to 10.14%, with a content of nitrogen ranging from 0.88 to 1.10%.

In making these analyses, the entire contents of a can were thoroughly pulped in a large mortar, great care being taken to scrape the interior of the can free from fat and jelly. The plan of taking a slice of the contents and regarding that as a fair sample Warden regards as fallacious.

For the determination of *moisture*, a weight of from 5 to 6 grm. of the sample was teased with forceps in a flat platinum dish, and dried first at 100° and subsequently at 120°. The samples were then moistened with absolute alcohol, and redried. The whole time of heating occupied from 8 to 9 hours. In another large platinum dish from 30 to 40 grm. weight of pulp was similarly heated, reduced to fine powder, and again heated. This dried pulp was preserved in a closely stoppered bottle and employed for the determination of fat, nitrogen, and aqueous extract. The ash and ash-constituents were conveniently determined on the undried pulp.

In estimating the *ash*, the portion of the pulp used for ascertaining the moisture was charred at a temperature below redness, crushed with a glass rod, exhausted with boiling water, and again ignited. The residue was again treated with boiling water, and the *insoluble ash* ignited and weighed. The aqueous extract was evaporated to dryness, the residue heated nearly to redness, and weighed to find the *soluble ash*. The *total ash* was regarded as the sum of the soluble and insoluble ashes determined as just described, and it was found that the figures thus obtained agreed well with determinations of the total ash by direct ignition, while avoiding the difficulty experienced in the latter case in effecting complete combustion of the carbon without losing a portion of the alkali-metal salts by volatilisation.

The soluble ash was used for the estimation of *potassium* and *sodium*, by dissolving it in water and adding in succession barium chloride, ferric chloride, and ammonia to the warm solution, the last reagent being employed in quantity sufficient to render the liquid just alkaline. The precipitate (consisting of barium sulphate, ferric phosphate and ferric hydroxide) was filtered off, the filtrate treated with ammonium carbonate and ammonium oxalate, and warmed for some time on the water-bath. The precipitate (consisting of barium carbonate and calcium oxalate) was removed by filtration, the filtrate evaporated to dryness in platinum and the residue gently ignited. The residue was redissolved in water, the solution filtered from a little barium carbonate, the filtered liquid treated with a drop of hydrochloric

acid, and evaporated with platinic chloride to effect a separation of the potassium and sodium.[1]

For the estimation of the *chlorine* and *phosphoric acid*, Warden and Bose mix 20 grm. of the freshly pulped meat with about 2 grm. of pure sodium carbonate, dissolved in sufficient water to cover the pulp. The resulting magma is evaporated to dryness, carbonised, extracted successively with water and with nitric acid, the residue again ignited and dissolved in nitric acid, and the chlorine and phosphates estimated in the mixed solution by the usual methods.

The *total nitrogen* was estimated in the dried pulp by Kjeldahl's process, and multiplied by the factor 6.25 to find the *proteins*.

The *extractive matter* was estimated by boiling 1 grm. of the dry pulp with distilled water in a 100 c.c. flask, and when cold diluting to 100 c.c. The liquid was passed through a dry filter, and an aliquot portion of the very faintly opalescent filtrate evaporated to dryness in a platinum dish and the *residue* weighed. The greater part of the filtrate was used for the estimation of *extractive nitrogen* by Kjeldahl's method.

The *fat* was estimated by treating about 0.5 grm. of the dried pulp in a small accurately-stoppered weighing bottle, and adding a measured volume of light petroleum-ether from a burette. The mixture was allowed to stand for 2 days, with occasional agitation, when a portion of the perfectly clear supernatant liquid was withdrawn by a small burette, and a carefully-measured volume discharged into a small beaker. The petroleum-ether was then distilled off, and the residual fat dried at 100° and weighed. This method, which is due to Dragendorff, was found by Warden and Bose to give results which agreed closely with those obtained by exhausting the substance with a solvent of fat in the usual way.

Warden and Bose have compared their analyses of canned meats examined by the foregoing process with the figures obtained by König by the analysis of fresh beef and mutton (page 262). They find that, while the percentage of moisture in the canned meat is usually lower than in fresh meat, the fat in canned meat as a rule exceeds the proportion in fresh. Taking the nitrogenous matters as representing the nutritive value of the meat, and ascertaining their amount by multiplying the total nitrogen by 6.25, they obtain the following amounts of albuminous matters in the anhydrous and fat-free samples of meat examined:

[1] *Note.*—The use of perchloric acid in such cases is greatly preferable (*Davis, J. Agric., Sci.*, 1912).

	Albuminous matters in anhydrous, fat-free meat.
Average of canned beef samples	87.06%
Average of canned mutton samples	87.19%
Average of all fresh cow- and ox-flesh	93.94%
Average of all fresh mutton	93.81%
Average of all canned meat samples	87.12%
Average of all fresh meat samples	93.87%

Analyses by König of 7 specimens of canned meat showed them to have the following average composition: Proteins, etc., 28.97; fat, 12.63; ash, 3.71; and water, 54.69%. These figures correspond to 10.33% of nitrogen and 27.27% of fat in the anhydrous samples, and to 88.63% of albuminous matters in the anhydrous and fat-free samples.

From the foregoing figures Warden and Bose conclude that canned meat has a sensibly lower nutritive value than fresh meat. The apparent difference is, however, increased by the salt which has evidently been added to some of the canned meat samples.

Preservatives in canned foods can be sought for and estimated by the methods given on pages 170 and 372.

The following table is compiled from Atwater and Bryant's figures.[1]

Food materials	Number of analyses	Refuse	Water	Protein		Fat	Total Carbohydrates	Ash	Fuel value per pound
				N×6.25	By difference				
BEEF, CANNED.		%	%	%	%	%	%	%	Cals.
Boiled beef, as purchased	1	....	51.8	25.5	24.4	22.5	.....	1.3	1,425
Cheek, ox, as purchased	1	....	66.1	22.2	22.3	8.4	.....	3.2	765
Chili-con-carne, as purchased	1	....	75.4	13.3	13.3	4.6	4.0	2.7	515
Collops, minced, as purchased	1	....	72.3	17.8	17.9	6.8	1.1	1.9	640
Corned beef:									
Minimum	15	....	43.2	20.7	19.6	11.7	.....	2.0	1,000
Maximum	15	....	58.3	35.1	34.2	31.1	.....	7.3	1,695
Average	15	....	51.8	26.3	25.5	18.7	.....	4.0	1,280
Dried beef, as purchased:									
Minimum	2	....	44.2	38.0	37.1	6.1	.....	9.8	955
Maximum	2	....	45.3	40.4	40.1	4.8	.....	12.6	965
Average	2	....	44.8	39.2	38.6	5.4	.....	11.2	960
Kidneys, stewed, as purchased:									
Minimum	2	....	70.9	14.6	.....	4.9	.....	2.1	580
Maximum	2	....	72.9	22.1	.....	5.4	4.3	2.8	620
Average	2	....	71.9	18.4	.....	5.1	2.1	2.5	600
Luncheon beef, as purchased	1	....	52.9	27.6	26.4	15.9	.....	4.8	1,185
Palates, ox, as purchased:									
Minimum	2	....	69.6	16.4	15.9	9.4	.....	0.4	750
Maximum	2	....	73.1	19.3	19.0	10.6	.....	2.0	755
Average	2	....	71.4	17.8	17.4	10.0	....	1.2	755
Roast beef, as purchased:									
Minimum	4	....	55.8	20.3	19.3	9.0	.....	1.2	935
Maximum	4	....	62.8	29.8	30.0	23.6	.....	1.4	1,375
Average	4	....	58.9	25.9	25.0	14.8	.....	1.3	1,105

[1] *U. S. Dept. Agric. Exp. Sta. Bull.* 28, 1899.

Food materials	Number of analyses	Refuse	Water	Protein		Fat	Total carbohydrates	Ash	Fuel value per pound
				N×6.25	By difference				
Beef, Canned. (*Con.*)		%	%	%	%	%	%	%	Cals.
Rump steak, as purchased	1	....	56.3	24.3	23.5	18.7	.....	1.5	1,240
Sweetbreads, as purchased	1	....	69.0	20.2	19.5	9.5	.....	2.0	775
Tails, ox:									
Edible portion	1	....	67.9	26.3	24.6	6.3	.....	1.2	755
As purchased	1	29.7	47.7	18.5	17.3	4.5	.....	0.8	535
Tongue, ground, as purchased:									
Minimum	6	....	42.5	20.1	20.2	21.6	.....	2.9	1,305
Maximum	6	....	54.9	23.6	22.8	32.6	.....	5.1	1,750
Average	6	....	49.9	21.4	21.0	25.1	.....	4.0	1,455
Tongue, whole, as purchased:									
Minimum	5	....	42.4	10.8	18.6	15.7	.....	3.0	865
Maximum	5	....	57.4	23.4	23.0	32.7	.....	6.3	1,725
Average	5	....	51.3	19.5	21.5	23.2	.....	4.0	1,340
Tripe, as purchased:									
Minimum	2	....	68.9	16.5	16.2	2.6	.....	0.4	425
Maximum	2	....	80.2	17.0	16.6	14.5	.....	0.6	920
Average	2	....	74.6	16.8	16.4	8.5	.....	0.5	670
LAMB, CANNED.									
Tongue, spiced and cooked:									
Edible portion	1	....	67.4	13.9	14.3	17.8	.....	0.5	1,010
As purchased	1	2.6	65.7	13.5	13.9	17.3	.....	0.5	980
MUTTON, CANNED.									
Corned, as purchased	1	....	45.8	28.8	27.2	22.8	.....	4.2	1,500
Tongue, as purchased	1	....	47.6	24.4	23.6	24.0	.....	4.8	1,465
PORK, CANNED.									
Brawn, boars' brains, as purchased:									
Minimum	2	....	44.3	20.1	18.2	12.9	.....	4.3	1,110
Maximum	2	....	53.7	30.3	28.5	33.2	.....	4.9	1,775
Average	2	....	49.0	25.2	23.4	23.0	.....	4.6	1,440
PORK, CANNED.									
Boars' heads, as purchased:									
Minimum	2	....	50.5	19.8	17.8	19.3	.....	2.8	1,180
Maximum	2	....	60.1	21.6	20.7	25.0	.....	3.8	1,455
Average	2	....	55.3	20.7	19.2	22.2	.....	3.3	1,320
Ham, deviled, as purchased:									
Minimum	6	....	38.4	16.5	16.9	29.5	.....	2.3	1,610
Maximum	6	....	49.4	21.4	20.5	38.9	.....	4.4	1,975
Average	6	....	44.1	19.0	18.5	34.1	.....	3.3	1,790
POULTRY AND GAME, CANNED.									
Chicken, sandwich, as purchased.	1	....	46.9	20.8	20.5	30.0	.....	1.6	1,655
Turkey, sandwich, as purchased	1	....	47.4	20.7	20.7	29.2	.....	2.7	1,615
Plover, roast, as purchased	1	....	57.7	22.4	.....	10.2	7.6	2.1	985
Quail, as purchased	1	....	66.9	21.8	.....	8.0	1.7	1.6	775

a Refuse liquid.

More recent work has been done on canned meats by Wiley and Bigelow.[1]

Metals in Canned Foods.—*Tin* is dissolved from the containing can very readily in the case of acid fruits, and sometimes to such an extent as to communicate a distinct metallic taste to the food. Canned meat, soup, and fish are less liable than fruits and vegetables

[1] *Bull.* 13, *Part* 10, *Bur. Chem. U. S. Dept. Agric.*, 1902.

to contamination from this cause, but the evidence on the subject is very conflicting. Thus, J. Attfield (*Pharm. Jour.*, [iii], **14**, 719) failed to find more than very minute traces of tin compounds in various canned foods, but states that he not unfrequently detected minute particles of metallic tin by carefully washing the external surfaces of a mass of meat just removed from a can. Out of 50 cans of preserved animal food, G. W. Wigner (*Analyst*, 1880, 219) found only one to contain tin in appreciable quantities.

On the other hand, A. E. Menke (*Chem. News*, 1878, **38**, 971) detected and determined tin in canned pine-apple, apple, and lobster. In 1880, G. J. Wishart (*Chem. News*, **42**, 47) found tin in canned pine-apples, apples, and greengages, in quantities ranging from 0.21 grain to 0.36 grain of SnO_2 per 2-pound can, together with much larger proportions of iron. The taste was distinctly metallic and the fruits were uneatable. In 1883, A. Wynter Blyth found tin in every one of 19 samples of canned fruit (apricots, pine-apples, tomatoes), the proportions ranging from 15 to 11 grains per pound.

In 1889, Sedgwick showed that poisonous effects were produced by pears which had been cooked in a tinned saucepan. Beckurts, in the same year, called attention to the formation of tin sulphide by the action of albuminous matters on tin, and Nehring recorded the presence of tin in preserved asparagus in quantities ranging from 0.19 to 0.31%. Bettink, in 1890, found from 19 to 72 mgrm. of tin per kgrm. of tinned lettuce and meat which had occasioned the illness of a number of soldiers. Kayser found 0.19% of tin in preserved eels which had proved injurious to several persons who had partaken of them. Allen found 0.21% of tin in tomato-sardines suspected to have occasioned colic and diarrhœa.

The amount of tin dissolved necessarily depends on the length of time the article of food has been in contact with the metal, but van Hamel Roos, in an extensive research on the subject, found all tinned foods, whether of animal or vegetable nature, to contain more or less tin (abst. *Analyst*, 1891, 195).

O. Hehner (*Analyst*, 1880, 219) found canned animal foods of almost every variety to contain more or less tin. The weight of tin found in one of the soups was 0.035 grm. in a 1-pound can; in a can of preserved milk, 0.008 grm.; and in a 1-pound can of preserved oysters 0.045 grm., in addition to a considerable quantity of copper[1]. The tin was found throughout the mass of the soups and pasty foods, but in the case of the hard meats existed chiefly on the surface. In many cases

[1] Copper has been found to occur naturally in oysters.

the cans were much discoloured and blackened on the inner surface, but in others the surface of the metal was perfectly bright, although there was an abundance of tin in solution.[1]

For the detection of the tin in the above analyses, Hehner incinerated about 30 grm. of the material in a platinum basin, and heated the ash with strong hydrochloric acid. The greater part of the acid was then boiled off, about 30 to 40 c.c. of water added, and the liquid filtered. The alternate treatment of the residue with acid and water was repeated until no more tin could be extracted. The clear (and, as a rule, colourless) solutions thus obtained were then treated with sulphuretted hydrogen, and any yellow precipitate of stannic sulphide further treated, if necessary, in the usual manner.

In the foregoing process it is assumed that boiling hydrochloric acid can be relied on to dissolve tin from the ash of food, but in the experience of Allen its complete solution is always difficult and sometimes impossible to effect. It is highly probable that the negative results obtained by some chemists when examining canned foods for tin (*e.g.*, J. Attfield, *Pharm. Jour.*, [iii], **14**, 719) have been due to the use of inefficient methods of analysis.

Bigelow and Bacon[2] call attention to the fact that while it has been customary to attribute the presence of tin salts in canned foods to the action of acids, several varieties of canned foods, such as fish, beets, lima beans, asparagus, pumpkin and shrimp, although of low acid content, have a marked solvent action upon the tin lining of the container in which they are preserved. Their results as shown in the following tables indicate that some agent other than acid is present and exerts a pronounced solvent action on the tin. In the case of shrimps they believe this substance to be monomethylamine and they suggest that this or a similar compound (amine or amino-acid) may be at work in other canned goods. Their conclusions are not astonishing in view of the well-known solvent action of alkalies and bases on tin.

Emery[3] has studied the effects of edible fats and oils upon metallic containers and concludes that under certain conditions, such as high acidity, presence of moisture, air and heat, metals such as iron, tin,

[1] Tin is not usually regarded as a very active poison, but much evidently depends on the condition, as to solubility or otherwise, in which the metal is exhibited, and whether it be in the stannous or the stannic condition. Hehner has found that freshly precipitated and moist stannous hydroxide, when given to a guinea-pig, acted as a powerful irritant poison, whereas freshly precipitated and moist stannic hydroxide was comparatively inert.

The action of tin on the animal organism has been systematically investigated by T. P. White (*Pharm. Jour.*, [iii], 17, 166), who concludes "that tin, though possessing decided toxic properties when introduced into the blood, is entirely devoid of danger when taken internally in any form that could arise from being in contact with fruit or vegetables."

In a case recorded by Luff (*Brit. Med. Jour.*, April 12, 1890), preserved cherries contaminated with tin appeared to act as an irritant and cardiac poison.

[2] *Circular 79, Bur. Chem. U. S. Dept. Agric.*

[3] *Annual Report U. S. Bur. Animal Ind.*, 1909, page 265.

ACIDITY AND TIN CONTENT OF CANNED GOODS ABOUT 6 MONTHS AFTER PACKING.

Substance	Acidity as acetic acid, %	Milligrams of tin per kilogram	Milligrams of tin per 100 mgrm. of acid
Salt fish	0.012	90	75.0
Salt fish	.012	112	93.3
Beets	.036	262	72.8
Corn	.012	33	27.5
Corn	.012	46	38.3
Pumpkin	.012	193	26.8
Lima beans	.017	36	21.2
String beans	.108	80	7.4
String beans	.108	98	9.1
Pumpkin	.156	117	7.5
Pumpkin	.156	93	6.0
Corn	.019	12	6.3
Peas	.126	69	5.5
Peas	.126	57	4.5
Peas	.025	13	5.2
Pears	.180	86	4.8
Pears	.180	79	4.4
Raspberries	.450	174	3.9
Tomatoes	.390	290	7.4
Tomatoes	.390	145	3.7
Tomatoes	.234	84	3.6
Cranberries	.534	180	3.3
Cherries	.966	146	1.5
Peaches	.486	90	1.9
Grapes	.510	61	1.2
Plums	.582	63	1.1

ACIDITY AND TIN CONTENT OF OLD CANNED GOODS.

Substances	Average of sample in years	Acidity as % acetic acid	Milligrams tin per kilogram.	Milligrams tin per 100 mgrm. acid
Yellow beets	Over 3	0.05	725	145
String beans	Over 10	.04	551	138
Corn	10	.11	563	51
Succotash	Over 3	.10	444	44
Mock turtle soup	Over 5	.10	306	30
Asparagus	2 to 3	.13	333	26
Tomatoes	16	.48	944	20
Peaches	Over 3	.43	786	19
Apples	Over 5	.22	364	17
Red kidney beans	Over 10	.23	313	14
Blackberry jam	8	.31	383	12
Roast beef	Over 10	.33	426	11
Beans (baked)	18	.34	388	11
Apricots	Over 3	.49	487	10
Lima beans	8	.19	170	9
Greengages	Over 3	.69	519	8
Apple butter	Over 5	.05	690	7

lead and copper are attacked to a considerable extent. The conditions within a can are nearly the reverse of those described and hence any great amount of corrosion would not be looked for.

Estimation of Metals in Canned Foods.

The following methods are suitable for the estimation of tin and certain other metals in organic products.

Allen devised the following process. With obvious modifications, the method can readily be applied quantitatively. The substance to be examined for heavy metals is placed in a porcelain capsule, and concentrated pure sulphuric acid dropped on it and incorporated with the aid of a glass rod. The acid should be in sufficient quantity to moisten the substance, but an excess should be avoided. The dish is then heated on a water-bath for a short time, after which the temperature is gradually raised to complete the decomposition of the chlorides. About 1 c.c. of strong nitric acid should now be added, and the heating continued till red fumes are evolved. Ignited magnesia in the proportion of 0.5 grm. for each c.c. of sulphuric and nitric acid previously used is now gradually added and incorporated with the material. The dish containing the mixture is then ignited at a *dull red* heat, preferably in a gas-muffle. After cooling, the ash is moistened with nitric acid and then gently reignited, this treatment being repeated till the carbon is wholly consumed. The residue is then treated with 8 to 10 drops of strong sulphuric acid, heated till fumes are evolved, cooled, boiled with water, diluted, *without filtration*, to about 100 c.c., and hydrogen sulphide passed through the liquid to saturation. The solution is then filtered, and examined according to the following scheme of analysis:

<table>
<tr><td colspan="3">Aqueous Solution may contain zinc, iron, earthy phosphates, etc. Add bromine-water to destroy sulphuretted hydrogen and ensure the existence of any iron in the ferric state, boil, then add excess of ammonia, boil again, and filter.</td><td colspan="3">Precipitate and Residue may contain PbS, SnO_2, SnS_2, CuS, $CaSO_4$, etc. Fuse in porcelain for 10 minutes with 2 grm. of mixed potassium and sodium carbonates and 1 grm. of sulphur. When cool, boil with water and filter.</td></tr>
<tr><td rowspan="2">Precipitate may contain iron, phosphates, etc.</td><td colspan="2">Filtrate, if blue, contains nickel. Divide into two portions:</td><td colspan="2">Residue. Boil with strong hydrochloric acid as long as hydrogen sulphide is evolved, add a few drops of bromine-water to complete the oxidation of the copper sulphide and filter if necessary. To the filtrate add excess of ammonia, when a blue colouration will be indicative of copper. Acidify the liquid with acetic acid and divide into two portions.</td><td>Filtrate. Acidify with acetic acid. A yellow precipitate of SnS_2 indicates Tin.</td></tr>
<tr><td>I. Heat to boiling and add potassium ferrocyanide. White precipitate or turbidity indicates Zinc.</td><td>II. If zinc be found in I., for its determination acidify the ammoniacal solution strongly with acetic acid, filter if necessary, and precipitate the zinc from the filtrate by hydrogen sulphide. Any nickel present will be co-precipitated.</td><td>I. Add potassium dichromate. A yellow precipitate of $PbCrO_4$ indicates Lead.</td><td>II. Add potassium ferrocyanide. A brownish precipitate or colouration indicates Copper.</td><td></td></tr>
</table>

Bigelow and Munson[1] have modified Allen's original method as follows. The directions are those of the A. O. A. C.[2]

(a) **Allen's Method Modified.**—Treat 100 grm. of the moist material, or 25 grm. of the dried material, with about 5 c.c. of concentrated sulphuric acid and 2 c.c. of nitric acid. After foaming has ceased, add 3 grm. of magnesium oxide and mix thoroughly. Ignite over a Bunsen burner or, preferably, in a muffle, until thoroughly charred. Grind in a mortar and again ignite to complete combustion. The addition of a few drops of nitric acid may be necessary toward the end to complete the operation. Add about 50 c.c. of hydrochloric acid (1:3) and heat to boiling or upon a steam bath for 1/2 hour. Nearly neutralise the acid with sodium hydroxide, dilute to 150 c.c. with water, precipitate with hydrogen sulphide, and filter, after heating for a few moments upon a steam bath to facilitate the separation of the precipitated sulphides. Dry the precipitate and insoluble ash residue, and then fuse in a porcelain crucible with a mixture consisting of 1 grm. each of sodium carbonate, potassium carbonate, and sulphur. Dissolve the fused mass with hot water and filter. Sulphides of lead and copper remain upon the filter. Acidify the filtrate with acetic acid to precipitate the tin sulphide. Collect the tin sulphide upon a filter. Wash thoroughly and then dissolve by the aid of heat in a concentrated solution of ferric chloride. The reduced iron salt is then titrated with potassium dichromate. 1 c.c. of $N/10$ potassium dichromate equals 0.00295 grm. of tin. The determination of the tin by igniting and weighing as stannic oxide is unreliable, owing to the precipitation of appreciable amounts of silica dissolved by the mixed carbonates from the porcelain crucible. Determine the copper and lead, which remain as insoluble sulphides after the fusion, and the zinc, which remains in the original filtrate, according to the following method:

(b) **Munson's Method.**—Treat 100 grm. of the moist sample after evaporating to dryness, or 25 grm. of the dry sample, in a 4-inch porcelain evaporating dish with sufficient concentrated sulphuric acid to thoroughly carbonise the mass. Gently heat over a Bunsen burner until all danger of foaming is past, which will require not more than 3 minutes; then transfer the dish to a muffle and keep it at a low red heat until all organic matter is destroyed. It is occasionally found necessary to add a few drops of nitric acid to completely destroy organic matter. When the material is completely ashed, allow the

[1] *J. Amer. Chem. Soc.*, 1900, **22**, 32.
[2] *Bull. 107 (Revised), Bur. Chem. U. S. Dept. Agric.*

dish to cool; add 25 c.c. of hydrochloric acid (1:8) and evaporate on a water-bath to dryness; take up with water and acidify with 2 or 3 drops of hydrochloric acid. Transfer to a beaker without filtering and treat with hydrogen sulphide. After heating upon a water-bath for a few minutes the precipitate and the insoluble residue are collected upon a filter. The precipitate and residue may contain sulphides of tin, lead, and copper, and oxide of tin; the filtrate will contain any zinc that is present.

Fuse the sulphide precipitate and insoluble ash residue with about 3 grm. of sodium hydroxide in a silver crucible for 1/2 hour to render soluble any insoluble tin compounds. Dissolve the mass with hot water and slightly acidify with hydrochloric acid. Again treat with hydrogen sulphide without filtering. By this treatment all the tin is thrown down as sulphide with the sulphides of copper and lead. Collect the precipitate upon a filter and wash thoroughly with hot water. The filtrate may be rejected. To separate the tin sulphide from those of copper and lead, wash several times upon the filter with separate portions of 10 c.c. of strong boiling ammonium sulphide. Usually 50 c.c. of ammonium sulphide will be found sufficient to completely dissolve all tin sulphide, but portions of the filtrate should be tested to prove this point. Acidify with hydrochloric acid to precipitate the tin sulphide, which, after standing for a few moments, is collected upon an ashless filter, ignited, and weighed as stannic oxide.

Treat the insoluble residue remaining from the ammonium sulphide washing with nitric acid, filter, wash, nearly neutralise the excess of mineral acid with ammonium hydroxide and add ammonium acetate, as there is usually a small amount of iron present. If an iron salt is precipitated, filter, wash, and divide the filtrate for the determinations of copper and lead. In the absence of lead, copper may be determined electrolytically, or it may be titrated with potassium cyanide. Unless added as a colouring agent, copper will seldom be present in sufficient quantity to warrant its determination.

Schryver has recently reported his investigations on tin in canned meats to the Local Government Board (*Report No. 7, Local Gov't. Board (Medical Department) of Great Britain*). In estimating tin he destroys the organic matter by heating with potassium sulphate and sulphuric acid as in the Kjeldahl method for the estimation of nitrogen. The acid liquid is diluted with water to 100 c.c. and the tin precipitated with hydrogen sulphide. After standing overnight the tin sulphide is filtered off. If the amount of tin is rather large (more than 0.03%) 50 grm. are treated as above and the tin converted into oxide and weighed

as such. For minute quantities Schryver devised a colourimetric method which is conducted as follows:

The sulphide precipitate, from 10 grm. of the sample, obtained in the above way, together with the filter paper, is transferred to a test-tube and boiled with 5 c.c. of concentrated hydrochloric acid. After digestion the solution is filtered off (on suction filter) and the filter washed with 2.5 c.c. of acid. A stream of carbon dioxide is introduced above the filtrate to replace the air and a standard strip of zinc foil (2×0.5 in.) added to the hot liquid. Two c.c. of the reagent (0.2 grm. of dinitro-diphenylamine sulphoxide $NH\langle^{C_6H_3O_2N}_{C_6H_3O_2N}\rangle SO$, in 100 c.c. of $N/10$ sodium hydroxide solution) are then added and the solution boiled for 1 or 2 minutes, diluted to 100 c.c. with water and again filtered by suction. In the presence of tin a violet colour is produced. A drop of ferric chloride solution is added to produce the full depth of colour. For comparison a standard solution of tin chloride is used. The depth of colour produced (Lauth's violet) is not exactly proportional to the amount of tin present.

Schryver obtained the following results using his methods.

Foodstuffs	Origin	Grains of tin per lb.
Bacon, sliced	U. S. A.	0.61
Beef essence	England (3 makers)	1.58 to 1.92
Beef extract	S. America (2 makers)	0.40 to 5.33
Curried rabbit	Australia	0.19
Fruits	London importer	0.33 to 1.03
	England	1.42 to 2.81
Jams	U. S. A. (tin pierced)	5.13
Lobsters	U. S. A.	2.39
Plum-pudding	England	trace
Pork-pie	England	2.92
Roast fowl	England	0.58 to 1.44
Salmon	British Columbia	0.4 to 0.6
Tomato soup	U. S. A.	3.5
Vegetables	Australia	1.51 to 2.19

Schreiber and Taber[1] make the following statements in regard to methods previously proposed for the estimation of tin.

"Ashing the material at a very low heat in a muffle will give correct results with some foods under some circumstances, but not always, and when the composition of the material is not known, it is not safe to apply this method. Moreover, tin and its salts are volatilised by heat in the presence of ammonium chloride and probably with other chlorides also, since it is well known that sodium chloride volatilises on heating strongly. When small amounts of a metal are to be

[1] *Circular 67, Bur. Chem. U. S. Dept. Agric.*, 1911. See also *Report No. 7, Local Gov't. Board (Medical Department) of Great Britain, On the presence of tin in certain canned goods.*

recovered from a large amount of organic matter, the danger of mechanical loss in the fumes is very great. After ashing, the ash must be fused with alkali hydroxide if all the tin is to be recovered, so that this process does not offer any advantage over the method herein proposed in regard to the time necessary for making the estimation."

"Munson has proposed carbonising the organic matter with sulphuric acid and heat, and ashing in a muffle with the aid of nitric acid. Estimations made by this method did not give satisfactory duplicates, and the results represented only about 50% of the tin present, as estimated by sulphuric acid digestion in Halenke's wet ashing method, as modified by Schryver. In the latter method the organic matter is destroyed by digestion with large amounts of sulphuric acid with the aid of potassium sulphate."

"Schryver has shown that the wet combustion method gives accurate results and good duplicates. However, this method has some very serious practical defects. When tin is to be estimated in meat, fish, or sirups, only 25 grm. of material can be digested in one flask. This necessitates making two digestions in order that an amount can be used which will avoid the greater multiplication of the analytical error which would occur if the result were calculated as mgrm. per kgrm. Using such small quantities of the sample increases the error of weighing and sampling, and, moreover, limits the amount of work which can be done under ordinary conditions. The flasks have a tendency to break during the digestion, which, together with the foaming of the material, requires constant attention and considerable experience before satisfactory results can be obtained."

"Our work indicated that the following was the most satisfactory method for making a sulphuric acid digestion: Weigh 25 grm. of fish or meat or 50 grm. of vegetables into a beaker, wash into an 800 or 1,000 c.c. Kjeldahl flask, add from 100 to 150 c.c. of water, 25 grm. of potassium sulphate, 50 c.c. of sulphuric acid (sp. gr. 1.84), and a few glass beads. Place over a small flame, rotate a few times till it boils, then increase heat and boil vigourously until the water is boiled off and fumes of sulphur trioxide appear in the neck of the flask and settle back, remove it from the flame before it foams or caking will ensue and the flask break in subsequent boiling. Add 50 c.c. of sulphuric acid (sp. gr. 1.84), turn the flame down so that it just touches the flask and heat gently until the mass boils quietly. Again increase the heat and boil vigourously until decolourised. If the flask is placed in an asbestos ring, cracking can often be avoided. This digestion requires at least 6 hours."

As a result of their work, Schreiber and Taber propose the following alkali fusion method for the estimation of tin in canned goods.[1] The most serious objection to the method is its length. When properly carried out it yields accurate results.

"Pass the sample through a meat-grinding machine and mix the resulting mass well so as to get as homogeneous a sample as possible. Weigh 100 grm. of the sample and 10 grm. of magnesium oxide into an 8-oz. wrought-iron crucible on a rough balance. Add 50 c.c. of an aqueous solution containing 150 grm. of sodium hydroxide and 100 grm. of sodium carbonate per litre, stir well with a short piece of stout glass rod, add 75 c.c. of 95% alcohol and stir again. Place on the steam bath and evaporate the alcohol. This must be done with care, stirring frequently at the beginning, else it may bump or foam over, but if the material be gradually heated there is no danger of loss. If the material foams, remove from the steam bath or hot plate for a moment, and then replace on the bath and it will usually boil quietly. Large lumps in the mass will cause bumping; therefore the sample should be as fine as possible. Fish liquefy on the steam bath and boil down quietly, without bumping. After danger from frothing has ceased, apply the full heat of the steam bath. This evaporation requires about 1 hour. Transfer to a hot plate covered with a thin sheet of asbestos and dry down gradually, running at from 130 to 160° at first (determine temperature by laying a thermometer on the asbestos); then raise the temperature and continue the boiling, finally using the full heat of the hot plate, which should be sufficient to boil off sulphuric acid. This requires from 1 1/2 to 2 hours. Place in a cold muffle, heat gradually until all volatile matter is driven off, and then burn until all of the carbon is destroyed. This requires from 2 to 3 hours. Remove from the muffle as soon as burned, cool, cover the residue with water, and let stand a few minutes. Using an iron spatula, scrape and wash the contents of the crucible into a 600 c.c. beaker. Cover the beaker and add gradually 40 c.c. of dilute sulphuric acid (1 : 1) and 10 c.c. to the crucible containing some water. Rotate, scrape the sides of the crucible with a spatula and wash into the beaker.

"When the reaction is ended, add 50 c.c. of sulphuric acid (sp. gr. 1.84) and 30 c.c. of nitric acid (sp. gr. 1.42). Cover with a watch glass and boil briskly on a hot plate, finally with the full heat of the hot plate, until the residue gives off fumes of SO_3. Allow to fume for 10 minutes, remove from the hot plate and allow to cool but not to solidify. Pass the stem of a funnel bent at an angle over the lip of the beaker and

[1] *Circular 67, Bur. Chem. U. S. Dept. Agric.*

under the cover glass and add successively small amounts of distilled water through the funnel from a wash bottle until violent action has ceased. Then add rapidly about 150 to 200 c.c. more water through the funnel and remove the funnel and watch glass, washing into the beaker with distilled water. Stir the cake in the beaker and wash into a 1-litre Erlenmeyer with distilled water.

"The total volume in the Erlenmeyer at this time should be about 300 to 400 c.c. Cool, pass in hydrogen sulphide for a few seconds, rotate the flask and add 28% ammonium hydroxide slowly until the black colour of the precipitated iron sulphide just persists on rotating the flask. Immediately make acid with 1 : 1 sulphuric acid and add 10 c.c. excess of the dilute acid. Dilute the contents of the flask to 1,000 c.c. with boiling water and continue passing in a rapid stream of hydrogen sulphide for 25 minutes more, cork, and let stand overnight. The next morning heat on the steam bath for about 1/2 hour, rotating the flask two or three times during the heating, partly cool by setting in cold water, so that the flask can be handled easily and filter onto a 12.5 cm. ashless white ribbon paper, No. 589, washing the filter with a solution consisting of 50 c.c. of glacial acetic acid and 100 c.c. of a saturated solution of ammonium acetate, made up to a litre with distilled water. Wash the precipitate six times with this solution filling the filter at each washing. Return the filter paper and precipitate to the Erlenmeyer, add 100 c.c. of 20% potassium hydroxide and boil over a free flame for a couple of minutes until the filter paper is broken up and the solution is clear. (The flask can be manipulated over the flame easily with a large wooden test-tube holder made of strips of pine wood and rubber bands.) Immediately decant through a double white ribbon filter paper of 12.5 cm. into a 400 c.c. beaker, washing the flask and filter with successive portions of hot water until the filtrate comes through colourless. The filtrate will have a volume of about 200 to 300 c.c. Add 20 c.c. of concentrated hydrochloric acid to this solution, stir, add a few drops of phenolphthalein and add concentrated hydrochloric acid from a burette until the dark colour of the solution disappears, then add 1 c.c. excess of the acid. Test with a strip of litmus and see that the solution is acid, stir well, place on a steam bath, heat for 20 minutes, cover and let stand overnight.

"In the morning test the supernatant liquor, which should be perfectly clear and brilliant, with a piece of blue litmus. If not acid, make so with concentrated hydrochloric acid and then add an excess of 1 c.c. If the supernatant liquor is acid and turbid, make alkaline with potassium hydroxide and then acid with 1 c.c. excess. The solu-

tion must neither be alkaline nor too acid, or there will be trouble in filtering and washing. Heat on a steam bath for 1/2 hour, stirring two or three times. Filter onto a 12.5 cm. white ribbon paper. The precipitate will sometimes run through and must be returned until the filtrate is perfectly clear and brilliant. If the solution to be filtered is stirred vigourously, allowed to stand until the precipitate clots, and then poured onto the filter, refiltering may usually be avoided. The filtrate must be perfectly clear or some tin will be lost. Wash alternately with distilled water and the ammonium acetate solution previously mentioned until the filtrate obtained from a washing with distilled water is free from chloride. (Do not mistake the precipitate given by the acetate solution and silver nitrate, which is soluble in water, for silver chloride.) This requires washing until the volume of the filtrate is 200 c.c. or more. Fill the filter at each washing. Place the moist filter in a porcelain crucible, dry and char on an asbestos gauze, and burn off all carbon over the free flame. Cool in desiccator and weigh as stannic oxide."

Preservation of Meat by Low Temperatures.

Like the other methods of preservation, this one has been known and understood in its simpler aspects since the earliest times. In cold climates it is necessarily a thing which is forced upon the attention of the inhabitants, for example by the freezing and consequent preservation of game. Taking advantage of climatic conditions and of natural ice and ice mixed with salt, meat has intentionally been preserved by low temperatures for hundreds or thousands of years.

The early knowledge of the subject of meats preserved by low temperature was of a general nature, fragmentary, and indefinite. During the last 10 years great additions to our knowledge of the subject have been made by several investigators.

For the purpose of considering the effects of low temperatures on meat, this may be considered as a material consisting of elongated cells or sacs containing in water solution proteins, extractives and salts. Ice begins to form in such a solution when the temperature is lowered to $-0.4°$.

The subject of preservation by low temperatures can best be considered with reference to two temperatures:

(1) The freezing-point of the meat juice (-0.4 (31.3° F.) for beef juice).

(2) The temperature at which meat is frozen solid—about $-10°$ (16° F.).

In practice, chilled meat—beef, mutton, pork—is stored at temperatures ranging from 0–3° (32–38° F.). Frozen meat is usually stored at about −9° although occasionally use is made of a higher temperature and for certain foods, especially fish, a lower temperature is desirable.

When meat is stored, as it usually is, above its freezing-point at temperatures between 0 and 3° all of the changes which usually occur at higher temperatures take place, but at a reduced rate. The chemical action of atmospheric oxygen on the fat (rancidity) goes on but at a slow rate, especially as meat is usually stored in the dark. Enzyme action in the fat which leads to the production of free fatty acids is also extremely slow. The proteolytic enzyme of the muscle fibre is definitely active and in 15–20 days produces a considerable effect on the meat judged organoleptically. The action of bacteria is retarded but not stopped at these temperatures. Their colonies make definite progress into the meat in two ways: by uniform extension inward from the surface and by extension and locomotion along veins and arteries, nerve sheaths, etc. Penetration by the first method is uniform and slow; by the second method, irregular and more rapid.[1] Large pieces of beef-sides and quarters can easily be kept in good condition for 30 days by this method of preservation, and are sometimes held by epicures for 60 days or even longer at the temperatures above mentioned (32–38° F.). (See also page 309.)

Meat solidly frozen represents a very different condition from that just described, inasmuch as in a solid medium the growth and reproduction of bacteria are prevented absolutely, the action of proteolytic enzymes is so slight as to be negligible and, at the temperature required, the oxidising action of the air on the fat goes on at a very slow rate. An examination of the effects of temperatures below the freezing-point upon meat discloses the following phenomena. With the reduction of the temperature ice begins to form at −0.4° (31.3° F.). In this reduction of the freezing-point the soluble proteins, since they are colloids, play little part. Strange to say the ice forms outside the cells (or muscle fibres) instead of inside.[2] As the temperature is lowered more ice separates and a more concentrated solution remains within the fibres. The effect of this is to force the fibres to assume a smaller and smaller bulk so that finally the fibres instead of lying adjacent to one another, as at higher temperatures, are shrunken to thin strands, and these are separated by relatively large ice masses. A cross section

[1] Richardson and Scherubel, *J. Ind. Eng. Chem.*, 1909, 1, 95.
[2] Richardson and Scherubel, *J. Am. Chem. Soc.*, 1908, 30, 1545.

under the microscope shows the grouped and individual muscle fibres irregular in outline, and separated by relatively large ice areas. If the temperature is reduced sufficiently, the whole mass will be solid, but before this point is reached, the solution within the cells becomes so viscous that it is for practical purposes solid.

It should be emphasised that it is the solid condition in this (and other similar cases) and not any definite temperature which limits and determines bacterial growth and activity, for certain bacteria, in suitable liquid media, thrive down to a temperature at least as low as the cryohydric point of water-salt, namely, -22°.

It has been suggested by the author that the term "cryabiotic point" be applied to the temperature at which any medium assumes a sufficiently solid or rigid condition so as to preclude bacterial growth and reproduction.

A convenient method for demonstrating the appearance of muscular tissue in the frozen condition, consists in immersing sawed cubes 1 cm. on an edge, in the frozen condition, in cold 95% alcohol. The alcohol extracts the ice and hardens the tissue in its frozen form and structure. The pieces can then be handled according to ordinary histological technique.

On the large scale chilled meats are held in "chill rooms" or "coolers" whose temperature is lowered by means of circulating salt brine cooled by means of boiling ammonia in a mechanical refrigerating plant. Frozen meats are held in "freezers" which are usually cooled by a direct-expansion ammonia system, but circulating calcium chloride brine may also be used. Formerly large quantities of chilled beef were shipped from the United States of America to Europe, especially England, but now (1912) this trade is almost nil, the current of trade being filled with frozen meat from Argentine.

Chilled and frozen meats are transported in refrigerator cars or in steamships provided with mechanical refrigerating plant.

The principal meats which are held in frozen condition are beef and the parts of beef, mutton, poultry and fish.

During the past few years there has been much discussion on the question of the wholesomeness and nutritive value of frozen meats. After investigation it now appears that under proper conditions it is one of the best methods for the preservation of meats and poultry.[1]

Pennington believes poultry can be kept in good condition frozen

[1] Pennington, Hearings before U. S. Committee on Manufactures relative to Foods Held in Cold Storage, 1911. Richardson, *loc. cit.* Richardson and Scherubel, *J. Am. Chem. Soc.*, 1908, 30, 1515. Richardson, Reports of *First Congress of Refrigeration*, Paris, 1909. Pennington, *Circular No. 70 U. S. Dept. Agric. Bur Chem.*, 1911.

for 1 year. Experiments of the author indicate that both beef and poultry under suitable conditions can be kept solidly frozen with their wholesomeness and nutritive value unimpaired for more than 3 years and probably much longer. Decomposition of both fat and lean, if it occurs, takes place either before freezing or after thawing.

Methods of Analysis for Frozen Meats.—The general methods of analysis do not differ from those applicable to fresh meats. The histologic picture is altered so much by the freezing process and by the rate of thawing, that histologic evidence in regard to chemical change in frozen meats is of no value. Staining reactions especially of nuclei and striæ are likely to be abnormal. The histologic method in the hands of an experienced observer may afford evidence of the frozen condition in some cases but not in all. Slow thawing tends to eliminate distortion and restore the tissues to the normal condition.

Maljean[1] bases a method for the detection of frozen meat upon an observation of G. Pouchet's, that when fresh blood is frozen at 10 to 15° the corpuscles are ruptured and after thawing do not again appear in normal form, if indeed they can be observed at all. The method consists in expressing a drop of juice or blood (preferably from a blood-vessel) onto a glass slide and examining it through the microscope. The normal juice or blood shows corpuscles of normal form floating in a colourless serum, but that from frozen meat show distorted corpuscles (or none at all), while the serum is dark. The observation is correct, but the method is difficult to apply, because it is often difficult or impossible to obtain juice or blood from chilled beef which shows corpuscles, especially where access to large pieces is not possible and thus in any given case chilled meat might be mistaken for frozen. The method must, therefore, be used with circumspection.

Chemical Composition of Frozen Beef.—In the following tables the results were obtained by using the general methods of analysis for fresh meats given on pages 295 *et seq.* The figures may be compared with the similar ones for fresh beef given on pages 306 and 307. All the estimations were made on beef "knuckles" (the crural triceps of anatomists). The last table is a recapitulation of the results obtained on frozen beef in comparison with fresh beef. The figures are essentially the same and show no progressive change of any kind.

Preservation of Meat by Antiseptics (including the ordinary curing materials).—Condimental antiseptics, especially salt, have long been in use for the preservation of meats. The non-condimental antiseptics, such as boric acid appear to have come into use in modern

[1] *J. Pharm. Chim.*, 1892, 25, 348.

PART I.—ANALYSES OF FROZEN BEEF KNUCKLES. AGE 33 TO 554 DAYS. TEMPERATURE OF FREEZER, 9–12° F. FIGURES ON BASIS OF ORIGINAL MEAT.

How stored	Lab. No.	Killed	Analysed	Age, days	Moisture %	Ash %	Fat pet. eth. ext. %	Total N. %	Amm. N. Method 1. %	Amm. N. Method 2. %	Cold water extract							
											Total solids %	Ash. %	Organic extractives %	Total N. %	Coag. N. %	Albumose N. %	Meat base N. %	Acid as lactic %
Open freezer	.....	10/12 1906	1/22 1907	102	77.02	1.20	1.80	3.64	0.030	.....	6.07	1.14	4.93	0.803	0.419	0.022	0.350	0.72
Open freezer	.....	10/12 1906	1/13 1908	458	75.74	1 23	2.12	3.42	0.024	0.012	5.59	0.96	4.53	0.733	0.361	0.023	0.337	0.92
Open freezer	.....	10/12 1906	1/13 1908	458	76.72	1.23	2.02	3.32	0.025	0.012	5.80	1.14	4.66	0.730	0.353	0.026	0.343	0.92
Open freezer	2925	10/12 1906	3/27 1908	529	76.59	1.24	1.73	3.50	.....	0.012	5.94	1.15	4.79	0.836	0.449	0.022	0.359	0.77
Open freezer	2926	10/12 1906	3/27 1908	529	75.32	1.29	2.13	3.61	0.029	0.013	6.27	1.09	5.18	0.861	0.468	0.021	0.360	0.74
Open freezer	3605	10/12 1906	4/15 1908	548	75.49	1.27	1.87	3.53	0.025	0.012	6.21	1.19	5.02	0.770	0.381	0.021	0.371	0.73
Open freezer	3606	10/12 1906	4/15 1908	548	76.83	1.25	1.05	3.47	0.024	0.012	5.98	1.18	4.80	0.828	0.458	0.025	0.379	0.66
Open freezer	3801	10/12 1906	4/21 1908	554	75.40	1.23	2.03	3.65	0.031	0.012	6.05	1.22	4.83	0.812	0.418	0.026	0.369	0.79
In hermetically sealed tinned pails	2546	2/11 1908	3/16 1908	33	76.76	1.20	1.17	3.31	0.026	0.009	5.56	1.16	4.40	0.766	0.373	0.023	0.359	0.72
In hermetically sealed tinned pails	3551	2/11 1908	4/14 1908	62	77.11	1.16	1.65	3.38	0.031	0.011	5.88	1.14	4.74	0.819	0.432	0.025	0.365	0.80
In closed glass jars	3735	8/20 1907	4/20 1908	212	76.66	1.22	1.31	3.65	0.030	0.010	5.98	1.09	4.89	0.795	0.420	0.021	0.350	0.88
In closed glass jars	3736	8/20 1907	4/20 1908	212	77.03	1.28	1.30	3.65	0.031	0.010	6.06	1.21	4.85	0.798	0.426	0.023	0.349	0.88
Maximum	.....	.....	.....	554	77.11	1.29	2.12	3.65	0.031	0.013	6.27	1.21	5.18	0.861	0.468	0.026	0.379	0.92
Minimum	.....	.....	.....	33	75.32	1.16	1.17	3.31	0.024	0.009	5.56	0.96	4.40	0.730	0.353	0.021	0.337	0.66
Average	.....	.....	.....	353	76.39	1.23	1.65	3.51	0.028	0.011	5.94	1.14	4.80	0.795	0.413	0.023	0.357	0.79

PART II.—ANALYSES OF FROZEN BEEF KNUCKLES. AGE, 33 TO 554 DAYS. TEMPERATURE OF FREEZER, 9–12° F. FIGURES CALCULATED TO MOISTURE-, ASH-, AND FAT-FREE BASIS.

How stored.	Lab. No.	Killed	Analysed	Age, days	Total N. %	Amm. N. Method 1 %	Amm. N. Method 2 %	Organic extractives %	Cold water extract				
									Total N. %	Coag. N. %	Albumose N. %	Meat base N. %	Acid as lactic %
Open freezer	.....	10/12 1906	1/22 1907	102	18.22	0.150	.....	24.67	4.02	2.10	0.110	1.75	3.60
Open freezer	.....	10/12 1906	1/13 1908	458	16.36	0.115	0.057	21.67	3.51	1.73	0.110	1.61	4.40
Open freezer	.....	10/12 1906	1/13 1908	458	16.58	0.125	0.060	23.27	3.64	1.76	0.130	1.71	4.59
Open freezer	2925	10/12 1906	3/27 1908	529	17.12	.....	0.059	23.43	4.09	2.19	0.108	1.76	3.77
Open freezer	2926	10/12 1906	3/27 1908	529	16.98	0.136	0.061	24.36	4.05	2.20	0.099	1.69	3.48
Open freezer	3605	10/12 1906	4/15 1908	548	16.52	0.117	0.056	23.49	3.60	1.78	0.098	1.74	3.41
Open freezer	3606	10/12 1906	4/15 1908	548	16.63	0.115	0.057	23.00	3.97	2.19	0.119	1.82	3.12
Open freezer	3801	10/12 1906	4/21 1908	554	17.10	0.145	0.056	22.63	3.80	1.96	0.121	1.73	3.70
In hermetically sealed tinned pails	2546	2/11 1908	3/16 1908	33	15.86	0.124	0.043	21.08	3.67	1.79	0.110	1.72	3.45
In hermetically sealed tinned pails	3551	2/11 1908	4/14 1908	62	16.83	0.154	0.055	23.60	4.08	2.15	0.124	1.82	3.98
In closed glass jars	3735	8/20 1907	4/20 1908	212	17.54	0.144	0.041	23.50	3.82	2.02	0.101	1.68	4.22
In closed glass jars	3736	8/20 1907	4/20 1908	212	17.90	0.152	0.049	23.78	3.91	2.09	0.112	1.71	4.31
Maximum	.....	.....	.....	554	18.22	0.154	0.061	24.67	4.09	2.20	0.130	1.82	4.59
Minimum	.....	.....	.....	33	15.86	0.115	0.041	21.08	3.51	1.73	0.098	1.61	3.12
Average	.....	.....	.....	353	16.97	0.133	0.049	23.20	3.93	1.99	0.111	1.73	3.83

SUMMARY OF RESULTS OF ANALYSES OF FRESH BEEF KNUCKLES AND FROZEN BEEF KNUCKLES. ALL ANALYTICAL FIGURES ARE PERCENTAGES. ON BASIS OF ORIGINAL MEAT.

	No. samples	Age, days	Moisture	Ash	Fat	Total N.	Amm. N. Method I.	Amm. N. Method II	Cold water extract							
									Total solids	Ash	Organic extractives	Total N.	Coag. N.	Albumose N.	Meat base N.	Acid as lactic
Fresh—Maximum	13	7	77.27	1.31	3.34	3.65	0.033	0.011	6.24	1.27	5.27	0.854	0.452	0.034	0.398	0.82
Minimum	.....	0	75.26	1.11	0.78	3.34	0.022	0.009	5.55	0.95	4.42	0.742	0.358	0.014	0.360	0.63
Average	.....	3.7	76.35	1.23	1.43	3.49	0.029	0.010	6.01	1.14	4.87	0.806	0.413	0.024	0.355	0.68
Frozen—Maximum	13	554	77.11	1.29	2.12	3.65	0.031	0.013	6.27	1.21	5.18	0.861	0.468	0.026	0.379	0.92
Minimum	.....	33	75.32	1.16	1.17	3.31	0.024	0.009	5.56	0.96	4.40	0.730	0.353	0.021	0.337	0.66
Average	.....	353	76.39	1.23	1.65	3.51	0.028	0.011	5.94	1.14	4.80	0.795	0.413	0.023	0.357	0.79

ON MOISTURE-, ASH- AND FAT-FREE BASIS.

	No. samples	Age, days	Total N.	Amm. N. Method 1	Amm. N. Method 2	Cold water extract					
						Organic extractives	Total N.	Coag. N.	Albumose N.	Meat base N.	Acid as lactic
Fresh—Maximum	13	7	17.05	0.154	0.053	26.12	4.15	2.20	0.162	1.86	3.79
Minimum	.....	0	16.22	0.107	0.043	21.51	3.56	1.74	0.069	1.61	2.63
Average	.....	3.7	16.63	0.139	0.049	23.22	3.84	1.99	0.117	1.77	3.25
Frozen—Maximum	12	554	18.22	0.154	0.061	24.67	4.09	2.20	0.130	1.82	4.59
Minimum	.....	33	15.86	0.115	0.041	21.08	3.51	1.73	0.098	1.61	3.12
Average	.....	353	16.93	0.133	0.049	23.20	3.93	1.99	0.111	1.73	3.83

NITROGEN FIGURES AS PERCENTAGES OF THE TOTAL NITROGEN.

	No. samples	Age, days	Amm. N. Method 1.	Amm. N. Method 2.	Cold water extract			
					Total N.	Coag. N.	Albumose N.	Meat base N.
Fresh—Maximum	13	7	0.927	0.318	24.75	13.10	0.983	11.18
Minimum	.....	0	0.641	0.262	21.45	10.35	0.419	9.73
Average	.....	3.7	0.834	0.291	23.11	11.83	0.707	10.64
Frozen—Maximum	12	554	0.917	0.361	24.23	13.20	0.783	10.92
Minimum	.....	33	0.702	0.271	21.43	10.56	0.575	9.56
Average	.....	353	0.791	0.325	22.68	11.76	0.661	10.19

times, and not long after their use as preservatives were either prohibited or their use limited by legislative enactments in various countries. On the subject of their harmfulness and harmlessness when used in small quantity much has been written and published.[1]

The condimental antiseptics as defined by the Regulations governing Meat Inspection of the U. S. Department of Agriculture, include salt, saltpetre, sugar, vinegar, wood smoke and spices, and the use of these substances for the curing of meat is allowed in the United States of America as well as in other countries. Of these, the most important is salt.

The methods of curing meat, as the application of these substances to meat is called, differ depending on whether the meat is whole or comminuted. The curing materials are applied to the surface of whole pieces of meat, whereas with the exception of smoke, they are mixed with hashed meat.

Methods of Curing Meat. Dry Salt Cure.—The simplest method of curing meat is the process known as dry-curing in which salt (or a mixture in proper proportion of salt, cane sugar and saltpetre) is sprinkled on the surface of the pieces which are then laid in piles, in chill rooms, whose temperature is held at a little above the freezing-point—usually at 2–3°. The curing materials penetrate the meat by diffusion and after the lapse of a length of time which varies with the thickness of the piece, enough of them will have reached the centre so that the meat is adjudged "cured"—that is, preserved so that it can be removed to warmer storage or to the smoke house without danger of spoilage. This method of curing is applied to backs and sides of pork and to a lesser degree to pork hams and bellies. The latter are usually smoked after they are cured; the former not.

Curing in Brine.—Sweet-pickle cure.[2]

In this process the pieces of meat are immersed in a brine containing salt alone, or, as is customary in recent practice, salt, sugar and saltpetre. The brine is known in the packing-house as "pickle" and when it contains sugar is called "sweet pickle." The meats are designated as "pickled" or "sweet-pickled" meats. This method of cure is applied to pork hams, shoulders and bellies, various cuts of beef which go to make barrelled beef, and dried beef, tongues, etc. The length of time in cure for pork bellies is usually 20–30 days, for

[1] Liebreich's *Treatise on the Effects of Borax and Boric Acid on the Human System.* Wiley, *Bull.* 84, *Bur. Chem. U. S. Dept. Agric.* Reports of the Referee Board, Ira Remsen, Chairman, *Bull.*

[2] See E. Polenske. Ueber den Verlust, welchen das Rindfleisch durch das Pökeln erleidet, sowie, über die Veränderungen salpeterhaltiger Pökellaken. *Arb. kais. Gesundh.*, 1891, 7, 471–4. *Ueber das Pökeln von Fleisch in salpeterhaltigen Laken*, *ibid.*, 1893, 9, 126–35; *ibid.*, *Jahresber. Nahr. u. Genussm.* 1891, 40.

pork hams 50–80 days, depending on the thickness of the piece. When fully cured the meats are first soaked in water in order to reduce the percentage of salt in the exterior portions of the meat and then smoked.[1]

Other Methods of Curing.—Modifications of the methods above described are in use, such as sprinkling pork bellies with the dry curing materials and piling them in special boxes, whereupon a brine is formed by the action of the curing materials upon the water in the meat. Again, small pieces of meat are sometimes sprinkled with the curing materials and packed in barrels so tightly that although diffusion and curing take place no brine is formed. The former process is applied to the manufacture of very mild cured bacon, the latter to the curing of meats intended for sausage manufacture at a later date.

Special Varieties of Hams.—Westphalian hams as cured in northwestern Germany are intended to be sliced and eaten raw. After they are cured according to one of the methods just described, they are smoked over a bright hardwood fire into which juniper berries and twigs are constantly thrown, for a considerable period (8 days) in order to dry the meat. The fumes from the juniper give a special piquancy to the product. So-called "Italian" hams, after curing, are pressed in order to flatten them, thickly sprinkled with ground black pepper and are then dried in large dry rooms after the manner of summer sausage (see below) the temperature being held at 10 to 15°. They are eaten raw. Hams are sometimes aged for periods ranging up to 3 years, either in the air or buried in wood ashes to develop special flavours. Hams of this sort are sliced and eaten raw as a relish.

The Curing of Sausage.—According to the standards adopted by the Association of Official Agricultural Chemists in 1908 sausage is defined as follows:

"Sausage, sausage meat is a comminuted meat from neat cattle or swine, or a mixture of such meats, either fresh, salted, pickled or smoked, with added salt and spices and with or without the addition of edible animal fats, blood and sugar, or subsequent smoking. It contains no larger amount of water than the meats from which it is prepared contain when in their fresh condition, and if it bears a name descriptive of kind, composition, or origin, it corresponds to such descriptive name. All animal tissues used as containers, such as

[1] Ref. McBryde, *A Bacteriological Study of Ham Souring. U. S. Dept. Agri. Bur. An. Ind. Bull.*, 132.

casings, stomachs, etc. are clean and sound and impart to the contents no other substance than salt.

"Blood sausage is sausage to which has been added clean, fresh blood from neat cattle or swine in good health at the time of slaughter."

This definition takes no account of the addition of starch or flour to sausage—a very general and world-wide practice. In the United States of America, corn flour is very commonly used and such sausage is labelled, in compliance with the regulations governing meat inspection under the Act of Congress, approved June 30, 1906, "Sausage with Cereal."

For the curing of sausage the same materials are used as for the curing of other meats, but since they are mixed with the hashed or ground product, the curing proceeds very rapidly. The curing materials, the spices and any amylaceous materials are mixed with the ground or hashed mixture of meats, and the resulting mixtures are allowed to cure in trucks or pans for a period varying from a few minutes to 3 days (summer sausage). It is then sold as sausage meat or stuffed into appropriate casings (intestines, bladder, weasand, etc., from the ox, sheep or swine) and sold raw or after cooking, smoking or drying or a combination of these as the case may be.

Varieties of Sausage.—The varieties of sausage are numerous and are different in different countries.

German sausages are extensively made from blood, liver, heart, brain, etc., and from fresh, dried, smoked, and salted meat. An addition of farinaceous material, usually in the form of flour or pea-meal, is very common.

The following description of the chief varieties of German sausage is an abstract of that of J. König:

Blutwurst or *Rothwurst* is made with hogs' blood, bacon, and pork, sometimes with the addition of heart or kidney, and either with or without flour. These sausages are similar to those known in England as "black pudding." They soon undergo decomposition.

Mettwurst (Bologna or thick sausage) is made from pork and lard, with an addition (*inter alia*) of beef or horseflesh.

Cervelatwurst or brain-sausage is somewhat similar to the last. The Italian sausage *Salamiwurst* receives an addition of red wine. *Knackwurst* is a hard sausage, of a similar composition to cervelat, but the meat is previously cooked.

Leberwurst or liver-sausage is composed of liver, lung, kidney, skin, and lard or suet, with or without flour. Liver-sausages readily undergo

change, and are then liable to occasion symptoms of irritant poisoning. *Trüffelwurst* is made from meat, fat, and flour, with an admixture of truffles.

Schwartenwurst, *Sülzenwurst*, and *Magenwurst* are German sausages made from skin, stomach, etc., boiled soft, and mixed with unsalted bacon and a little blood.

Bratwurst is made from fresh raw pork and bacon, flavoured with salt, pepper, and lemon-peel or cumin.

Frankfort and *Vienna sausages* are small, filled into sheep's gut, and composed of raw, slightly smoked pork, flavoured with salt, nitre, and pepper.

Reiswurst and *Grützwurst* are sausages commonly manufactured in North Germany from oat- or buckwheat meal, blood, soft-boiled skin, bacon, spices, etc.

Erbswurst is composed of beef-suet, bacon, peas, onions, spices, etc. Sausages of this kind, when of French manufacture, often contain much coarse meal and husk. Hence the woody fibre is high (4.3%) as compared with that in German pease-sausages (0.88 to 1.08%). G. Heppe examined three samples of *Erbswurst* which contained 7.32% of flesh constituents. F. Hofmann found in one sample a mere trace of animal proteins, while another contained 16.45 of total proteins, of which from 2 to 3% was of animal origin.

The following analyses also show the variation in the composition of pease-sausages:

Authority	Date	Water	Nitrogenous matters	Fat	Starch, etc.	Salts
Ritter	1870	29.25	16.02	29.70	11.94	13.19
König	1884	11.00	19.65	15.52	41.05	11.88

The so-called blood- and liver-sausages often contain more flour than blood, liver, or flesh.

A specimen of cooked Lorraine sausage, examined by Allen, contained smoked meat, gristle, pea-meal, and onions, and gave the following figures on analysis: Water, 46.04; fat, 25.67; proteins, 15.49; gristle, 3.65; starch, 4.00; and ash (sulphated), 6.36%.

Sausages which are intended to be kept should not contain milk, flour, bread or brains and little water, 35% or better, 30% being the proper amount.

The following analyses of different varieties of German sausages are by J. König.

	Water	Nitrogenous matters	Fat	Carbo-hydrates	Ash	Sum of constituents
Cervelatwurst (brain-sausage)	37.37	17.64	39.76	.....	5.44	100.21
Mettwurst (Bologna or thick sausage)	20.76	27.31	39.77	5.10	6.95	99.89
Frankfurter Würstchen (Frankfort small sausages)	42.79	11.69	39.61	2.25	3.66	100.00
Blutwurst (black pudding), best quality	49.93	11.81	11.48	25.09	1.69	100.00
Blutwurst (black pudding), ordinary quality	63.61	9.95	8.87	15.83	1.76	100.00
Leberwurst (liver-sausage), best quality	48.70	15.93	26.33	6.38	2.66	100.00
Leberwurst (liver-sausage), third quality	47.58	10.87	14.43	20.71	2.87	96.46
Leberwurst, without flour	35.89	16.13	45.51	.....	3.72	101.25
Sülzenwurst	41.50	23.10	22.80	.....	12.60	100.00
Knackwurst	58.60	22.80	11.40	.....	7.20	100.00
Erbswurst (German pease-sausage)	6.53	15.46	37.94	31.38	8.69	100.00
Trüffelwurst, best quality	43.29	13.06	41.27	.....	2.41	100.03
Schinkenwurst (ham-sausage)	46.87	12.87	24.43	12.52	3.31	100.00

English sausages are generally very different from those of German manufacture. As sold (with the exception of "polonies"), they are made of uncooked and unsmoked meat, and are intended to be cooked and eaten while quite fresh. The addition of dry bread or biscuit is very common, but by no means invariable.

The following analyses of sausages obtained from respectable dealers in Sheffield were made in A. H. Allen's laboratory:

Description of sausage	Price per lb.	Water	Fat	Proteins	Gristle, etc.	Starch	Ash
Pork	9d.	54.99	21.04	12.28	0.67	1.05	3.52
"Cambridge" pork	9d.	51.54	29.72	9.45	0.72	2.20	3.47
Mutton	1s.	55.58	30.51	1.89	3.11	3.90	2.50
German	8d.	46.54	17.87	16.38	1.13	15.00	4.47
Polony	10d.	45.57	32.66	17.26	0.54	2.30	2.80

In these analyses, a weight of 10 grm. was dried at 105° for the estimation of the *water*. The dried substance was then extracted with ether in a Soxhlet tube, the solution evaporated, and the residual *fat* weighed. The residue insoluble in ether was moistened with sulphuric and nitric acids, ignited, again moistened with sulphuric acid, reignited, and the *sulphated ash* weighed.

For the estimation of the *gelatinoids*, a weight of 20 grm. of the sausage was disintegrated by stirring it in a basin with cold water, the excess of water drained off, and the fragments of gristle picked out with a pair of forceps with the aid of a lens. They were then washed in succession with methyl alcohol and with ether, dried at 100°, and weighed. The

nitrogen contained in the *gristle*, etc., thus found, was then determined by Kjeldahl's process, and the amount deducted from the total nitrogen found by the same process in the original sausage. The difference was regarded as *protein* nitrogen, and multiplied by 6.3 to find the proportion of these compounds present.

The *starch* was determined by Mahrhofer's process (see below). No allowance was made for that derived from the pepper, or other spices. No wheat-starch could be observed by the microscope in the case of the first two samples. The dry bread used in the manufacture of sausages may be taken as containing 60% of starch.

A. W. Stokes, in a communication to A. H. Allen,[1] stated that in 1894 he found that sausages which were being extensively sold on street-stalls in the east of London contained 10% of flesh, 20 of fat, and 70% of bread. On being placed in water they disintegrated, the meat sinking rapidly, so that a fairly good separation of the constituents could be effected by elutriation. No proceedings were taken against the vendors, owing to the absence of any legal or authoritative definition of a sausage.

French Sausages.—French sausages differ from those of English and American manufacture chiefly because they contain large amounts of horse flesh. To some extent, horse flesh is a recognised ingredient of sausage throughout the continent of Europe. According to Mitchell[2] the following are some varieties of French Sausage.

"*Saucisses* consist of a skin of pig's intestine filled with raw or smoked minced flesh (usually pork), and seasoned with salt, pepper, pimento, etc. They are termed saucisses longues or saucisses plates, according to their form.

"*Saucissons* only differ from saucisses in being larger, more compact, and generally more highly seasoned. There are many varieties, such as *Saucissons de Lyons, Saucissons cru,* etc.

"*Cervelas* are large, short sausages containing salted and spiced flesh. They appear to be analogous to the English *saveloys.* According to L. Baillet, French sausages are not intended to be kept for more than a few days. While still firm to the touch and sound, they gradually acquire on keeping a sharp but not unpleasant flavour, and are then termed *piqué* by the manufacturers. At a more advanced stage of alteration the exterior assumes an earthy tint, and is sometimes perceptibly moist to the hand, these changes being accompanied by the production of an acid and disagreeable odour. This condition is

[1] See second edition, Vol. 4, page 281.
[2] Flesh foods, page 128.

termed *échauffé*. Baillet states that in the east of France, the addition of starch (up to 15%) is a common practice."

American Sausages.—In America, especially in the larger establishments, many different kinds of sausage are manufactured corresponding to German, Italian, Dutch and other formulæ. The following classification includes the principal kinds:

I. *Domestic Sausage. For prompt consumption.*

a. Fresh pork sausage.

b. Cooked sausage: Liver and blood sausage.
Head cheese and souse.

c. Smoked-cooked sausage.

1. Bologna sausage.

2. Frankfurts sausage.

II. *Summer sausage.*—Well dried (moisture 30–40%). Perishability reduced to a minimum.

a. *Smoked.*—Cervelats and German Salami, Farmer, Holsteiner, Goteburg, Roma, Mecklenburger, Mortadella, Capicolla.

b. *Unsmoked.*—Italian Salami, Milanese, D'Arles, Genoa, Lyon, Pepperoni.

III. Various cooked and smoked meats packed in sausage form, ready to be sliced and eaten. Various spicings and textures.

The Water in Sausage.—The less water there is in sausage the better will the product keep. To the total moisture content the lean meat contributes about 76% of its weight, fatty tissue 3 to 8%, starch and flour 10–15%. It is a very common practice to add water to sausage meat during the manufacture as an aid in stuffing the meat into delicate casings, and it is claimed that the use of excessive amounts of amylaceous substances is for the purpose of enabling the manufacturer to incorporate excessive amounts of water in his product. The moderate use of starch, flour, etc., (called "binders") is for the purpose of causing the various ingredients to cohere, thus forming a mass of homogeneous texture. By various means known to sausage-makers lean meat can be made to absorb more than its normal content of water. It is, of course, obvious that water is not added to those sausages—such as the various summer sausages—which are intended for long keeping and which are dried.

Use of the Non-condimental Antiseptics as Preservatives.—The use of the non-condimental antiseptics such as borax, boric acid, sulphurous acid and the sulphates, benzoic acid and the benzoates reached a maximum during the years 1890–1905 and has since declined, chiefly because of the legal restrictions and agitation against their

use. At present there are very decided and antagonistic views held by the highest authorities on both sides of the question of the wholesomeness of foods containing small quantities of them.[1] Borax and boric acid are used on the surface of cured meats for export shipment.

The Function of Salt in Cured Meats.—Salt, sodium chloride, is the principal preserving material used in the curing of meats as commonly practised, but when used alone it does not produce the most satisfactory or best preserved meat. For the proper curing of meat not only salt, but sugar and saltpetre are required, and also the use of low temperatures and finally smoking and drying. Salt appears to have little directly harmful effect on bacteria. In small quantity it is added to nearly all culture media. At higher concentrations it inhibits, but does not prevent, the growth of bacteria, by increasing the osmotic pressure beyond that most favourable for bacterial growth. Some kinds of bacteria will not develop in salt solutions of greater concentrations than 10%, others thrive in a saturated solution, even at low temperatures, and the curing pickle from meat in process of cure always abounds with a great variety of bacteria. However, the chemical activities of these organisms must be greatly reduced by the combination of low temperature and salt, as otherwise the meat would spoil or "sour" as the expression is, whereas the percentage of meat which spoils or sours in process of cure is very low. The effect of salt in lessening the vitality of bacteria can be shown by growing gelatin-liquefying bacteria in nutrient gelatin media containing salt; if the percentage of salt is high enough, growth is retarded and the liquefying power lost altogether. In estimating the effectiveness of salt as an antiseptic in cured meats, it should be calculated as a percentage of the moisture present. Thus in a ham whose lean showed by analysis, moisture 65% and salt 5.5%, the concentration of the salt in the water would be 8.46%. The total osmotic effect of the solution in the meat, however, would be due not only to the salt, but to all the soluble substances present.

The Function of Sugar in Cured Meats.—Sugar, sucrose, is used as a curing material primarily for the sake of the flavour which it contributes to the meat. It probably, also, exerts a slight beneficial effect in protecting the meat against proteoclastic decomposition by bacteria, in the same way that milk sugar protects casein in milk. The sucrose, by fermentation, raises the acidity of pickle and meat slightly.

[1] Liebreich's Treatises on the Effects of Borax and Boric Acid on the Human System. Wiley. *Bull.* 84, *U. S. Bur. Chem.*, Remsen (Chairman Referee Board), *Bull. H., U. S. Dep. Agric.*

The Function of Saltpetre in Cured Meats.—Saltpetre performs several functions in the curing of meat. It maintains the red colour of the meat by the conversion of hæmoglobin into a stable nitroso-compound. This is brought about directly by the action of nitric oxide, which is formed by the action of nitrate-reducing bacteria on the saltpetre. It maintains aerobic conditions within the meat, thus tending to prevent the development of the typical anaerobic bacteria which cause putrefaction.[1] While it is not an antiseptic in the sense ordinarily understood, it appears to protect the nitrogenous tissue against bacterial attack, for meat cured without saltpetre produces many more sour pieces than meat cured with saltpetre.[2] Sodium nitrate is just as effective as potassium nitrate. The maintenance of a red colour in meat through the reduction of saltpetre by bacteria involves several chemical changes. There are, in all probability, several red nitroso derivatives of hæmoglobin, chief of which are nitrosohæmoglobin and nitrosohæmochromogen, any one of which can impart the red colour to meat. When no saltpetre is used in the curing of meat the sodium chloride very quickly causes the red colour of fresh meat to disappear, and the meat to turn dull grey. When saltpetre is used in the curing this grey colour, sooner or later, depending on temperature and other conditions, gives place to the red colour of the nitroso derivatives. The nitric oxide which brings about this reaction is produced according to the following equations:

Reduction of saltpetre to nitrite by bacteria.

(1) $KNO_3 + 2H = KNO_2 + H_2O$.
(2) $KNO_2 + HR = HNO_2 + KR$
(3) $2HNO_2 = 2H_2O + NO + NO_2$

Reaction 2 is brought about by the lactic acid in meat resulting from *rigor mortis*. It is stated by some authorities that nitrates are reduced to nitrites by the direct action of meat.[3] The author working on perfectly fresh meat with careful technique finds that meat does not bring about this reduction. Most samples of saltpetre give the test for nitrites with Griess's reagent. The surface of old meat is always covered with bacteria and hashing the meat distributes them throughout the mass and the reduction experiments have been made on hashed meat. One or both of these causes have probably contributed to the reports that meat alone reduces nitrate to nitrites. If meat reduced nitrates the

[1] Richardson, *J. Am. Chem. Soc.*, 1907, 29, 1757–67.
[2] *Annual Report U. S. Bur. Animal Ind.*, 1908, p. 301.
[3] *Annual Report U. S. Bur. Animal Ind.*, 1908, 307; Abelous and Gérard, *Compt. rend.*, 1899, 129 (iii), 164–66.

nitroso derivative could be produced by injection of a suitable nitrate into meat, but this is not the case.

The experiments of Haldane,[1] of Hoagland,[2] and the experiments of the writer, point to the following explanation in connection with the action of saltpetre in the curing of meats. When salted or pickled, the oxyhæmoglobin in a piece of meat, is changed first to methæmoglobin, then in the interior to hæmochromogen. The nitrate is reduced to nitrite chiefly in the pickle and being produced in small quantity penetrates the meat more slowly than the saltpetre, so that meat is often found whose centre contains only hæmochromogen (which is converted to hæmatin on exposure to the air, and turns grey or drab) whereas the outer parts are coloured red by nitrosohæmoglobin. In the presence of a little acid, under reducing conditions (absence of air) nitrites always produce a bright red nitroso derivative when acting upon hæmoglobin, hæmochromogen or hæmatin.

Nitrates and nitrites gradually disappear in pickle and cured meats, probably according to the equations already given and the following:

$$(4)\quad HNO_2 + NH_3 = NH_4NO_2$$
$$(5)\quad NH_4NO_2 = 2H_2O + N_2$$

These reactions take place when a nitrite is in contact with ammonia or an amino compound, at boiling temperature, or at ordinary temperatures, under the influence of denitrifying bacteria. In summer sausage, after 60 days, usually no nitrates and only traces of nitrites are found. If more than a certain quantity of saltpetre (2–3 oz. per 100 pounds) is used in curing meat, especially in sausage, after a time the meat becomes pitted, porous and spongy owing to the gases formed, NO and N_2. The odour and flavour of such meat is pungent. Some of the flavour of old hams, and summer sausage, prized by epicures, is due to this cause.

The NO derivatives in cured meats often, but not invariably, yield a bright red solution when the meat is extracted with ether, or alcohol and ether. This colour might be mistaken for an artificial colouring matter in the examination of sausage for dyes, and is, therefore, important to the analyst. This colour dissolves in the fat present in sausage, colouring it red, but the colouring matters of some spices, notably pimento, have the same property. The nitroso derivative upon standing in ether solution turns dark brown. Addition of ammonia precipitates it as a red layer at the bottom. This colour

[1] *J. Hygiene*, 1901, 1, 115.
[2] *Annual Report U. S. Bur. Animal Ind.*, 1908, 301.

is found usually in old summer sausage cured with considerable saltpetre.

The Smoking and Drying of Meats.—After large pieces of meat are cured they are soaked in water for different lengths of time, depending upon the size of the piece and the temperature, and are then smoked. Sausages are of course smoked directly. The smoking is accomplished by hanging the meat on racks in a chimney below which a slow fire, fed by hardwood and sawdust, is kept burning. The usual duration of the smoking is from 1 to 10 days. The smoke deposits certain creosotic or empyreumatic substances upon the surface of the meat, thus giving it a distinctive flavour, and at the same time the surface is dried. The drying is rather more important than the smoking so far as the preservation of the meat is concerned, and is assisted in some cases by steam coils built into the smoke houses.

Composition of Cured Meats.—The composition of mild-cured and smoked pork hams handled according to customary American practice, and the analyses made according to the general methods of analysis given on page 372 is shown in the following table. In order to make a comparison between the composition of fresh and cured meats it is very necessary to calculate to the moisture-ash- and fat-free basis or for a still more accurate comparison, to the moisture-ash and curing materials- and fat-free basis. It can readily be seen that the analyst must be prepared to find considerable variations from these figures in individual examples, owing to the use of more or less curing materials, differences in the time of curing, etc.

AVERAGE COMPOSITION OF MILD-CURED, SALTED AND SMOKED PORK HAMS—COMPILATION.

Section	Salt, %			Sugar, %			Saltpetre, %		
	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.
1	7.0	4.0	5.5	1.85	0.80	1.20	0.30	0.18	0.22
2	6.0	3.5	5.0	1.05	0.55	0.70	0.25	0.16	0.20
3	5.5	3.0	4.5	0.80	0.40	0.55	0.20	0.14	0.17
4	5.0	2.0	4.0	0.70	0.30	0.45	0.15	0.11	0.13
5 (Fat)	1.2	0.5	0.7	0.10	0.05	0.08	....	.....	....
Butt	7.0	4.0	6.0	.....	.....	....	0.30	0.20	0.25
Whole of lean portion.....	.....	.....	5.0	.....	.....	1.00	.....	.....	0.20

Moisture, lean portion. Max., 65; Min., 60. (Dried ham, 30). Average 62 %.

AVERAGE COMPOSITION OF MILD-CURED BACON.

	Salt, %			Sugar, %			Saltpetre, %		
	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.
Lean	12.0	6.0	9.0	2.5	1.70	1.90	0.60	0.30	0.4
Fat	1.8	0.7	1.0	0.08	0.04	0.05	0.10	0.05	0.07
Whole piece	5.0	2.0	2.5	1.7	0.60	0.80	0.16	0.02	0.06
Moisture, whole piece	26	15	20	.....	.....	....	.....	.....	....

AVERAGE COMPOSITION OF CURED AND SMOKED DRIED BEEF HAMS.[1]

	Salt, %			Sugar, %			Saltpetre, %		
	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.
Outside	13.5	10.0	11.5	1.20	0.75	1.00	0.40	0.20	0.30
Inside	13.0	9.5	10.5	1.00	0.50	0.85	0.30	0.06	0.24
Whole	13.3	10.0	11.0	1.10	0.60	0.95	0.35	0.20	0.30

Moisture, whole. Max., 55.0; min., 48.0; av., 51.0%.

Methods of Sampling Cured Meats.—In general the methods of sampling for meats on page 293 are applicable here, but for certain purposes the samples in the case of ham, bacon and dried beef are selected from circumscribed areas and do not represent the whole piece. For factory control purposes as well as for specific information it is desirable to ascertain the distribution of curing materials and for this purpose the following methods were devised.

Sampling of Ham.—If an average of the whole piece is desired it is cut and sawed into parallel slices 1/2 in. thick, the bone, gristle, etc., separated from the lean and the latter handled as in the case of fresh meats. To ascertain the distribution of salt, a slice about 1 in. thick is cut out of the thickest part of the ham and a strip removed as shown in the illustration. This strip is divided into 5 parts numbered from 1 to 5 and the salt in each of these estimated separately. Sometimes a sixth sample is taken from near the bone as shown, and a seventh from the opposite side. If it is desired to estimate the distribution of sugar and saltpetre as well as salt, parallel slices are cut from the thick part of the ham and a wider strip taken from each slice. Each strip is then sampled separately as described above and the pieces bearing the same number combined for the estimations.

[1] Three kinds are commonly cured in large establishments, the "knuckle," "outside" and "inside," all derived from the "round."

Sampling of Bacon.—Either the whole piece is taken for the sample and hashed, or the piece is cut into 1 in. slices and the lean dissected from the fat, each being ground and analysed separately.

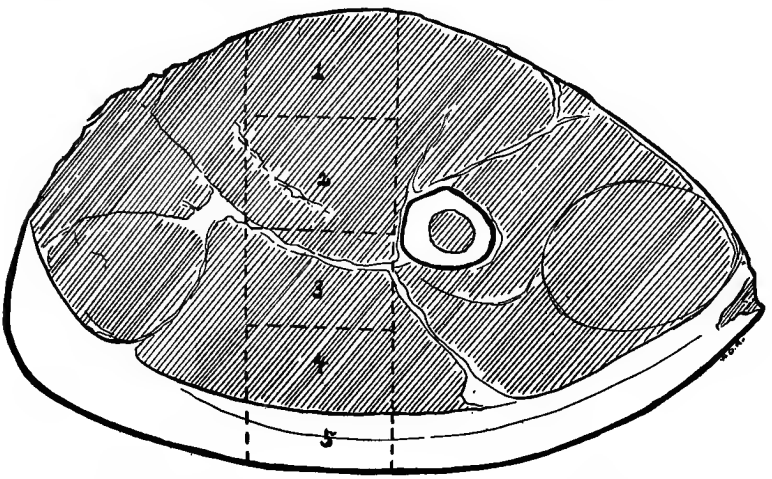


FIG. 26.—Method of sampling cured pork ham. Slice through thickest part.

Sampling of Dried Beef.—The pieces which are cured and smoked for dried beef are from the round and are designated as "outside," "inside" and "knuckle." The whole piece or a slice through the

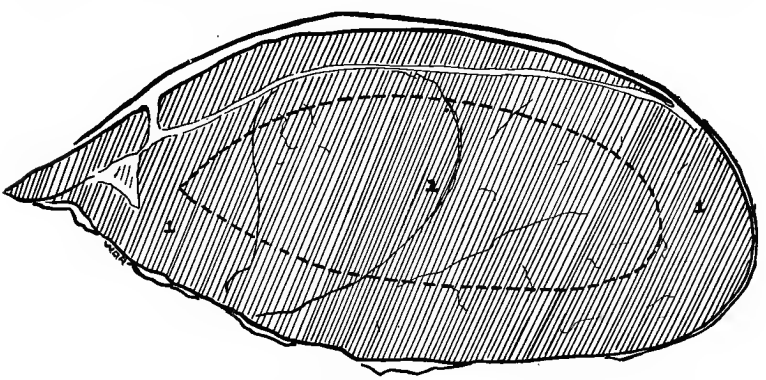


FIG. 27.—Method of sampling cured dried beef. (Cut shows an "outside" piece.) Slice through thickest part.

thickest part is usually analysed; but if it is desired to ascertain the distribution of curing materials, the piece is cut into parallel slices and

a ring or band about 1–1 1/2 in. wide is cut from the circumference of each slice and numbered 1. The interior is numbered 2. Samples bearing the same numbers from the different slices are combined.

Sampling of Sausage.—In sampling sausage it is sufficient to remove the casing and grind or hash the contents.

Methods for the Analysis of Pickle and Cured Meats. *Analysis of Pickle.*—If the pickle is new, it consists of water (and the salts naturally present in it) salt, sugar, and saltpetre. The salt can be determined by titration of the chlorine against silver nitrate using potassium chromate as indicator. If the sugar used is a high-grade white or brown sugar, it can be determined with sufficient accuracy by the polarimeter, although the salts present influence the reading to some extent. The saltpetre may be determined by the Schloesing-Wagner method (see below).

If, however, the pickle has been used, it will contain besides the substances mentioned, the various extractive matters of meat, and in this case the pickle may be considered as a cold-water extract and analysed accordingly. The coagulable, albumose, and meat-base nitrogen may be estimated as described on page 301. The principal estimations for used pickles follow.

Nitrogen.—The Nitrogen in nitrates is determined by the Schloesing-Wagner method as follows.[1]

A flask of about 250 c.c. is provided with a rubber stopper with two holes. Through one of them is passed a small separatory funnel (100 c.c.). The other has a delivery tube, the end of which is so bent as to pass easily under the mouth of the measuring burette and is covered with a piece of rubber tubing.

Fifty cubic centimetres of saturated ferrous chloride solution and the same amount of 10% hydrochloric acid are placed in the flask. The ferrous chloride is made by dissolving nails in warm 20% actual HCl and is kept tightly stoppered. The contents of the flask are boiled until the air is driven off. The delivery tube is then placed under the measuring burette which is filled with a 40% sodium hydroxide solution, and is supported in a large evaporating dish three-fourths full of the same solution.

Twenty-five cubic centimetres of a saltpetre solution containing 5 grm. of C. P. potassium nitrate to the litre are placed in the separatory funnel and with continued boiling, allowed to pass drop by drop into the flask. When almost all has run out the funnel is washed three times with 10 c.c. of 10% hydrochloric acid, each 10 c.c. being allowed to

[1] Wiley, *Principles and Practice of Altgricuural Analysis*, Vol. 2, page 397.

pass into the flask. When no more nitric oxide is evolved the measuring burette is transferred to a large cylinder filled with water.

Next 25 c.c. of the pickle to be examined are taken and placed in the separating funnel (50 c.c. of ferrous chloride solution and 50 c.c. 10% HCl are first placed in flask). The contents of the flask are boiled until all air is expelled, the delivery tube inserted under the measuring burette and the pickle allowed to drop into the flask. The separating as funnel is washed out three times with 10 c.c. 10% hydrochloric acid above and the burette transferred to the cylinder. After allowing it to remain there for about 3 minutes, the volume of nitric oxide is read by bringing the level within and without the burette to the same point. This volume is compared with the volume given by the 25 c.c. of saltpetre solution and the calculation made as follows:

Twenty-five cubic centimetres of saltpetre solution = 0.125 grm. of saltpetre. 0.125 divided by volume of nitric oxide from 25 c.c. = amount of potassium nitrate that corresponds to 1 c.c. volume.

% saltpetre in pickle =

$$100\left(\frac{\text{amount of } KNO_3 = \text{to 1 c.c.} \times \text{vol. given by 25 c.c. pickle.}}{\text{sp. gr.} \times 25}\right)$$

The nitrogen excluding nitrates is estimated by the Kjeldahl Gunning method using mercury (page 297) after removing nitrates by adding to 50 c.c. of the pickle 10 c.c. saturated solution of ferrous chloride and 10 c.c. strong hydrochloric acid and boiling a sufficient length of time. This is necessitated by the fact that the modified Kjeldahl method, to include nitrates, does not yield correct results in the presence of much salt.

The total nitrogen is estimated by taking the sum of the nitric-nitrogen and nitrogen exclusive of nitrates.

Sugar.—Either Allihn's method or Pavy's method may be conveniently used for this estimation. The Pavy method is conducted as follows:

The sp. gr. of the solution is first determined after which 100 c.c. of the pickle are transferred to a 200 c.c. volumetric flask. 10 c.c. of strong hydrochloric acid are now added and the sugar inverted by Clerget's method—heating to 68° in water, taking 15 minutes to reach this temperature. The flask is removed, cooled and made almost neutral to methyl orange with sodium hydroxide. (150 grm. NaOH to 500 c.c. water.) The sugar solution is now made up to volume and the sugar estimated as follows:

Solutions:

(1) 34.65 grm. crystallised copper sulphate in 500 c.c.

(2) 173 grm. potassium sodium tartrate and 160 grm. potassium hydroxide in 500 c.c.

120 c.c. of each of these solutions are placed in a 2,000 c.c. flask, 740 c.c. ammonia water, sp. gr. 0.90, added, made up to volume and standardised as follows. (Compare Vol. 1.)

1 grm. of pure sucrose is placed in a 200 c.c. volumetric flask, dissolved in 100 c.c. water, 10 c.c. strong hydrochloric acid added and inverted by Clerget's method, after which the flask is made up to volume. A small boiling flask of 200–250 c.c. capacity is fitted with a two-hole rubber stopper. Through one of the holes the tip of a 25 c.c. burette is inserted, and through the other the upper end of a bent delivery tube, leading under water in a beaker and fitted with pinch cock and Bunsen valve at lower end. The burette of this sugar apparatus is filled with the pure invert sugar solution. 100 c.c. of the Pavy copper solution is placed in the boiling flask and boiled until no more air bubbles rise through the water from the Bunsen valve at end of the delivery tube. The flame is now removed, the pinch cock closed and the sugar solution added, a little at a time until the blue colour of the copper solution is discharged. The number of cubic centimetres of sugar solution multiplied by 0.005 equals the value of 100 c.c. of the copper solution in terms of sucrose.

The same procedure is followed in estimating the sugar in the inverted pickle solution. Nearly the total amount required for decolourising the copper solution is added all at once, to avoid protracted boiling and loss of ammonia. The amount to be added can be estimated by a preliminary approximate titration. The copper solution will stand a total continuous boiling of 5 minutes. The calculation is made as follows:

Weight of sugar in 100 c.c. pickle equals 200 times sugar value of 100 c.c. copper solution divided by cubic centimetres pickle solution required to decolourise.

$$\text{Sugar } \% = \frac{\text{wt. of sugar in 100 c.c. pickle}}{\text{sp. gr.}}$$

Salt, Sodium Chloride.—If the pickle is sufficiently pure it may be titrated after dilution with water, against $N/10$ silver nitrate solution using potassium chromate as indicator. Or the pickle may be evaporated to dryness and charred, the char extracted with water and the extract titrated as above.

Or the titration may be carried out according to Volhard's method, using thiocyanate as indicator in the presence of nitric acid.

Nitrites.—Weigh out 10 grm. pickle into a 500 c.c. volumetric flask. If the pickle is red, add dilute acetic acid until the colour is discharged, and make up to volume. Into each of several Nessler tubes place 2 c.c. of each of the following solutions. (Griess-Ilosvay reagent):

(1) 1 gm. sulphanilic acid is dissolved by heating in 14.7 grm. glacial acetic acid diluted with an equal amount of water. By the gradual addition of water this solution is made up to 315 c.c.

(2) 0.2 grm. α-naphthylamine is dissolved by heating in 14.7 grm. glacial acetic acid and mixed with twice this amount of water and then diluted gradually to 350 c.c.

After the reagent has been added to the Nessler tubes add from 1/10 to 2 c.c., or a suitable quantity, of the diluted pickle, to different tubes and compare with standards in the usual way.

Boric Acid.—Boric Acid was at one time used as a curing agent in pickles, but its use has now been abandoned. It may be estimated by the following method based on that of Thompson.[1]

Borates are calculated as boric acid. New pickle containing no organic matter (other than sugar) and no phosphates may be titrated directly.

The sp. gr. is first determined. Then 50 c.c. of pickle are measured into a 3 or 4-in. porcelain evaporating dish, 25–30 c.c of a 5% barium hydroxide solution added and evaporated to dryness on the steam bath. The residue is ignited, ground with a small agate pestle in the same dish, and extracted with 30–50 c.c. of hot water. The solution, is decanted through a filter into a 400 c.c. Erlenmeyer flask, and the extraction repeated several times. Finally the char is transferred to the filter and thoroughly washed with boiling water. The filtrate is made very slightly acid to methyl orange, boiled 1 minute, cooled and neutralised with sodium hydroxide solution. 100 c.c. C. P. glycerin are now added and the solution titrated against $N/2$ sodium hydroxide using phenolphthaleïn as indicator. A blank of 100 c.c. glycerin is titrated and subtracted from the previous titration. Mannitol may be used in place of glycerol. The quantitative estimation should always be preceded by a qualitative test with tumeric paper.

1 c.c. $N/2$ NaOH = 0.04775 grm. borax.
1 c.c. $N/2$ NaOH = 0.02525 grm. anhy. borax.
1 c.c. $N/2$ NaOH = 0.031 grm. cryst. boric acid.

[1] *Anal.*, 1893, 18,184.

Analysis of Cured Meats.

The general methods for the analysis of meat are applicable to cured meats also and, therefore, only methods of special application are given here.

Salt, Sodium Chloride.—15–20 grm. of the hashed, minced, or finely cut sample are weighed out into a 3-in. porcelain evaporating dish and dried in the oven. (For quick work the sample is incinerated directly.) The sample is charred, but is not heated sufficiently to volatilise sodium chloride, ground with small pestle in the same dish, and transferred with hot water to a 200 c.c volumetric flask. The solution is cooled, made up to volume and an aliquot of 20 c.c. titrated against $N/10$ silver nitrate using potassium chromate as indicator. This method is sufficiently accurate for all practical purposes.

Sugar.—100 grm. of the prepared sample are weighed into a 10-in. porcelain evaporating dish, 250–300 c.c. distilled water added (or just enough for easy ebullition) and boiled for 10 minutes. The solution is decanted through muslin (starch free) if the meat is lean, or through wet filter paper if the meat contains much fat, into an evaporating dish. In the latter case the contents of the dish may be chilled before each decantation to solidify the fat. The meat is extracted in this way three times and is finally transferred to the muslin and squeezed. The combined extracts are concentrated to about 400 c.c. and transferred to a 500 c.c. flask. 15 c.c. of lead subacetate solution (U. S. P.) are added and the volume made up to the mark. The solution is filtered through a folded filter and 400 c.c. (two 200 c.c. flasks) of the filtrate concentrated to 40 or 50 c.c. A few drops of lead subacetate solution are now added, then 10 c.c. of a saturated solution of sodium sulphate. The solution is filtered through a 9 cm. filter into a 100–110 c.c. sugar flask, the precipitate well washed with small quantities of hot water, the flask cooled, made up to 100 c.c. and inverted by Clerget's method. The solution is cooled, neutralised, made up to 200 c.c. in a second volumetric flask and the sugar burette (see Analysis of Pickle-*Sugar*) filled with the solution. From this point the analysis is conducted as for sugar in pickle. Polarisation of the sugar solution is interferred with by the salt and extractives present. Fresh and cured meats containing no added sugar usually reduce an amount of the Pavy solution equivalent to 0.07–0.10% sucrose.

Saltpetre.—100 grm. of the prepared sample are extracted as for sugar, allowing the extraction to take place in the cold 10–15 minutes before the first boiling. The extract is concentrated to very small

bulk on the steam bath and all of it used for the estimation, which is conducted by the Schloesing-Wagner method as in the case of pickle, the extract being introduced drop by drop from the separating funnel into the boiling ferrous chloride solution to avoid foaming.

Boric Acid.—This is determined as in pickle working on a 100 grm. sample for quantities up to 0.2%. 20 c.c. of 5% barium hydroxide solution are added before evaporating and charring.

Starch in Sausage.—The direct estimation of starch in sausage is difficult of manipulation and requires much practice on the part of the analyst to insure success. The following is based on the A. O. A. C. method but has been modified by A. Lowenstein[1] and the writer. The A. O. A. C. method is based on Mayrhofer's method (*Forschungs-Ber. Lebensin*, 1896, **3**, 141; 1897, **4**, 47).

Fat interferes with the estimation and if the sample contains much fat this should first be removed as follows: 10–20 grm. are weighed into a 300 c.c. casserole and dried in the vacuum oven at low temperature for 1.5–2 hours. The dried sample is boiled with petroleum ether and washed until all the fat is extracted and removed. The remaining solvent is then driven off. If the sample is sufficiently lean the removal of the fat may be dispensed with. In either case the sample is next treated with 50–100 c.c. of an 8% potassium hydroxide solution, covered with a water glass and heated on the steam bath with occasional stirring until the meat is all dissolved. The solution is cooled and 50 c.c. of 95% alcohol are added and thoroughly mixed in. The precipitate is filtered off on asbestos using a Hirsch funnel (a hump of asbestos in the middle hastens the filtration) washed twice with a hot solution of 4% potassium hydroxide in 50% alcohol, then several times with 50% alcohol. The precipitate and asbestos are returned to the original casserole and digested with 60 c.c. (more if the starch content is high) of normal potassium hydroxide which dissolves the starch. The asbestos is filtered off on a Hirsch funnel and well washed with hot water. The filtrate is strongly acidified with acetic acid, made up to 250 c.c. and filtered through a Buchner funnel. An aliquot of the filtrate is precipitated with an equal volume of 95% alcohol. A Gooch crucible is prepared with a thin layer of asbestos and on this a layer of prepared sea sand 2–4 mm. thick is placed. The crucible is then dried and weighed. The starch is filtered off on the Gooch crucible, washed successively with 50% alcohol, absolute alcohol and finally ether. The crucible is then dried to constant weight at 100–105°, cooled and weighed.

[1] Private communication.

Approximate Estimation of Starch.—A method which can make no claims for accuracy but which is only roughly approximate is that of Ambühl (*Pharm. Centralh.*, 1881, **22**, 438). It is convenient in combining a qualitative method with an approximate quantitative method. 2 grm. of the sausage are macerated with 100 to 200 c.c. water, then boiled for 30 minutes and diluted to 200 c.c. in a volumetric flask. Aliquots are pipetted or strained off, cooled, treated with iodine solution in potassium iodide and the depth of colour compared with standards containing known amounts of the same kind of starch as that in the sample (ascertained microscopically), and boiled for 30 minutes also. The usual allowance must be made when corn flour or other raw product is present in the product in place of pure starch.

Price's Method.—Price has reviewed the various methods for estimating starch in meat food products (*Circular* 203, *Bur. Animal Industry, U. S. Dept. Agric.*) and has finally developed the following method based on Bigelow's modification of Mayrhofer's method (*Bull.* 13, *Part* 10, *Bur. Chem., U. S. Dept. Agric.*) and Perrier's method (*Bull. Scien. Pharm.*, 1908, 305). This method has been used as a practical routine method in the meat inspection laboratories in the United States.

In a 200 c.c. beaker treat 10 grm. of finely divided meat with 75 c.c. of an 8% solution of potassium hydroxide in 95% alcohol, and heat on the steam bath until all the meat is dissolved. This will require from 30 to 45 minutes. Add an equal volume of 95% alcohol, cool, and allow to stand at least 1 hour. Filter by suction through a thin layer of asbestos in a Gooch crucible. Wash twice with warm 4% potassium hydroxide in 50% alcohol and then twice with warm 50% alcohol. Discard the wash water. Endeavour to retain as much of the precipitate in the beaker as possible until the last washing. Place the crucible with contents in the original beaker, add 40 c.c. of water and then 25 c.c. of concentrated sulphuric acid. Stir during the addition of the acid and see that the acid comes in contact with all the precipitate. Allow to stand about 5 minutes, add 40 c.c. of water, and heat just to boiling, stirring constantly. Transfer the solution to a 500 c.c. graduated flask, add 2 c.c. of a 20% aqueous solution of phosphotungstic acid, allow to cool to room temperature, and make up to mark with distilled water. Filter through a starch-free filter paper, and determine the dextrose present in a 50 c.c. portion of the filtrate with Fehling's solution after neutralizing the acid, using Low's method, as given in *Bureau of Chemistry Bulletin* 107 (revised), page 241, for the determination of the copper in the cuprous oxide precipitate. The amount of dextrose multiplied by 0.9 gives the equivalent in starch.

Low's method is as follows:

(*a*) *Standardisation of the Thiosulphate Solution.*—Prepare a solution of sodium thiosulphate containing 19 grm. of pure crystals to 1,000 c.c. Weigh accurately about 0.2 grm. of pure copper foil and place in a flask of 250 c.c. capacity. Dissolve by warming with 5 c.c. of a mixture of equal volumes of strong nitric acid and water. Dilute to 50 c.c., boil to expel the red fumes, add 5 c.c. strong bromine water, and boil until the bromine is thoroughly expelled. Remove from the heat and add a slight excess of strong ammonium hydroxide—7 c.c. is about the right amount. Again boil until the excess of ammonia is expelled, as shown by a change of colour of the liquid, and a partial precipitation. Now add a slight excess of strong acetic acid (3 or 4 c.c. of 80% acid) and boil for a minute. Cool to room temperature and add 10 c.c. of a solution of pure potassium iodide containing 300 grm. of potassium iodide to 1,000 c.c. Titrate at once with the thiosulphate solution until the brown tinge has become weak, then add sufficient starch liquor to produce a marked blue colouration. Continue the titration cautiously until the colour due to free iodine has entirely vanished. The blue colour changes toward the end to a faint lilac. If at this point the thiosulphate be added drop by drop and a little time be allowed for complete reaction after each addition there is no difficulty in determining the end point within a single drop. 1 c.c. of the thiosulphate solution will be found to correspond to about 0.005 grm. of copper.

(*b*) *Determination of Copper.*—After washing the precipitated cuprous oxide, cover the gooch with a watch glass and dissolve the oxide by means of 5 c.c. of warm nitric acid (1 : 1) poured under the watch glass with a pipette. Catch the filtrate in a flask of 250 c.c. capacity, wash watch glass and gooch free of copper; 50 c.c. of water will be sufficient. Boil to expel red fumes, add 5 c.c. of bromine water, boil off the bromine, and proceed exactly as in standardising the thiosulphate.

Because of the fact that not only different kinds of starch but also different kinds of flour as well as dry bread crumbs are sometimes mixed with ground meats and sausage, it is difficult for the analyst to estimate the total amount of vegetable matter added in this way unless a sample of the original material added is obtainable. Since this is seldom possible the analyst can only report the percentage of starch actually found in the majority of cases.

Horse Flesh Sausages.[1]—On the continent of Europe, and especially

[1] For identifying kinds of meat see also *The Precipitin Method*, page 271.

in France, horse flesh is extensively used for the manufacture of sausages, the vendors of which are required to indicate the nature of the articles they sell. Hence a means of recognising horse flesh, and detecting it when mixed with the flesh of other animals, is of practical importance.

The physical characters of horse flesh have been employed for its recognition (see page 273) but are not very conclusive under any circumstances, and are useless when the flesh is minced and mixed with other kinds of meat, as in sausages.

Horse flesh is remarkable for the comparatively large proportion of *glycogen*[1] usually contained in it, and this fact has been utilised by

[1] **Glycogen** $(C_6H_{10}O_5)n$, was first found in the liver, but has been more recently met with in many other parts of the body. It has been termed "Animal Starch" from its close analogy to soluble starch. The physiological significance of glycogen has been the subject of much controversy, and the question is not yet settled.

Glycogen may be prepared by rapidly cutting up the liver of an animal killed immediately previously, and throwing the fragments into five times their weight of boiling water. After boiling for a short time, the fragments of liver are mixed with sand and reduced to powder in a mortar, and then returned to the water, which is again boiled. The liquid is strained, and faintly acidified with acetic acid while still hot. The filtrate from the coagulated proteins is rapidly cooled, and the remaining proteins precipitated by the alternate addition of hydrochloric acid and potassio-mercuric iodide. The filtered liquid is mixed with such a volume of strong alcohol as to make it contain 60% of absolute alcohol, when the precipitated glycogen is filtered off, washed first with 60% alcohol, and then with absolute alcohol and ether.

Kistiakoffsky (*J. Russ. Chem. Soc.*, **25**, 60; abst. *Trans*, 1893, **62**, 618) prepares glycogen from the liver and muscles, taken as before immediately after the death of the animal, by cold extraction. The material is ruhbed up in an iron mortar, cooled to a very low temperature to prevent fermentation, and the homogeneous mass then extracted with ice-cold water containing 1 to 2% hydrochloric acid. This operation is repeated until the last extract ceases to give the glycogen reaction with iodine. If it is not essential that the whole of the glycogen should be extracted, water containing from 0.2 to 0.7% of acid may be used. The solution obtained is coloured by hæmoglobin, and contains albuminous matters, which are preciptated by means of mercuric iodide. This precipitate is filtered off and washed with dilute mercuric iodide solution until free from glycogen. The glycogen is precipitated from the filtrate and washings by the addition of about one and a half volumes of alcohol. It is collected on a filter, and washed first with 75% alcohol, and then with ether-alcohol. After being dried over sulphuric acid, the product forms a white, amorphous powder, containing no nitrogenous compounds, and leaving only traces of ash on ignition. If dried in the air, a resinous mass difficult to powder is obtained. This method may be used for the estimation of glycogen in animal tissues, the results obtained being somewhat under those found by Brücke's method. (See further, Kistiakoffsky, abst. *Trans.*, 1896, 69, 80.)

Brücke advocates the use of 0.1 to 0.3% solutions of alkali for the extraction of glycogen, instead of the 2% alkali solutions as sometimes used. If the extraction of the glycogen be effected with boiling water, the aqueous liquid contains, besides glycogen, alkali albuminates, glutin, and traces of peptone (Kistiakoffsky, abst., *Trans.*, 1896, 69, 80). These are all precipitated by hydrochloric acid and potassio-mercuric iodide as already described

Of the substances proposed for the extraction of glycogen, boiling water, trichloracetic acid, sulpho-salicylic acid, and formaldehyde all extract albumoses, in some cases in considerable quantities, from the animal substances used. The separation of the albumoses is attended with some difficulty. Their presence may be conveniently recognised by treating the glycogen solution with a reagent containing 100 parts of sodium tungstate, 50 parts of phosphoric acid, 10 parts of concentrated hydrochloric acid, and 500 parts of water. On treatment with Millon's reagent, the dried precipitate will show the presence of 0.02% of albumose. The presence of glycogen does not interfere. This test is due to D Huizinga (*Pflüger's Archiv.*, **61**, 32; abst, *Trans.*, 1896, **68**, 6), who finds that the best results are obtained when the liver or other animal substance is treated with a mixture of equal parts of a saturated solution of mercuric chloride and Esbach s reagent, made by dissolving 10 grm. of picric acid and 20 grm. of citric acid in water, and making the solution up to 1,000 c.c. This treatment does not extract the whole of the glycogen from the animal substance.

Pure glycogen is a snow-white, amorphous powder, readily soluble in water to form a solution which is usually, but not invariably, opalescent, and wh ch becomes more limpid on adding acetic acid or an alkali. Glycogen is precipitated from its aqueous solution by alcohol whenever the alcohol amounts to 60% of the liquid. If the solution be quite free from salts, the separation is sometimes very difficult, but takes place instantly on adding a minute quantity of common salt. The precipitation of glycogen in liquids containing 60% of alcohol distinguishes it from the different varieties of dextrin, none of which are precipitated by alcohol of less than 85% strength. On the other hand, glycogen exactly simulates erythro-dextrin in its behaviour with a solution of iodine, which produces a port-wine colour, disappearing on heating, and returning as the liquid cools.

Brautigam and Edelmann (*Chem. Centralb.*, 1894, **1**, 485; abst. *Analyst*, 1894, **19**, 24) for its detection in sausages. They state that 10%. of horse flesh or horse-liver can be detected by this means, the proportion of glycogen therein ranging from 0.37 to 1.07%, while the flesh of other animals used for food contains little or none—ox flesh coming next with 0.20%. On the other hand, the flesh of the fœtus, both of man and of the lower animals, is rich in glycogen. M. Humbert (*J. Pharm. et Chim.*, 1895, 195; abst. *Analyst*, 1895, **20**, 95) confirms the value of the observation of Brautigam and Edelmann and recommends the following method of procedure: About 50 grm. weight of the muscular tissue should be cut into small pieces and boiled for an hour with 200 c.c. of water. After cooling, nitric acid is added in the proportion of 5 c.c. to 100 c.c. of broth, and the liquid filtered. To a portion of the filtrate contained in a test-tube iodine-water[1] is added drop by drop, so as not to mix the liquids, when the formation of a reddish-violet zone at the junction of the two strata will indicate the presence of glycogen in the original sample. The reaction is said to be quite characteristic of horse flesh. Humbert states that of 10 samples of horse flesh obtained from different dealers in Paris, seven showed the colour very plainly, in two it was less pronounced but distinct, while in one case it was doubtful. In no instance was there any colouration with beef, veal, mutton, or pork. Beef-broth left in contact with the iodine for 10 days gave no reaction. The flesh of the ass gave a negative result, but with that of the mule the reaction was the same as with horse flesh. A mixture of equal parts of horse flesh, beef, mutton, veal, and pork showed the colouration, but it was less pronounced than with horse flesh alone. (Compare p. 284.)

Courlay and Coremons (abst. *Analyst*, 1896, page 231) recommend a slight modification of the foregoing test. About 50 grm. of the substance, in as fresh a state as possible, should be finely divided and boiled for 15 to 30 minutes with 20 c.c. of water. After cooling (*no* addition of nitric acid being made) the broth is filtered and then tested with a few drops of iodine solution, prepared by dissolving 2 parts of iodine and 4 of potassium iodide in 100 parts of water. A brown colouration, disappearing on warming to 80° and reappearing on cooling, shows the presence of horse flesh.[2] In the presence of starch (*e.g.*, in sausages) the blue reaction with iodine may entirely mask the brown

[1] The reagent should be recently prepared by saturating hot water with iodine. In the original form of the test, Brautigam and Edelmann employed hydriodic acid instead of the hot iodine water recommended by Humbert.

[2] Courlay and Coremons state that, by their modification of the iodine test, no glycogen reaction was obtained from the flesh of calves, pigs, dogs, or cats; but that this observation does not apply to the fetus of any of these animals.

colouration due to glycogen; but this is obviated by treating the broth with two or three times its measure of strong acetic acid, filtering, and applying the iodine test to the filtrate.

W. Niebel (*Chem. Centralb.*, 1893, page 323; abst. *Analyst*, 1895, **20**, 252) criticises the foregoing methods on the ground that the reaction with iodine is uncertain, since glycogen is also found in the flesh of dogs, cats, and very young calves, in the livers of cattle, and in meat-extract to the amount of 1.5%. With old sausages from horse flesh Brautigam and Edelmann always obtained the glycogen reaction, although that substance would usually be completely decomposed under these circumstances. There is also an uncertainty in the reaction caused by the fact that the erythro-dextrin formed from the starch gives a similar colouration with iodine, and no means of removing it is known. Niebel considers that the red colour with iodine is not sufficient proof of the presence of glycogen, which should be isolated in a pure condition. Nevertheless, the iodine colouration, and the occurrence of more than 1% of glucose in the fat-free substance, points to the presence of horse flesh in a sample, even when all the glycogen has been decomposed. The red colour only fails in the case of the flesh of young foals.

The figures given on p. 282 were obtained by Bujard more recently. Some of the samples were analysed both by the method of Niebel and Salkowsky and by that of Mayrhofer (*Forsch. Ber.*, 1896, 141), to which he gives the preference on the ground of simplicity. In the latter process, the finely divided flesh is dissolved in aqueous potassium hydroxide, the proteins precipitated by hydrochloric acid and Nessler's solution, and the clear filtrate treated with alcohol. This throws down the glycogen, which is filtered off, washed with dilute alcohol and with ether, and dried at 110°.

In Bujard's opinion, his figures show that only in exceptional cases (where the amount is large) can the glycogen be taken as conclusive of the presence of horse flesh, especially when the latter is mixed with other kinds of flesh.

The methods of Pflüger have now to a large extent superseded the older methods for the estimation of glycogen. These are given in detail on pages 284 and 285.

James Bell has pointed out (*Chem. News*, **55**, 15) that the fat of the horse differs materially in its characters from that of the ox. Thus the fat isolated from different parts of the horse—such as the round, flank, ribs, kidneys, and heart—was found to have a sp. gr. at 38°

ranging from 0.9086 to 0.9088. The intramuscular fat had a sp. gr. of 0.9084 at the same temperature, and hence was not greatly different in character from that obtained directly from the adipose tissue. The horse-fat melted to a clear oil at 70° and the amount of solid fat deposited at a lower temperature was comparatively small. A series of similar experiments made with beef-fat showed a m. p. of 110 to 116° F. The sp. gr. was taken at 120° F., and when the results were corrected to 100°, to render them comparable with those obtained with horse-fat, the sp. gr. was found to range from 0.9036 to 0.9040. These figures show a substantial difference between beef-fat and horse-fat, and the distinction is still more marked in the case of mutton-fat. The low melting-point of horse-fat is an important characteristic, and in cases where the flesh is not of mixed origin ought usually to be conclusive as to its nature.

R. Frühling (*Zeit. ang. Chem.*, 1896, 352; abst. *Analyst*, 1896, **21**, 231) was led to study the fat of horse flesh, being unable to obtain conclusive results by the reaction for glycogen. He found the fat extracted from sausages made wholly from horse flesh to have an iodine-absorption of 72.5%, while that from sausage composed of horse flesh with 15% of pork gave 62.3; and from sausage composed of equal parts of horse flesh and pork, 57.2%. As pure pork-fat (lard) shows an iodine-absorption averaging about 60, it is evident that the method is inconclusive.

H. Bremer (*Forsch. Ber.*, 1897, **4**, 1; abst. *Analyst*, 1897, **22**, 104) has reviewed the work of other chemists, and proposed a method of detecting horse flesh, based on the characters of the intramuscular fat.

The subjoined table gives the results obtained by his method:

	Iodine No. of intramuscular fat	Iodine No. of liquid fatty acids
I. Horse flesh sausage without bacon.....	75.8	108.1
II. Horse flesh sausage with about 6% of bacon....	74.0	104.1
III. Horse flesh cervelat (brain) sausage with about 22% of well-smoked bacon.	53.7	92.4
IV. Horse flesh cervelat sausage with about 25% of bacon.	74.1	102.1
V. Ordinary sausage with some bacon.....	57.6	94.2
VI. Thuringian cervelat sausage with about 65% of pig's fat.	64.3	95.8
VII. Mixture of I. and V. in equal parts.....	66.4	103.1
VIII. Mixture of IV. and VI. in equal parts.....	65.2	99.5

It is stated that whenever horse flesh is present the petroleum-ether extract has a red to reddish-brown colour, and that even the liquid fatty acids have a more or less reddish-yellow shade. On the other hand, bull's flesh gives a similar colour, so that too much reliance must not be placed on this fact, except as a confirmatory test. When, however, this is found to be the case, when at the same time glycogen is detected, and when the iodine number of the intramuscular fat exceeds 65, and that of the liquid fatty acid is considerably over 95, there can be but little doubt as to the presence of horse flesh.

Artificial Colour in Sausage.—Artificial dyes are now less used than formerly to colour sausage meat. In the large establishments in the United States which are under government inspection, under certain restrictions the 7 certified colours Amaranth (107), Ponceau 3R(56), Erythrosin (517), Orange 1 (85), Naphthol Yellow S(4), Light green S. F. Yellowish (435), and Indigodisulpho acid (692) as well as annatto can be used to colour sausage casings (but not the meat) according to the rules and regulations for enforcing the Meat Inspection Act. The methods of identifying colours when used in food products, and especially in meat are imperfect, even when it is certain that an added dye is present and the subject is complicated by the difficulties of extraction, and the colour found in meat which has been cured with saltpetre. Ground meat when exposed to the air rapidly turns to a dirty drab, especially in the presence of salt, and the artificial colour was formerly added to maintain a red colour. Saltpetre accomplishes the same result (page 364).

Red ochre is said to have been used, but would make its presence evident by excessive iron in the ash of the material.

For the detection of *cochineal-carmine* in sausages, Klinger and Bujard (*Zeit angw. Chem.*, 1891, **4**, 515; abst. *J. Chem. Soc.*, 1893, **63**, 56) recommend that about 20 grm. of the cut-up sausage should be heated in a water-bath with a mixture of equal parts of water and glycerin. In the absence of this colouring matter only a slight yellow colour is produced, but in its presence the liquid becomes decidedly reddish in colour. The filtered solution is heated, if necessary, with another 20 grm. of the sausage. The clear liquid is then examined in the spectroscope, when cochineal-carmine can be readily recognised by its characteristic absorption-bands lying between b and D. A preferable plan is to precipitate the colouring matter as a lake, and, after washing, to dissolve it in a little tartaric acid. A more concentrated solution is thus obtained, and the spectroscopic test is consequently more satisfactory.

H. Bremer (abst. *Analyst*, 1897, **22**, 216) confirms the value of the foregoing method for the detection of cochineal-carmine. He heats the finely divided sausage for several hours on the water-bath with two volumes of the slightly acidified glycerin-water mixture. The yellow solution is freed from fat and filtered, and the colouring matter precipitated as a lake by the addition of alum and ammonia. The absorption-bands lying between b and D, which are characteristic of carmine-lake, may be readily observed in the spectroscope. In one instance Bremer found a cervelat sausage coloured with cochineal-carmine to have all the appearance of good meat when cut, but further examination showed it to be quite unfit for food, the "acidity number" of the fat being 76.0 (*e.g.*, the separated fat required 7.6% of potassium hydroxide for its neutralisation). *Aniline Red* has been detected, but certain varieties of **Benzopurpurin** (Vol. 5) are now most commonly employed. This colouring matter is allied to Congo Red, and has the property of dyeing vegetable fibres without a mordant. It is added to the meat together with the dry bread or biscuit, which it effectually colours.

Aniline Red, if present, can be detected by extracting the finely divided sausage with methyl alcohol, evaporating the solution after straining or filtering, taking up the residue with water, and immersing white wool in the boiling liquid, when the fibre will be dyed red if Rosaniline is present.[1]

Weller and Riegel (*Forsch. Ber.*, 1897, **4**, 204; abst. *Analyst*, 1897, **22**, 324) state that the hæmoglobin of pig's blood is converted by nitre into a modified form which dissolves with red colour in diluted glycerin, alcohol, amyl alcohol, and ether, but can be distinguished from cochineal-carmine by its absorption-spectrum after reduction by ammonium sulphide. (See Vol. 5.)

In attempting to extract vegetable and coal tar dyes from meat it is often necessary to try several solvents before a satisfactory one is found. Besides those already enumerated, acidified glycerin or alcohol, a 5% solution of salicylic acid and dilute ammonia will be found useful. After filtering off the solvent it is concentrated to small bulk, acidified with HCl and white, grease-free wool dyed in it. If the wool is dyed distinctly red a coal tar dye is probably present and should be examined further.

Very dilute extracts may be concentrated by precipitation of the dye

[1] See *Circulars* 25, 35, *and* 63, *Bur. Chem. U. S. Dept. Agric. Bull.* 107 *Rev. Ibid.*, page 190. and *Allen*, Vol. 5, p. 623 *et seq.*

as a lake, dissolving in hydrochloric acid and making alkaline with ammonia water.

Methods of the A. O. A. C.—The Association of Official Agricultural Chemists recommends several methods for the detection of artificial colours of which the following are applicable to meat products.

Methods of Sostegni and Carpentieri.[1]—Dissolve or extract from 10 to 20 grm. of the sample in 100 c.c. of water, filter if necessary, acidify with from 2 to 4 c.c. of a 10% solution of hydrochloric acid. In this solution immerse a piece of woolen cloth, which has been washed in a very dilute solution of boiling potassium hydroxide and then washed in water, and boil for from 5 to 10 minutes. Remove the cloth, thoroughly wash it in water, and boil in a very dilute hydrochloric acid solution. After washing out the acid dissolve the colour in a solution of ammonium hydroxide (1:50). With some of the dyes solution takes place quite readily while with others it is necessary to boil for some time. Take the wool out, add a slight excess of hydrochloric acid to the solution, immerse another piece of wool, and boil it again.

With vegetable colouring matter this second dyeing gives practically no colour, and there is no danger of mistaking a fruit colour for one of coal-tar origin. It is absolutely necessary that the second dyeing should be made, as some of the coal-tar dyes[2] will dye a dirty orange in the first acid bath which might be easily passed for vegetable colour, but after solution in alkaline bath the second acid bath dyes a bright pink.

Arata's Method.[3]—This method gives results comparable with those of the first dyeing of the preceding method. It was recommended for detecting coal-tar colours in wine, and has been used by Winton[4] for fruit products.

Boil from 20 to 30 grm. of the sample dissolved in 100 c.c. of water for 10 minutes with 10 c.c. of a 10% solution of potassium hydrogen sulphate and a piece of white wool or woolen cloth, which has been previously heated to boiling in a very dilute solution of sodium hydroxide and thoroughly washed in water. After removal from the solution, wash the wool in boiling water and dry it between filter papers.

[1] *Zts. anal. Chem.*, 1896, 35, 397. The method of Sostegni and Carpentieri and Aratas' method for coal-tar colours are not reliable in the presence of archil, archil derivatives, and sulphonated indigo, as these substances give dyeing reactions not to be distinguished from coal-tar colours. It is, however, comparatively easy to detect archil. A red colour turning purple with dilute ammonium hydroxide, reduced by zinc and hydrochloric acid and easily reoxidizing in the air, is either archil or a closely related colour. (Tolman, *J. Amer. Chem. Soc.*, 1905, 27, 25.) Archil (orchil) can be extracted from an ammoniacal solution by amyl alcohol, but the sulphonated archil colours now on the market do not respond to the test. Indigo is used in many green and violet colours and can be recognised as described in Vol. 5, 392, *et seq.*

[2] U. S. Dept. Agr., Bureau of Chemistry, *Bul.* 66. p, 24.

[3] *Zts. anal. Chem.*, 1889, 28, 639; Allen 5, 642

[4] Conn. Agr. Exper. Stat. Rept., 1899, Part 2, p. 131.

In addition to this, it is advisable in all cases to dissolve out the colouring matter with ammonium hydroxide as in the first method and dye again after acidifying with a few drops of hydrochloric acid, since Arata's method gives practically the same results as the first dyeing in hydrochloric-acid bath and needs to be confirmed by the second dyeing.

Another advantage in the second dyeing is that if a large piece of woolen cloth is used in the first dyeing, and a small piece in the second dyeing, small amounts of colouring matter can be brought out much more decidedly in the second dyeing, where practically all of the vegetable colouring matter has been excluded. The colouring matter can be identified to a certain extent by the schemes of Witt,[1] Allen, Weingärtner,[2] Dommergue,[3] Girard and Dupré,[4] and Rota.[5] The tests can be made directly on the dyed fabric or the dye can be dissolved out.[6] To remove the colour, wash the wool with dilute tartaric acid and then with water and dry between filter paper. Saturate the wool with strong sulphuric acid, press out the colour with a glass rod after from 5 to 10 minutes, and dilute to 10 c.c. with water.

Remove the wool, make solution alkaline with ammonium hydroxide, and when cold extract with from 5 to 10 c.c. of amyl alcohol. Separate the amyl alcohol, evaporate it to dryness, and test the residue with strong sulphuric acid.

Ponceau R, 2R, 3R, S, and 3S give yellow red to carmine red.

Ponceau S and Tropæolin O give yellow to orange yellow.

Biebrich scarlet gives a green; Bordeaux red and Crocein Scarlet give blue; Tropæolin OOO and Solid Red give violet.

If the wool is well dyed, most of these colours may be obtained on the fabric.

These are the reactions of only a few of the more common colours: in order to carry the work further the more complete works mentioned must be used.

Method for the Separation of the Seven Permitted Coal-tar Colours.—Price (Circular 180, Bur. Animal Industry, U. S. Dept. Agric.) proposes the following method for the separation of the seven colours, Amaranth, Ponceau 3R, Erythrosin, Orange I, Naphthol Yellow S, Light Green S F Yellowish, and Indigo Disulpho Acid.

[1] *Zts. anal. Chem.*, 1887, 26, 100.
[2] *Zts. anal. Chem.*, 1888, 27, 232–249.
[3] *Zts. anal. Chem.*, 1890, 29, 369–377.
[4] Analyse des matières alimentaries, etc., 583–593
[5] *Analyst*, 1899, 24, 41.
[6] *Zts. anal. Chem.*, 1889, 28, 639; Borgmann, Analyse des Weines, p. 91; Winton, Conn. Agr. Exper. Stat. Rept., 1899, Part 2, p. 131.

The regulations of the Secretary of Agriculture governing meat inspection allow the use of these dyes on casings of sausages and other meat food products when the character of the casing is such that the dye will not penetrate into the meat food product contained therein.

Reagents.—It is of the utmost importance that the reagents be employed of the strength indicated below:

Ammonium Sulphate Solution.—A saturated solution of ammonium sulphate is prepared by agitating ammonium sulphate in water at steam bath temperature until the water is completely saturated. This will require several hours. The solution is then allowed to cool at room temperature and should always contain an excess of undissolved ammonium sulphate crystals. The solution should always be agitated previous to using.

Sodium Chloride Solution.—A saturated solution of sodium chloride is prepared in the same manner as the saturated ammonium sulphate solution described above. The solution should stand in contact with undissolved crystals of the salt.

Calcium Chloride Solution (25%).

25 grm. of calcium chloride, C. P. granulated, in
100 c.c. of water.

Dilute ammonia (1:100).

1 c.c. of concentrated ammonia in
100 c.c. of water.

Dilute acetic acid (5%).

5 c.c. glacial acetic acid.
95 c.c. of water.

Stannous Chloride Solution.—Dissolve 30 grm. of granulated tin, free from iron, in 125 c.c .of hydrochloric acid (1.19 sp. gr.); dilute to 250 c.c. and filter through asbestos. To the filtrate add 250 c.c. of hydrochloric acid (1.124 sp. gr.) and 500 c.c. of water.

Acetone.

Alcohol (95%).

Amyl alcohol.

Acetic ether (*ethyl acetate*).

Ether, sulphuric (*ethyl ether*).

Hydrogen peroxide (3%).

Preliminary Test.—The well-known method of scattering the powdered dyestuff upon water, alcohol, and sulphuric acid should be used to determine whether one or more colours are present.

Method of Separation.[1]—Approximately 0.2 of a grm. of the sample is rubbed up in a mortar with about 25 c.c. of saturated ammonium due on the filter is washed with small portions of the saturated amsulphate solution, filtered, and if the filtrate is coloured red the resimonium sulphate solution until the washings are no longer coloured red. The filtrate and washings should contain Amaranth, together with some Naphthol Yellow S. The filtrate and washings are combined and shaken with separate portions of acetic ether until the acetic ether is no longer coloured yellow. The acetic ether removes the Naphthol Yellow S, and the Amaranth remains behind in the ammonium sulphate solution. The acetic ether extracts containing the Naphthol Yellow S need not be preserved, as this colour will be removed in a pure form later. The ammonium sulphate solution containing the Amaranth is shaken with acetone, which extracts the Amaranth. The acetone is then filtered to remove the excess of salt, diluted with a small amount of water, and the acetone driven off on the steam bath. The water solution is then tested for Amaranth.[2]

The undissolved portion of the original sample is removed from the filter, mortar, and pestle by washing with water. This water solution is acidified with acetic acid and shaken with separate portions of ether until the ether is no longer coloured. The ether will remove the Erythrosin. It is washed several times with water and finally shaken up with dilute ammonia solution. The Erythrosin is taken up by the ammonia solution. The excess of ammonia is driven off on the steam bath and the solution tested for Erythrosin. Observation should be carefully made to see that this solution when very dilute has no fluorescence, as this property indicates the presence of prohibited colours having similar reactions.

The water solution which has been extracted with ether is warmed on the steam bath until the ether is removed. It is then saturated at the steam bath temperature with sodium chloride, cooled, and filtered through a folded filter paper 18 cm. in diameter. This filtration should be carried out so as to keep the precipitate in the bottom of the filter. The precipitate is washed with saturated sodium chloride solution until the washings are colourless. The filtrate and washings should contain Light Green S F Yellowish, Naphthol Yellow S, and possibly traces of Orange I. They are combined and shaken with

[1] See Vol. 5, 646, *et seq.*

[2] For the identification of all the separated colours the reaction as given by Loomis (*Circular* 63, *Bureau of Chemistry, U. S. Department of Agriculture*, 1911) should be used.

[3] *Zts. anal. Chem.*, 1889, 28, 639; Borgmann, Analyse des Weines, p. 91; Winton, Conn. Agr. Exper. Stat. Rept., 1899, Part 2, p. 131.

acetone, which removes the Naphthol Yellow S. This acetone solution should be washed several times by agitation with saturated sodium chloride solution in order to remove any traces of green colour held mechanically by the acetone. A small amount of water is added and the acetone driven off on the steam bath. The water solution is acidified with acetic acid and shaken with amyl alcohol, which removes any traces of Orange I that may be present. The water solution is freed from amyl alcohol by heating on the steam bath and is then tested for Naphthol Yellow S.

The solution containing Light Green S F is evaporated to a small bulk and the residue is taken up in alcohol and filtered. This removes excess of salt. The alcohol is driven off and the solution then tested for Light Green S F Yellowish.

The precipitate on the filter that has been well washed with the supersaturated sodium chloride solution should contain Orange I, Ponceau 3R, and Indigo Disulpho Acid. This precipitate is dissolved in water and shaken with several successive portions of acetic ether. Orange I is taken up by the acetic ether. It is not necessary, however, to continue this extraction with acetic ether until all of the Orange I is removed. About three extractions will remove enough Orange I to make all the necessary tests for this colour. The acetic ether portions are combined and washed with supersaturated sodium chloride solution several times or until no more colour is removed. Now shake with water; this removes the Orange I from the acetic ether. The water solution is freed from acetic ether by heating on the steam bath, and is then used to identify the colour.

The water solution containing Ponceau 3R, Indigo Disulfo Acid, and any Orange I not removed by the acetic ether is warmed on the steam bath until free from acetic ether. It is cooled, made up to 125 c.c. with water, about 40 c.c. of 25% calcium chloride solution added, and allowed to stand about 15 minutes. Ponceau 3R and some Indigo Disulpho Acid are precipitated. 15 c.c. of stannous chloride solution are added and stirred until all the blue colour disappears. It should then be filtered immediately, as too long standing will destroy Ponceau 3R. Ponceau 3R remains on the filter paper. Wash with calcium chloride solution until the blue colour that may have become reoxidized during filtration is washed out; dissolve in dilute ammonia solution; drive off the excess of ammonia, and test for Ponceau 3R.

The filtrate, which should be practically colourless, is treated with hydrogen peroxide. If Indigo Disulpho Acid is present the solution will become deep blue in colour.

The following table gives an outline of the above-described method of separation. It is desirable in carrying out this separation to follow the table, referring from time to time to the description. Analysts who contemplate applying this method to the separation of mixtures of colours should first familiarise themselves with the method and with the behaviour of the permitted colours by attempting the separation of mixtures of known permitted colours. It will probably be simpler to begin with two or three colours and finally to apply the method to a mixture containing all seven. The presence of an unpermitted colour will be indicated by the effect on the reactions of the permitted colours with standard reagents, and the unpermitted colour may also interfere materially with the scheme of separation as outlined above.

Holtzendorff (Milwaukee Meeting Am. Chem. Soc., March, 1913) proposes the following method for the rapid routine separation of the seven permitted coal-tar colours:

The colour in approximately 50 grm. of the food is double-dyed on wool after the well-known method of Sostegni and Carpentieri, the colour removed from the wool by means of 2% ammonia water and the aqueous solution of colour thus obtained acidified with 5% acetic acid. The acid solution is agitated with successive portions of ethyl ether in a separatory funnel until colour ceases to come away in the ether layer. The acid aqueous solutions are united and reserved.

The ether extract is washed with 2% ammonia water, the ammonia driven off by heat and the residue tested for Erythrosin (conc. H_2SO_4 on dry colour = orange; after dilution orange ppt.).

The original acid aqueous solution is divided in two unequal portions, the larger of which is reserved. To the smaller portion, a small amount of zinc dust is added and sulphur dioxide gas run in for 10 minutes. If the solution remains coloured at the end of this time, the presence of an unpermitted colour is assured. If on the other hand decolourisation is complete, the colour is probably one or more of those permitted.

The reserved acid aqueous solution is shaken with successive portions of amyl alcohol in a separatory funnel until colour ceases to come off in the alcohol layer. The acid aqueous solution is reserved. The amyl alcohol extracts are washed with 2% ammonia water, the ammonia driven off and the residue tested forOrange I (conc. sulphuric acid on dry colour = magenta, after dilution = crimson changing to orange).

The reserved acid aqueous solution is saturated on the water bath with sodium chloride, cooled, filtered and washed with a saturated sodium chloride solution until filtrate is colourless, the filtrate made

SCHEME FOR SEPARATING THE SEVEN PERMITTED COAL-TAR COLOURS

Rub approximately 0.2 grm. of sample in a mortar with saturated $(NH_4)_2SO_4$ solution; filter and wash with the $(NH_4)_2SO_4$ solution until washings are no longer coloured red.

<table>
<tr><td colspan="2">Filtrate contains Amaranth and small amount Naphthol Yellow S. Shake with acetic ether.</td><td colspan="6">Residue contains Erythrosin, Naphthol Yellow S, Light Green S F Yellowish, Orange I, Ponceau 3R, and Indigo Disulpho Acid. Dissolve in H_2O, acidify with CH_3COOH, and shake with ether.</td></tr>
<tr><td rowspan="2">Acetic ether removes Naphthol Yellow S. This solution is discarded.</td><td rowspan="2">$(NH_4)_2SO_4$ solution contains Amaranth. Shake with acetone, which removes the Amaranth. Dilute with water and drive off the acetone.</td><td rowspan="2">Ether contains Erythrosin. Wash with H_2O and then shake with dilute NH_4OH, which removes the Erythrosin. The excess of NH_3 is then driven off.</td><td colspan="4">Water solution is freed of ether and saturated with NaCl on steam bath, cooled, filtered, and washed with saturated NaCl solution until washings are colourless.</td></tr>
<tr><td colspan="2">Filtrate contains Light Green S F Yellowish and Naphthol Yellow S. Shake with acetone. Separate acetone and salt solution.</td><td rowspan="2">Precipitate is dissolved in H_2O and shaken three times with acetic ether.</td><td colspan="2" rowspan="2">The H_2O solution is warmed on steam bath until free from acetic ether, cooled, made up to 125 c.c with H_2O, 40 c.c of 25% $CaCl_2$ solution added, and allowed to stand about 15 minutes, 15 c.c. of $SnCl_2$ solution added and stirred until the blue colour is destroyed. Filter.</td></tr>
<tr><td>Acetone removes Naphthol Yellow S. Wash with saturated NaCl solution; add H_2O to the acetone, and drive off the acetone on steam bath. Acidify with acetic acid; shake with amyl alcohol, which removes any traces of Orange I present, and then test the acidified water solution for Naphthol Yellow S.</td><td>NaCl solution contains Light Green S F Yellowish. Remove excess of salt by evaporating and taking up with alcohol; drive off alcohol and test for Light Green S F Yellowish.</td><td>Acetic ether contains Orange I. Wash with saturated NaCl solution and then shake with H_2O, which takes out the Orange I.</td><td>Filtrate contains Indigo Disulpho Acid. It is treated with H_2O_2, which should give a deep blue color if Indigo Disulpho Acid is present.</td><td>The precipitate contains Ponceau 3R. It is washed with $CaCl_2$, solution until free from blue colour. Dissolve in dilute NH_4OH. The excess of NH_3 is then driven off.</td></tr>
<tr><td></td><td>Amaranth.</td><td>Erythrosin.</td><td>Naphthol Yellow S.</td><td>Light Green S F Yellowish.</td><td>Orange I.</td><td>Indigo Disulpho Acid.</td><td>Ponceau 3R.</td></tr>
</table>

acid with 2 to 3 c.c., 1 to 5 sulphuric acid and shaken in a separating funnel with several portions of amyl alcohol. It is then washed with 2 to 3 portions of 50 c.c., each of water acidified with 2 c.c. 1 to 5 H_2SO_4, then with pure distilled water. The colour returns to the aqueous layer, which is separated and tested for Naphthol Yellow S (10% sodium hydroxide solution gives yellow ppt.).

The reserved acid salt solution is concentrated on the water bath and the colour washed out with 95% ethyl alcohol. The alcohol is driven off and the solution tested for Light Green S. F. Yellowish (conc. sulphuric acid on dry colour-yellow changing to green on dilution: aqueous solution is decolourised by 10% sodium hydroxide with violet ppt.).

The precipitate left on filter after saturation with sodium chloride is dissolved in water, filtered and about 50 c.c. of 10% barium chloride solution added, allowed to stand 10 to 15 minutes and filtered. The filtrate is reserved.

The barium precipitate is boiled with 5% ammonia water and filtered, the ammonia driven off and residue tested for Ponceau 3R (conc. sulphuric acid on dry colour = cherry red, after dilution changing to orange).

The reserved filtrate is divided into two portions, one of which is treated with a small amount of zinc dust and sulphur dioxide gas until colourless, filtered, made acid with acetic acid and boiled. A blue colour indicates Indigo Disulpho Acid (conc. sulphuric acid on dry colour = dark blue, after dilution blue).

To the second portion ammonia water is added until sharply alkaline and boiled for 5 minutes, the ammonia driven off and residue tested for Amaranth (conc. sulphuric acid on dry colour = purple violet; after dilution magenta changing to crimson).

Extracts of Meat.

A variety of preparations occur in commerce, under the titles of meat-extract, fluid beef, beef-juice, etc. These articles, while useful and valuable in their way, do not justify the extravagant claims made respecting certain of them.

Meat extracts, meat peptones, gelatin, etc., are defined as follows by the Committee on Food Standards of the Association of Official Agricultural Chemists (October, 1907):

1. Meat extract is the product obtained by extracting fresh meat with boiling water and concentrating the liquid portion by evaporation after the removal of fat, and contains not less than 75% of total solids,

of which not over 27% is ash, and not over 12% is sodium chloride (calculated from the total chlorine present), not over 0.6% is fat, and not less than 8% is nitrogen. The nitrogenous compounds contain not less than 40% of meat bases and not less than 10% of creatine and creatinine.

2. Fluid meat extract is identical with meat extract except that it is concentrated to a lower degree, and contains not more than 75 and not less than 50% of total solids.

3. Bone extract is the product obtained by extracting clean, fresh, trimmed bones of animals in good health at the time of slaughter with boiling water and concentrating the liquid portion by evaporation, after removal of fat, and contains not less than 75% of total solids.

4. Fluid bone extract is identical with bone extract except that it is concentrated to a lower degree, and contains not more than 75 and not less than 50% of total solids.

5. Meat juice is the fluid portion of muscle fibre, obtained by pressure or otherwise, and may be concentrated by evaporation at a temperature below the coagulating point of the soluble proteins. The solids contain not more than 15% of ash, not more than 2.5% of sodium chloride (calculated from the total chlorine present) not more than 4 nor less than 2% of phosphoric acid (P_2O_5), and not less than 12% of nitrogen. The nitrogenous substances contain not less than 35% of coagulable proteins and not more than 40% of meat bases.

6. Peptones are products prepared by the digestion of protein material by means of enzymes or otherwise, and contain not less than 90% of proteoses and peptones.

7. Gelatin (edible gelatin) is the purified, dried, inodorous product of the hydrolysis, by treatment with boiling water, of certain tissues, as skin, ligaments, and bones, from sound animals, and contains not more than 2% of ash and not less than 15% of nitrogen.

The following are the requisites for a meat-extract given by Liebig.[1]

1. A good extract should contain no albumin and no fat (the latter not above 1.5%).

2. The water content should not exceed 21%.

3. About 60% should be soluble in 80% alcohol.

4. The nitrogen content should be from 8.5 to 9.5%.

5. The ash should vary from 15 to 25 %, which, besides a little sodium chloride, consists principally of phosphates.

[1] Röttger, Lehrbuch der Nahrungsmittel-Chemie, p. 135 (Leipzig, 1907).

Liebig's Extractum Carnis.—The oldest preparation of the nature of a meat-extract was that of Justus von Liebig.[1] According to the original directions, the extract was to be prepared by treating lean beef (chopped fine) with 8 times its weight of cold water, straining from the insoluble fibrous matter, heating the liquid to a temperature sufficient to coagulate the dissolved albumin, filtering, and evaporating the filtrate to a syrupy condition. It is evident that both protein and gelatinoid substances are excluded from an extract prepared in the cold in the above manner. But this method of preparation was admitted by Liebig to be impracticable on a manufacturing scale, and in 1865 he stated that the only available plan was to mix the chopped flesh with water free from gypsum, and to raise the temperature of the mixture to 82° (*Pharm. Jour.*, [*iii*], **13**, 414).

In the following passage, actual boiling with water is recommended by Liebig: "Those who may feel inclined to prepare extract of meat as an article of commerce, will entirely miss their aim, unless they most carefully and conscientiously seek to avoid the errors of those who have hitherto attempted it. Half an hour's boiling of the chopped meat with 8 or 10 times its weight of water suffices to dissolve all the active ingredients. The decoction must, before it is evaporated, be most carefully cleansed from all fat (which would become rancid), and the evaporation must be conducted in the water-bath. True extract of meat is never hard and brittle, but soft, and it strongly attracts moisture from the atmosphere" (Liebig's *Letters on Chemistry*).

Liebig himself has stated that 34 pounds of meat are required to produce 1 pound of extract, a fact which shows how completely the real nutritive portion of the meat must be excluded. In short, an extract of meat prepared according to Liebig's original directions is practically free from albumin, gelatin, and fat, and may be said to comprise the saline and extractive matters of the meat.[2] Among these constituents, creatinine, lactic acid, phosphates, and potassium salts occupy a prominent position. The true nature and value of Liebig's extract is now becoming generally recognised. Though not strictly of alimentary value, it possesses marked stimulant and restorative properties, which render it useful in exhausted states of the system.

[1] Extract of meat was first described by Proust, in 1801, but the method of manufacturing it on a commercial scale is due to Liebig, and was described by him as early as 1847. The Liebig's Extract of Meat Company was established in 1865, but the article itself has been extensively made and sold under the designation of Liebig's Extract since the year 1856.

[2] Various recent analyses of the extract of meat manufactured by the Liebig Company show that the commercial preparation contains material quantities of gelatinoid substances and soluble, non-coagulable proteins.

Like tea and coffee, it is a food-adjunct rather than a true food.[1] Being rich in the flavouring matter of cooked meat ("osmazome"), Liebig's extract is often used for flavouring soups.[2]

Druitt (*Trans. Obstetr. Soc.*, 1861, p. 143), in describing the characters of a liquid essence of beef which had been prepared according to his instructions, states that it exerted a rapid and remarkable stimulating action on the brain, and proposed it as an auxiliary to, and partial substitute for, brandy in all cases of great exhaustion or weakness attended with cerebral depression or despondency. Similar stimulating effects have been observed as resulting from a copious employment of Liebig's extract. The effect of a feast of animal food on savages whose customary diet was almost exclusively vegetable has been observed to be similar to the administration of an intoxicating spirit or drug.

Commercial Meat-extracts.—A great number of preparations having the general nature of Liebig's extract of meat are now articles of commerce.[3] Some of these have received additions of gelatin, blood-albumin, meat-fibre, etc., while in certain cases the albumin has been more or less peptonised.[4] It is claimed on behalf of these preparations

[1] In a letter to *The Times* (October 1, 1872) Liebig wrote: "Neither tea nor extract of meat is nutriment in the ordinary sense; they possess a far higher importance by certain medicinal properties of a peculiar kind. The physician does not employ them as specific remedies. They serve the healthy man for the preservation of his health. Taken in proper proportions they strengthen the internal resistance of the body to the most various external injurious influences, which combine to disturb the general vital processes, and adjust these latter. . . . It is surely a grave offence against all the laws of physiology to compare tea, coffee, and extract of meat with the more common articles of food, and, because they are not that, to draw the inference, as Dr. Edward Smith has done, that they are nothing at all . . . Extract of meat is beef-tea made from fresh meat—not roasted—in the purest state, condensed to the consistency of a thick honey, to which nothing whatever is added by the manufacturer. The assertion that common salt is added to the extract is an unjustifiable invention. . . . The necessity for the consumption of meat is considerably lessened when extract of meat is added to the vegetable food; in addition to the nutritive value which vegetables possess in themselves, they acquire in the soluble component parts of meat those substances which give a meat-diet its peculiar effect."

[2] Kemmerich failed to keep animals alive on a diet of meat-extract, and the urine contained an abnormal proportion of carbon. It is not clear, however, that sufficient extract was ingested to correspond to ordinary food in the carbon and nitrogen content. M. Rubner found that the urine of dogs fed on Liebig's preparation acquired on concentration the peculiar odour of meat-extract. He concludes that the meat-extract does not contribute to the bodily heat, that the waste of tissue is neither hastened nor retarded by it, and that it passes away unaltered in composition (abst. *J. Chem. Soc.*, 1885, 409).

[3] The term "Liebig's extract" has now a wide significance and has been decided by High Court of Justice to be public property. Hence, it does not always imply an article manufactured by the Liebig's Extract of Meat Company.

[4] An interesting light on the methods of manufacturing meat-extracts and pseudo-peptones is afforded by the following process, which forms the subject of a patent by Etienne and Delhaye (1890, No. 19, 961). After removing the tendons and grease, the meat is chopped and mashed, mixed with about half its weight of water, and heated by steam under pressure for 1 hour to a temperature ranging between 150° and 175°. A portion of the albuminoid matter is rendered soluble, and goes into solution with the extractives. The residue, when pressed, forms a friable mass amounting to about one-third of the fresh meat used. This residue is treated on the water-bath with an equal weight of concentrated hydrochloric acid, until the fibro-muscular tissue is quite disintegrated and decomposed, when the liquid is filtered. The insoluble residue is sold as manure. The liquors are neutralised with sodium carbonate, and then contain "peptone" and sodium chloride in solution. If pure "peptone" is required, the liquid is decolourised with animal charcoal, and dialysed to remove the salts; but if only a meat-extract is required, the liquors from the steam treatment of the meat and the neutralised liquors from the acid treatment are mixed and evaporated in a vacuum until sufficiently concentrated.

that the various additions and methods of treatment give them value as real foods, but this is true in but a very limited sense, since the amount of such preparations which would require to be taken to furnish the carbon and nitrogen requisite to support life is enormously beyond the quantity of any of the preparations which could be consumed without upsetting the system, to say nothing of the extravagant cost of all such preparations if used in quantity necessary to sustain life.[1]

It appears, therefore, that meat-extracts have a true value as stimulants and restoratives, the proportions of meat-bases, extractives, and salts present being an index of their value in this respect. On the other hand, all attempts to give them the characters of true nutritive concentrated foods can meet with but a very limited success.

A failure to appreciate these facts has caused very delusive values to be placed on such preparations, and the errors have been further magnified by the discordant methods of judging of the value of such articles. Thus Stutzer has expressed the opinion that the albumoses and peptones are the only constituents of value in a meat-extract, and he ignores any meat-fibre, gelatin, or coagulable albumin which may be present. Another well-known analyst regards the matters precipitated by alcohol as being the only constituents of value; but such a contention is clearly untenable, since the precipitate formed by alcohol contains a variable but very considerable percentage of non-nitrogenous extractives and salts.[2]

In the opinion of Allen, the following are the chief considerations on which a judgment should be formed of the value of a meat-extract:

The percentage of *water* should first be taken into account. Thus a preparation which contains only 10% of solid matters must evidently have less than half the food-value of the meat from which it is derived; and it might happen that, exclusive of the meat-bases (valuable merely as stimulants) and the gelatin (of questionable nutritive value), such a preparation contained a smaller proportion of nitrogenised organic matter than is present in ordinary beer.

[1] In judging of the amount of credence to be attached to statements or the nutritive value and concentration of meat-extracts and similar preparations, it should be borne in mind that fresh lean meat contains about 20% of nutritive matter and 75% of water. Hence by the desiccation of 4 pounds weight there will be obtained 1 pound of dry substance, of which 80% is nutritive protein matter, the remaining 20% consisting of fat, meat-bases, salts, etc. By no possible means can further material concentration of the nutritive matter be effected. Statements that meat-extracts, meat-essences, fluid meats, etc., contain the nutritive matter of 30, 40, or 50 times their weight of fresh meat are injustifiable. Preparations still containing nearly half their weight of water, but of which a table-spoonful is said to be equal in nutritive value to a full meal of fresh lean meat, and meat-lozenges and tablets weighing less than a grm. 1 or 2 of which are alleged to suffice for a meal, are evidently quite inefficient for their pretended purpose as concentrated forms of food.

[2] On treating an aqueous solution of Liebig's extract of meat with excess of strong alcohol, Allen obtained a precipitate weighing 31.8% of the original extract, and containing 11.7% of ash. The nitrogen in the precipitate corresponds to 10.6% of proteins, leaving 9.5 for non-nitrogenous extractive matters.

It is usual in analyses of meat-extracts to state the whole of the *chlorine* in terms of sodium chloride. This convention is not scientifically accurate, since the chlorine derived from the meat exists chiefly, if not entirely, in the form of potassium chloride, the balance being as sodium chloride, added in the form of common salt. Making an allowance of, say, 0.06% of sodium chloride for every unit % of dry solid matter present in the preparation, any excess may be fairly regarded as having been added in the form of *common salt.* Thus, if a meat-extract contain 25% of water (=75% of solids) and 10% of chlorine in terms of sodium chloride, the allowance for natural chlorides would be 4.50% (=75×.06); and 5.50, that is, the difference between this figure and 10.00, will represent the added common salt of the sample. Added salt should, of course, be deducted in estimating the effective concentration of the preparation.

The *bases* are among the most important of the natural constituents of meat-extracts, but unfortunately the existing methods for their estimation are far from satisfactory. The amount of meat-bases in a preparation is often deduced from the percentage of nitrogen over and above that found to exist in other forms. Apart from the errors attendant on this indirect method of estimation, it is difficult to fix on a suitable factor for calculating the actual amount of meat-bases from the nitrogen ascribed to that form of combination. Stutzer adopts the factor 3.12, which would be correct if the bases were wholly creatine. Hehner prefers to use the albumin-factor (6.25) for all nitrogenous constituents of meat-extracts for convenience of comparison, with the knowledge that it is too high in the case of the meat-bases, but he points out that by adopting it the figure obtained (by difference) for the non-nitrogenised extractive matters is much lower and probably a better approximation to the truth than when Stutzer's factor is employed. Still, with the exception of leucine, tyrosine, and carnine, the factors for calculating the nitrogen to the bases are all lower than that for creatine.[1]

[1]The following are the factors corresponding to the chief bases, etc., of muscle:

Substance	Formula	Proportion of nitrogen	Factor
Creatine	$C_4H_9O_2N_3$.	42 in 131	3.12
Creatinine	$C_4H_7ON_3$.	42 in 113	2.69
Xanthine	$C_5H_4O_2N_4$.	56 in 152	2.71
Xanthocreatinine	$C_5H_{10}ON_4$.	56 in 142	2.54
Hypoxanthine	$C_5H_4ON_4$.	56 in 137	2.44
Carnine	$C_7H_8O_3N_4$.	56 in 196	3.50
Leucine	$C_6H_{13}O_2N$.	14 in 131	9.36
Tyrosine	$C_9H_{11}O_3N$.	14 in 181	12.93
Urea	CH_4ON_2.	28 in 60	2.14
Uric acid	$C_5H_4O_3N_4$.	56 in 168	3.00

The added *albumin* and *meat-fibre* present in some commercial meat-extracts have, of course, a true food-value, but the amount of these constituents present in such a quantity of a meat-extract as is usually, or could be, taken at a time is too insignificant to give it any appreciable value as nutriment.

The same remark applies to *gelatin*, which is present in some preparations as a product of the hot water or superheated steam employed for the extraction, and in other cases has been added as such. In fact, that gelatin has any value as a food is open to question, and has been the subject of much dispute.

The *albumoses* and *peptones* present in some meat-extracts have the advantage of being readily assimilated, and so far as they go are desirable constituents of such preparations. It is, however, almost certainly the fact that the proportion of the peptones has been greatly overestimated, and that some preparations in which certain analyses show a material proportion of peptones are in reality almost, if not entirely, devoid of such constituents.

Manufacture of Commercial Meat-extracts.—Bigelow and Cook (*U. S. Dept. Agric. Bur. Chem., Bull.* 114, 1908) describe the manufacture of meat-extracts in the larger establishments as follows:

"Up to a few years ago the soup liquor obtained from meat which was parboiled in the process of preparing canned meat was entirely wasted, but this liquor is now extensively utilised in the manufacture of extracts and preparations of meat. In preparing canned meat pieces of meat are placed in iron baskets which are suspended in large tanks containing cold water. Steam is admitted and the meat heated about 1/2 hour (30 to 40 minutes). The liquor, which is the source of meat-extracts, is pumped into triple-effect vacuum pans and heated at 71° for about 4 hours. Then the solution is transferred to a single-effect finishing kettle and heated 8 hours until the water content approximates 22%.

"A first-grade extract of beef is prepared from beef alone and is usually sold in jars. An extract of the trimmed bones, to which considerable meat adheres, is also made. The trimmings include odds and ends of meat, muscle tissue, bone, etc., and the product is a second-grade article. In preparing this extract the bones are heated, not boiled, for 30 to 40 minutes, and the liquor evaporated to the consistency of extract. The extract prepared from corned beef liquor constitutes another second-grade product. This extract has a high content of nitrates and sodium chloride. In addition there is an extract prepared from pork and other meats, sold under the general term of meat-extract.

Mixtures of the various meat and bone extracts are often made. A fluid meat-extract is usually a 50% solution of a solid extract.

"Assuming that beef extract contains 21.7% of water, there is obtained from 100 pounds of "soup liquor" 1.94 pounds of commercial meat-extract. These figures are high, as they are calculated from the total solids present in soup liquor. The manufacturers claim that 100 pounds of "soup liquor" will yield 1 pound of meat-extract."

The cured-meat-extract made in the larger establishments from liquors in which corned-beef is parboiled previous to canning naturally contains much salt as well as saltpetre and sugar. The salt content is reduced to conform to the accepted standards for meat-extract in the evaporators and also by means of centrifugal machines, and the extract when finished conforms to the standards in the other respects also.

Composition of Commercial Meat-extracts.[1]—Numerous analy-

[1] The following list of papers, treating of meat-extracts and commercial peptones, was compiled at Allen's request by A. R. Tankard and others. Many of the references are to abstracts of foreign papers in English journals, since these are more readily accessible than the originals.

Year	Author	Reference	Remarks
1880	A. Stutzer.....	*J. Chem. Soc.*, 38, 676	Estimation of "protein compounds" by cupric hydroxide.
1880	M. Rubner..... ...	*J. C. S.*, 38, 904; 40, 451.	Nutritive value of fluid meat.
1881	C. Estcourt.....	*Analyst*, 6, 201.	Composition of meat-extracts.
1881	S. Darby.....	*J. C. S.*, 40, 450.	Fluid meat.
1881	T. Defresne.....	*Pharm. Jour.*, [3], 12, 8.	Estimation of peptones.
1881	C. Tanret.....	*J. C. S.*, 40, 832.	Character of peptones.
1882	A. Stutzer...	*J. C. S.*, 42, 1239.	Precipitation of proteins by cupric hydroxide.
1882	A. H. Chester.....	*Analyst*, 7, 124.	Composition of various meat-extracts.
1882	Justice Field (Judgment).	*Pharm. Jour.*, [3], 13, 412.	Liebig Company *v.* Anderson.
1885	O. Hehner.....	*Analyst*, 10, 221.	Analyses of beef-tea.
1885	F. Szymanski.....	*J. C. S.*, 48, 822.	Characters of peptone.
1885–6	A. Stutzer.....	*Analyst*, 10, 57, 73; *J. S. C. I.*, 5, 37.	Analysis and composition of various meat-extracts.
1886	H. Weiske.....	*J. C. S.*, 50, 1087.	Peptones are not precipitated by cupric hydroxide.
1886	Kühne & Chittenden	*J. C. S.*, 50, 818.	Estimation of albumose and peptone.
1886	S. H. C. Martin... .	*J. C. S.*, 50, 636.	Separation of peptones from other proteins by ammonium sulphate.
1888	E. Schumacher-Kopp.	*J. S. C. I.*, 7, 130.	Analyses of Maggi's meat preparations.
1888	J. Kōnig.....	*J. S. C. I.*, 7, 449.	Valuation of peptones.
1889	Kōnig & Kisch.....	*J. C. S.*, 56, 803.	Estimation of albumose and peptone.
1890	G. Bruylants.....	*J. C. S.*, 58, 1351.	Analysis of peptones.
1890	A. Denaeyer.....	*J. C. S.*, 58, 1351; *Analyst*, 15, 101.	Analysis of peptones.
1891	A. Denaeyer.....	*Analyst*, 16, 98, 234	Analysis of peptones.
1891	Etienne & Delhaye..	English Patent, 10,961, 1890.	Improvements in preparation of peptonised soluble meat and peptone.
1892	Heaton & Vasey....	*Analyst*, 17, 28; *J. C. S.*, 62, ii. 1535	Analysis of peptones and review of literature.
1892–3	S. Riva-Rocci.....	*J. C. S.*, 62, ii. 1136; *Ch. News*. 67, 254.	Estimation of albumose and peptone in stomach contents.
1892–3	L. A. Hallopeau.....	*Ph. J.*, [3], 23, 181; *J. C. S.*, 64, ii, 104.	Estimation of peptones by precipitation with mercuric nitrate.

ses of meat-extracts and allied preparations have been published, but they have little value unless the exact method of analysis has been specified, and are useless for comparison, except where the various analyses of a series have been made by the same method. It must be borne in mind that preparations bearing the same names, and produced by the same firms at different dates, are liable to considerable variations in their character.

The following table p. 398 shows the composition of the chief commercial meat-extracts, according to results obtained in the years 1896 and 1897, by Otto Hehner. The analyses were made by Stutzer's method (page 420) except that in some of the later analyses the albumoses were precipitated by zinc sulphate instead of by ammonium sulphate. The peptones were precipitated by phosphotungstic acid.

Year	Author	Reference	Remarks
1893	A. Stutzer	*J. C. S.*, 64, ii. 146.	Estimation of nitrogenous constituents of commercial peptones.
1893	W. Kühne.....	*J. C. S.*, 64, i. 233.	Characters of albumoses and peptones.
1894	E. Kemmerich....	*J. C. S.*, 66, ii. 150.	Composition of South American meat-extract and meat-peptone.
1895	E. O. Beckmann....	*Analyst*, 20, 44; *J. C. S.*, 68, ii. 375.	Estimation of gelatin and albumin in peptone.
1895	L. Hugounenq.....	*Analyst*, 20, 94.	Analyses of adulterated peptones.
1895	A. Stutzer	*Analyst*, 20, 182; *J. C. S.*, 68, ii. 543	Composition of various meat-extracts.
1895	A. Stutzer,.....	*Analyst*, 20, 246; *J. S. C. I.*, 14, 897.	Nitrogenous constituents of meat-extracts and commercial peptones.
1895	—Dutto.....	*J. C. S.*, 68, ii. 468.	Assay of peptones by precipitation with potassiobismuth iodide.
1896	König & Bömer.....	*Analyst*, 21, 17; *J. C. S.*, 70, ii. 82.	Composition of various meat-extracts.
1896	A. Bömer..... ...	*Analyst*, 21, 16; *J. C. S.*, 70, ii. 83.	Precipitation of albumoses by zinc sulphate.
1896	A. Stutzer.....	*Analyst*, 21, 19; *J. C. S.*, 70, ii. 84.	Estimation of gelatin in meat-extracts and peptones.
1896	L. de Koningh.....	*J. C. S.*, 70, ii. 552.	Estimation of solids in beef-tea.
1897	G. Bruylants.....	*J. S. C. I.*, 16, 640.	Analysis of meat-extracts.
1897	A. Denaeyer.....	*Ph. J.*, [4], 1897, ii. 3.	Value of peptones.
1897	Rideal & Stewart...	*Analyst*, 22, 231.	Precipitation of proteins by chlorine.
1897	Allen & Searle.....	*Analyst*, 22, 258.	Precipitation of proteins by bromine.
1897	H. Schierning.....	*Zeits. anal. Chem.*, 1897, p. 643.	Precipitants of proteins.
1900	H. Schgirning.....	*Z. Anal. Chem.*, 1900, 39, 545.	Tannin—salt method.
1905	O. Folin.....	*Am. J. Physiol.*, 1905, 13, 48.	Estimation of creatinine.
1905	O. Folin.....	*Z. physiol. chem.*, 1904, 41, 223.	Estimation of creatinine.
1906	Bigelow and Cook...	*U. S. Dept. Agric. Bur. Chem. Bull.* 73.	Estimation of peptones.
1906	Bigelow and Cook...	*J. Am. Chem. Soc.* (1906), 28, 1485.	Estimation of peptones.
1907	Grindley and Woods.	*J. Biol. Chem.*, 1907, 2, 309.	Estimation of creatinine.
1907	Emmet and Grindley	*J. Biol. Chem.*, 1907, 2, 491.	Estimation of creatinine.
1908	Bigelow and Cook...	*U. S. Dept. Agric. Bur. Chem., Bull.* 114.	Composition of meat-extracts.
1909	A. Lowenstein	*J. Ind. Eng. Chem.* (1909), 1, 252.	Estimation of mocatine in meat-extracts.

ANALYSES OF CHIEF COMMERCIAL MEAT EXTRACTS (HEHNER).

Number	Description	Water	Fat (petroleum-ether) extract	Gelatin	Albumin	Meat-fibre and coagulated albumin	Albumoses	Peptones	Meat-bases	Ash	Difference	Sodium chloride	Phosphoric acid	Total nitrogen
1	Liebig Company's *Extractum Carnis*.	15.26	0.34	5.18	.....	2.12	2.01	8.06	39.32	23.51	4.20	5.81	6.97	9.07
2	Armour's Extract of Meat....	15.97	0.21	3.31	.....	.....	1.75	5.13	41.12	29.36	3.15	9.74	6.76	8.21
3	Brand & Co.'s *Extractum Carnis*.	17.85	0.38	4.56	.....	1.81	4.19	10.16	38.90	18.80	2.87	3.31	5.16	9.80
4	Liebig's Extract (Bovril & Co.'s make).	22.24	0.29	5.50	.....	1.30	3.62	8.44	38.58	20.45	—0.42	5.14	5.50	9.19
5	Brand & Co.'s Meat-juice	55.48	0.10	0.69	1.00	.....	1.06	2.50	12.50	11.06	15.61 Glycerin	4.43	1.52	2.84
6	Valentine's Meat-juice.....	55.53	0.10	0.75	0.25	.....	2.00	2.87	12.48	12.01	14.01	2.35	2.85	2.92
7	Wyeth's Meat-juice.....	61.61	0.08	1.12	5.62	.....	1.08	1.86	9.44	14.78	4.41	6.96	3.01	3.06
8	Borthwick's Bouillon.....	36.19	0.25	1.37	.....	4.00	1.16	11.09	24.25	17.93	3.76	6.09	3.58	6.70
9	Vitalia Meat-juice.....	70.19	0.32	0.45	16.44	0.37	0.05	0.37	2.82	6.65	2.34	5.11	0.37	3.28
10	Brand & Co.'s Essence of Beef	89.68	0.06	5.12	.....	.....	0.19	0.57	3.43	1.00	—0.05	0.33	0.40	1.49
11	Bovril Company's Fluid Beef	28.34	1.02	3.81	.....	5.37	8.38	13.18	19.38	17.67	2.85	9.07	4.05	8.02
12	Bovril Fluid Beef (unseasoned	44.75	0.62	1.06	.....	7.31	2.38	6.25	17.12	19.90	0.61	11.42	3.34	5.46
13	Bovril for Invalids.....	24.34	1.07	4.56	.....	5.87	5.56	6.44	34.07	16.50	1.59	5.23	3.35	9.20
14	Bovril for Invalids.....	17.47	0.51	2.56	4.43	15.25	1.06	8.82	31.89	16.30	1.91	2.46	1.43	10.21
15	Caffyn's *Liquor Carnis*.....	48.46	0.11	0.25	2.19	0.94	3.65	0.98	11.30	9.95	22.17 Glycerin	4.43	0.62	3.09
16	Extract of Meat with Vegetable Extract.	30.03	0.10	1.69	6.12	.....	1.74	4.85	16.97	23.47	15.05 Carbohydrate	11.56	3.02	5.02

The various nitrogenised matters were in all cases calculated from the nitrogen found by the factor 6.25 (compare page 394). As all the analyses and calculations were made by the same method, the results are comparable.

The following table shows the amounts of nitrogen existing in different forms in the foregoing preparations:

Number	Description	Nitrogen existing as						
		Gelatin	Albumin	Meat-fibre and coagulated albumin	Albumoses	Peptones	Meat-bases	Total nitrogen
1	Liebig's *Extractum Carnis*....	0.83	.....	0.34	0.32	1.29	6.29	9.07
2	Armour's Extract of Meat....	0.53	.. ...	.....	0.28	0.82	6.58	8.21
3	Brand & Co.'s *Extractum Carnis*	0.73	.....	0.29	0.67	1.69	6.42	9.80
4	Liebig's Extract (Bovril Co.'s make).	0.88	.....	0.21	0.58	1.35	6.17	9.19
5	Brand & Co.'s Meat-juice	0.11	0.16	.....	0.17	0.40	2.00	2.84
6	Valentine's Meat-juice... ...	0.12	0.04	.....	0.32	0.46	1.98	2.92
7	Wyeth's Meat-juice.....	0.18	0.90	.....	0.17	0.30	1.51	3.06
8	Borthwick's Bouillon.....	0.22	.....	0.64	0.19	1.77	3.88	6.70
9	Vitalia Meat-juice.....	0.07	2.63	0.06	0.01	0.06	0.43	3.28
10	Brand & Co.'s Essence of Beef	0.82	.....	.....	0.03	0.09	0.55	1.49
11	Bovril Fluid Beef.....	0.61	.. ...	0.86	1.34	2.11	3.10	8.02
12	Bovril Fluid Beef (unseasoned)	0.17	... ..	1.17	0.38	1.00	2.74	5.46
13	Bovril for Invalids.....	0.73	.....	0.94	0.89	1.03	5.61	9.20
14	Bovril for Invalids.....	0.41	0.71	2.44	0.17	1.41	5.07	10.21
15	Caffyn's *Liquor Carnis*.....	0.04	0.35	0.15	0.58	0.16	1.81	3.06
16	Extract of Meat and Vegetables.	0.27	0.98	.....	0.28	0.77	2.72	5.02

The foregoing preparations may be roughly classified as: concentrated meat-extracts, represented by analyses 1 to 4 on the table; articles of the bouillon and "meat-juice" class, represented by Nos. 5 to 10; and preparations which have received an addition in material quantity of a substance not naturally a constituent of a meat-extract. Thus, the finished preparations of the Bovril Company contain a variable percentage of finely divided meat-fibre and sometimes added albumin. "Bovril" is stated to contain "the entire nourishment of prime ox-beef." "Invalid Bovril" "differs from ordinary bovril in being more concentrated and quite devoid of seasoning," and is described as "the most perfect form of concentrated nourishment at present known."

The following analyses of Bovril preparations are by A. Stutzer (abst. *Analyst*, 1895, p. 182).

	Bovril fluid beef	Bovril fluid beef (seasoned)	Bovril for invalids	Bovril beef-jelly	Bovril lozenges
	%	%	%	%	%
Water	29.14	44.42	28.13	89.15	9.47
Sodium chloride	14.12	10.72	4.57	0.26	1.63
Other salts	3.38	7.60	11.50	1.04	5.71
Organic matter	53.36	37.26	55.80	9.55	83.19
Total nitrogen	8.25	5.12	8.69	1.46	11.94
Meat-fibre nitrogen	0.73	0.90	0.70	.....	0.57
Gelatin nitrogen	0.09	0.09	0.15	0.29	0.70

Samples 15 and 16 are other examples of meat-extracts to which additions have been made.

The following are additional published analyses of preparations which appear in the table on page 398. The analyses are somewhat wanting in detail, but are of interest as confirmations of the general character of the articles in question, and as illustrating their variation from time to time.

	Valentine's meat-juice			Wyeth's beef-juice		Brand & Co.'s essence of beef		
	R. R. Tatlock	R. H. Chittenden, 1891	O. Hehner 1893	R. R. Tatlock	R. H. Chittenden, 1891	A. Dupré, 1886	R. R. Tatlock	O. Hehner, 1893
Water	51.40	60.31	55.24	56.13	57.88	89.45	90.48	91.23
Ether extract	0.04	0.78	4.80	Trace.	0.85	Trace.	Trace.	0.18
Gelatin and albumin	7.66	0.55 (Albumin.)	0.93	11.16	0.47 (Albumin.)	5.88	4.83	3.79
Peptone		None	1.55		None			
Creatine and meat extractives	18.56	29.15	18.27	10.04	35.08	4.49	4.98	3.96
Non-nitrogenous extractives	11.96		8.08	7.23			0.47	None.
Sodium chloride	10.38	11.30	4.62	15.44	17.52	1.25	1.24	0.45
Other mineral matters			8.51					0.39
Containing P_2O_5	.... ..	4.00	.....	.....	3.94	0.59	.....	.....

Tatlock's and Dupré's analyses were probably made by some modification of the alcohol process (page 420). The figures of R. H. Chittenden are from his address to the Philadelphia County Medical Association (May, 1891). The results show generally that the preparations contain a large % of water, and, though of value as stimulants or food-adjuncts, that they cannot be regarded as concentrated forms of nutrient food.

The "Perfected Wyeth Beef-juice" is stated by the manufacturers to contain "not only the hæmoglobin but also the valuable nutritive

albuminous elements of beef active and unchanged. It is carried to a very high degree of concentration, each tablespoonful containing the nutrient and stimulating principles of three-quarters of a pound of fresh lean beef. It contains many times more pure serum-albumen than any of the ordinary preparations of this class, and it does not owe any of its nitrogenous material to added egg-albumen." An analysis quoted by the manufacturers states the preparation to contain: Moisture, 44.87%; organic matter (including 4.57 of nitrogen), 38.01% and mineral matter, 17.12%.

Among the more recent analyses of meat-extracts and similar preparations, those by Bigelow and Cook[1] are important, and are reproduced in the following tables. These analyses represent samples on the market in the winter of 1905–1906.

Analysis of Meat-extracts, Commercial Peptones, Etc.—The complete analysis of extracts of meat and allied preparations is both difficult and tedious, and in some respects cannot be effected satisfactorily by existing methods. The following processes are those which, in the experience of the writer, are the most satisfactory for their intended purpose.

Water and *Total Solid Matters* in meat-extracts may be estimated by evaporating a known weight of the sample to dryness at 100° and drying the residue till constant preferably in the vacuum oven. From 3 to 25 grm. should be employed, according to the nature of the preparation. Stutzer weighs the quantity intended for the estimation of water into a thin basin of tin-foil (about 20 mm. high and 55 mm. in diameter), dissolves it in a little hot water, and adds sand (previously ignited and freed from fine dust by a sieve) in sufficient quantity to absorb the liquid almost completely. The basin is then heated in the water-oven until the weight is constant. The weight of the tin-foil and sand being deducted, the *solid matter* of the extract is obtained. The tin basin and its contents are then used by Stutzer for the determination of the gelatin (page 415).

In the case of samples containing gelatin, or which from other circumstances cannot be readily dried, L. de Koningh treats the preparation with a weighed quantity of tannin containing a known amount of dry matter. The mixture is evaporated and dried in the water-oven till constant, when the weight of dry tannin is deducted from the residue obtained.

A method for estimating moisture in products of a viscous or semi-solid consistency, by the use of alcohol, which is applicable to meat-

[1] U. S. Dept. Agr. Bur. Chem., Bull. 114, 1908.

ANALYSIS OF SOLID MEAT-EXTRACTS BY BIGELOW AND COOK.

Maker	Mineral constituents						Acidity		Nitrogen as—									Ether extract	Undetermined	Sample	
	Moisture	Total ash	Chlorine as sodium chloride in ash	Total phosphoric acid	Organic phosphoric acid	Inorganic phosphoric acid	N/10 sodium hydroxide	As lactic acid	Total nitrogen	Insoluble and coagulable	Proteoses	Peptones	Total meat bases	Kreatine and kreatinine	Xanthine bases	Meat bases other than kreatine and xanthine	Ammonia			Net weight	Price
	%	%	%	%	%	%	*cc per grm.*	%	%	%	%	%	%	%	%	%	%	%	%	*Grm.*	*C.*
Cudahy Pkg. Co.	26.50	24.06	8.54	2.29	0.35	1.94	6.67	6.01	7.30	0.32	1.65	1.57	3.56	0.87	0.38	2.31	0.20	1.30	8.66	55.8	45
Liebig Co.	21.14	21.03	3.11	2.40	.61	1.79	9.04	8.13	9.07	.19	2.01	2.68	3.82	1.14	.03	2.65	.37	.94	5.89	57.8	45
Armour and Co.	21.66	20.46	5.47	4.55	.49	4.06	9.35	8.42	7.66	.48	2.02	1.90	3.05	.75	.04	2.26	.21	.50	11.67	45.7	40
Libby McNeal and Libby.	21.86	30.92	18.32	2.53	.24	2.29	5.72	5.15	6.02	.29	.77	1.33	3.20	1.01	.11	2.08	.43	.53	16.11	131.4	53
Swift and Co.	20.16	27.28	13.51	2.89	.18	2.71	4.61	4.15	6.60	.35	1.02	1.09	3.43	.81	.45	2.17	.71	.43	21.04	115.2	57
Hammond Co.	12.39	31.68	13.25	3.19	.21	2.98	7.16	6.44	6.86	.06	.86	1.48	4.21	1.24	.52	2.45	.25	.43	20.61	119.9	60

NITROGENOUS CONSTITUENTS OF SOLID MEAT-EXTRACTS (CALCULATED FROM ABOVE).

Maker	Nitrogenous substances									Nitrogenous substances expressed in terms of total nitrogen							
	Total proteins *a*	Insoluble and coagulable protein	Proteoses	Peptones	Total meat bases	Kreatine and kreatinine	Xanthine bases	Meat bases other than kreatine, kreatinine, and xanthine	Ammonia	Insoluble and coagulable protein	Proteoses	Peptones	Total meat bases	Kreatine and kreatinine	Xanthine bases	Meat bases other than kreatine, kreatinine, and xanthine	Ammonia
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Cudahy Pkg. Co.	22.12	2.00	10.31	9.81	11.11	2.71	1.03	7.21	0.24	4.38	22.60	21.51	48.77	11.92	5.21	31.64	2.74
Liebig Co.	30.50	1.19	12.56	16.75	11.92	3.56	.08	8.27	.45	2.09	22.16	29.55	42.12	12.57	.33	29.22	4.08
Armour and Co.	27.51	3.00	12.63	11.88	9.52	2.34	.11	7.05	.26	6.27	26.37	24.80	39.82	9.79	.52	29.50	2.74
Libby McNeal and Libby	14.93	1.81	4.81	8.31	9.98	3.15	.30	6.49	.52	4.82	12.79	22.09	53.16	16.78	1.83	34.55	7.14
Swift and Co.	15.38	2.19	6.38	6.81	10.70	2.53	1.22	6.77	.86	5.30	15.45	16.52	51.97	12.27	6.82	32.88	10.76
Hammond Co.	15.01	.38	5.38	9.25	13.14	3.87	1.41	7.64	.30	.87	12.54	21.57	61.37	18.08	7.58	35.71	3.64

a The sum of insoluble and coagulable proteins, proteoses, and peptones.

ANALYSIS OF FLUID MEAT-EXTRACTS BY BIGELOW AND COOK.

Maker	Mineral constituents						Acidity		Nitrogen as—									Ether extract	Undetermined	Sample	
	Moisture	Total ash	Chlorine as sodium chloride in ash	Total phosphoric acid	Organic phosphoric acid	Inorganic phosphoric acid	*N*/10 sodium hydroxide	As lactic acid	Total nitrogen	Insoluble and coagulable protein	Proteoses	Peptones	Total meat bases	Kreatine and kreatinine	Xanthine bases	Meat bases other than kreatine and xanthine	Ammonia			Net weight	Price
	%	%	%	%	%	%	*cc per gram.*	%	%	%	%	%	%	%	%	%	%	%	%	*Grms.*	*Cents.*
Armour and Co.	57.75	17.23	8.27	2.32	0.26	2.06	3.46	3.11	2.85	0.04	0.34	0.70	1.66	0.38	0.23	1.05	0.11	0.09	9.75	105.7	40
John Wyeth and Bro	58.84	16.21	6.71	3.27	.04	3.23	4.35	3.92	3.15	*a*.46	.10	.47	1.92	.26	.26	1.40	.20	.23	8.12	68.6	50
Valentine Co.	57.64	10.26	1.77	3.41	.45	2.96	5.04	4.53	3.06	.03	.10	.77	1.94	.35	.22	1.37	.22	.50	15.12	69.7	75
Armour and Co.	49.94	15.91	7.02	3.29	.46	2.83	5.29	4.76	3.87	*a*.29	.69	.74	2.02	.48	.17	1.37	.13	.04	12.14	63.5	35
Cudahy Pkg. Co.	55.99	16.99	8.48	2.48	.38	2.10	5.51	4.92	3.95	*b*.17	.54	.41	2.63	.80	.04	1.79	.20	.05	6.60	73.7	35
Cibild Co.	64.63	16.13	11.38	.95	.14	.81	2.70	2.43	3.18	*c*.31	.44	.89	1.36	.50	.09	.77	.18	.06	2.04	102.2	45
Mosquera–Julia Food Co.	68.97	13.85	10.05	.80	.18	.62	2.45	2.20	2.41	*a*.08	.31	.91	.98	.26	.08	.64	.13	.09	3.54	127.4	50

a All coagulable. *b* Largely insoluble. *c* Partly insoluble.

NITROGENOUS CONSTITUENTS OF FLUID MEAT-EXTRACTS (CALCULATED FROM ABOVE).

Maker	Nitrogeneous bodies								Nitrogenous bodies expressed in terms of total nitrogen								
	Total proteins *a*	Insoluble and coagulable proteins	Proteoses	Peptones	Total meat bases	Kreatine and kreatinine	Xanthine bases	Meat bases other than kreatine, kreatinin, and Xanthine	Ammonia	Insoluble and coagulable protein	Proteoses	Peptones	Total meat bases	Kreatine and kreatinine	Xanthine bases	Meat bases other than kreatine, kreatinin, and xanthine	Ammonia
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Armour and Co.	6.76	0.25	2.13	4.38	5.18	1.19	0.62	3.28	0.13	1.40	11.93	24.56	58.25	13.33	8.07	36.84	3.86
John Wyeth and Bro.	6.45	2.88	.63	2.94	5.99	.81	.71	4.37	.24	14.60	3.17	14.92	60.95	8.25	8.25	44.44	6.35
Valentine Co.	5.63	.19	.63	4.81	6.05	1.09	.60	4.27	.27	.98	3.27	25.16	63.40	11.44	7.19	44.47	7.19
Armour and Co.	10.75	1.81	4.31	4.63	6.30	1.50	.46	4.27	.16	7.49	17.83	19.12	52.20	12.40	4.39	35.40	3.36
Cudahy Pkg. Co.	7.00	1.06	3.38	2.56	8.21	2.50	.11	5.58	.24	4.30	13.67	10.38	66.58	20.25	1.01	45.32	5.06
Cibild Co.	10.25	1.94	2.75	5.56	4.24	1.56	.24	2.40	.22	9.75	13.84	27.99	42.77	15.72	2.83	24.21	5.66
Mosquera–Julia Food Co.	8.13	.50	1.94	5.69	3.06	.81	.22	2.00	.16	3.32	12.86	37.76	40.66	10.79	3.32	26.56	5.39

a The sum of insoluble and coagulable proteids, proteoses, and peptones.

MEAT JUICES PREPARED IN LABORATORY (BIGELOW AND COOK.

Serial No.	Preparation of juice	Composition of sample					
		Water in juice	Ash	Chlorine as sodium chloride in ash	Phosphoric acid (P_2O_5)	Ether extract	Acidity as lactic acid
		%	%	%	%	%	%
17091	Round beef, cold pressed....	85.76	1.53	0.12	0.37	0.27	0.27
17092	Chuck beef, cold pressed....	86.85	1.86	.20	.31	.30	.32
17091	Round beef pressed at 60°...	90.65	1.36	.15	.36	.19	.15
17092	Chuck beef pressed at 60°...	91.90	1.29	.19	.29	.64	.20
19766	Juice from beef chuck at 60°.	89.56	1.27	.16	.37	....	.....
19767	Juice pressed from sirloin steak and water.	91.10	1.40	.12	.18	.....	.....
19785	Juice extracted from sirloin steak by cold pressure.	96.13	.46	.05	.14	.....	.....
19786	Juice extracted from beef chuck by cold pressure.	96.58	.43	.05	.11	.....	.....
19787	Juice extracted from beef chuck by cold pressure after 6 hours at 60°–100°.	98.11	.39	05	.12	....	....

Serial No.	Preparation of juice	Composition of sample						
		Total nitrogen	Insoluble nitrogen	Coagulable nitrogen	Proteose nitrogen	Peptone nitrogen	Amino nitrogen	Undetermined matter
		%	%	%	%	%	%	%
17091	Round beef, cold pressed.....	2.08	0.16	1.37	0.06	0.16	0.33	0.47
17092	Chuck beef, cold pressed.....	1.74	.29	.98	.07	.11	.29	1.03
17091	Round beef pressed at 60°.....	1.16	.68		.04	.01	.43	1.90
17092	Chuck beef pressed at 60°...	1.09	.12	.41	.07	.21	.27	.40
19766	Juice from beef chuck at 60°	1.09	.49		.42	.....	.18	2.92
19767	Juice pressed from sirloin steak and water.	1.18	.54		.20	.18	.26	.94
19785	Juice extracted from sirloin steak by cold pressure.	.48	.34		Trace.	None.	.14	.85
19786	Juice extracted from beef chuck by cold pressure.	.43	.34		Trace.	None	.09	.59
19787	Juice extracted from beef chuck by cold pressure after 6 hours at 60°–100°.	.24	0		Trace.	.12	.08	.25

Serial No.	Preparation of juice	Results in terms of total nitrogen					Nitrogenous bodies				
		Insoluble protein	Coagulable protein	Albumoses	Peptones	Amido bodies	Insoluble protein	Coagulable protein	Proteoses	Peptones	Amido substances
		%	%	%	%	%	%	%	%	%	%
17091	Round beef, cold pressed	7.69	65.87	2.88	7.69	15.87	1.00	8.56	0.38	1.00	1.03
17092	Chuck beef, cold pressed	16.66	56.32	4.02	6.32	16.66	1.81	6.13	.44	.69	.90
17091	Round beef pressed at 60°	58.62		3.45	.86	37.07	4.25		.25	.06	1.34
17092	Chuck beef pressed at 60°	11.01	37.61	6.42	19.26	24.77	.75	2.56	.44	1.31	.84
19766	Juice from beef chuck at 60°	44.95		38.53	.....	16.51	3.06		2.63	.....	.56
19767	Juice pressed from sirloin steak and water	45.76		16.95	15.25	22.03	3.38		1.25	1.13	.81
19785	Juice extracted from sirloin steak by cold pressure.	70.83		.....	.....	29.17	2.13		Trace.	None.	.44
19786	Juice extracted from beef chuck by cold pressure.	79.07		.....	.....	20.93	2.13		Trace.	None.	.28
19787	Juice extracted from beef chuck by cold pressure after 6 hours at 60°–100°	0		.....	50.00	33.33	0		Trace.	.75	.25

extracts has been proposed by Lowenstein.[1] 2 grm. or more of the sample are weighed into a small metal dish about 2.5 in. in diameter, together with a short glass stirring rod flattened at one end to facilitate spreading the material over the bottom of the dish. About 15 c.c. of 95% or, better, absolute alcohol are added and thoroughly incorporated with the sample. The dish is then placed on the steam bath and the alcohol evaporated with constant stirring; another portion of 15 c.c. is then added and evaporated in a similar manner. In case the sample is very difficult to dry, 4 applications of alcohol may be made. Drying is continued on the steam bath for 30 minutes when the sample is transferred to the drying oven and dried at 105° to constant weight. A vacuum oven can be used to advantage for the final drying.

If the moisture residue is to be used for another estimation, such as nitrogen, it is convenient to use thin lead or tin-foil dishes or Hofmeister glass dishes which can be cut or broken, and placed in the apparatus used with the samples.

Ash.—The ash in meat-extracts may be estimated by charring a convenient quantity (2–10 grm.) extracting the char with hot water, ashing the char and adding the extract which is then evaporated to

[1] *J. Ind. Eng. Chem.* (1909), 1, 252.

dryness, dried and weighed. (See General Methods for the Analysis of Meat, page 295.)

Chlorine and Sodium Chloride.—Chlorine may be determined by Volhard's method or gravimetrically by precipitation as silver chloride, working on a solution of the ash.

For factory control work, approximate estimations are made by charring a convenient quantity in a porcelain dish, grinding the char with a small agate pestle, and transferring the ground material by means of hot water to a 250 c.c. graduated flask. After making up to volume, the chlorine in an aliquot is titrated with silver nitrate using neutral potassium chromate as indicator. The result is usually calculated to sodium chloride.

When the ash is to be used for the sodium chloride estimation, care should be taken to keep the ignition temperature as low as possible in order to avoid volatilisation of sodium chloride.

Total Phosphorus.—The method described on page 295 may be used in the case of meat-extracts, the solution being made in nitrohydrochloric acid and the phosphorus weighed as magnesium pyrophosphate.

Fat.—The fat content of meat-extracts is usually very low, but it is difficult to extract. Petroleum ether is the best solvent to use, as this extracts the fat only whereas ethyl ether may dissolve other substances as well. If ethyl ether is used for the extraction it should be anhydrous and alcohol-free. The extraction is performed as for meat (see page 296). Tin-foil or a glass dish can be used in which to dry the sample and the drying should be done according to one of the methods given above, after which the sample is ground with sand and transferred to the extraction shell. A second drying in the vacuum oven is advisable at this stage. The extraction proceeds in the Soxhlet apparatus for 16–24 hours, and the final drying of the fat should take place in the vacuum oven for accurate results. In all cases the fat should be tested for solubility in petroleum ether.

Total Nitrogen.—The sample if pasty or solid can be conveniently weighed out on a small piece of tin-foil and the whole transferred to an 800 c.c. Kjeldahl flask. If the preparation is liquid, the weighing bottle should of course be used. The estimation is carried out in the same way as for meat (see page 297). If saltpetre be present (as it is in the case of "cured" meat-extracts from corned-beef cooking-liquors) it should be removed before the estimation of organic nitrogen is carried out, by adding 10 c.c. of freshly prepared saturated ferrous chloride solution and 5 c.c. strong hydrochloric acid and boiling a sufficient length of time.

Nitrate Nitrogen.—In cases where saltpetre is present, the nitrate nitrogen is estimated by the Schloesing-Wagner method (see page 368). The sample is weighed out dissolved in hot water and made up to volume in a graduated flask. An aliquot is used for the estimation.

No analysis of a meat-extract or similar preparation can be regarded as affording reliable information as to the quality of the sample which does not make some distinction between the different forms in which the nitrogen exists. Thus while the extractive matters and meat-bases have a special value of their own, they are not nutritive. Albumin, albumose and peptone, on the other hand, are true nutritive compounds, and are superior in value to gelatin.

Proximate Analysis and Determination of the Nitrogenised Constituents of Meat-extracts, etc.—In the fullest possible analysis of a meat-extract, an attempt will be made to discriminate between and estimate the amount of nitrogen existing in the various forms of meat-fibre and insoluble albumin, coagulable albumin, acid-albumin, albumoses, peptones, coagulable gelatin, gelatin-peptone, meat-bases, amino-compounds and ammonia. Such an analysis is necessarily tedious and rarely necessary, but some of the more important of the above estimations can be effected with reasonable ease and accuracy, and are not uncommonly required of the analyst.

In consequence of the uncertainty attaching to the composition of certain of the nitrogen containing constituents of meat-extracts, it is often convenient to state simply the amounts of nitrogen found to exist in the various forms, and in cases where it is preferred to state the actual amounts of the nitrogen-containing substances present the corresponding amounts of nitrogen should always be given in addition.

Ammoniacal nitrogen should be estimated by distilling the aqueous solution of a known weight of the preparation with barium carbonate, which is preferable to magnesia.

Unaltered Proteins and Meat-fibre.—Bovril and certain allied preparations contain finely powdered meat-fibre. This may be detected by treating the meat-extract with cold water, and examining the insoluble portion under the microscope. If meat-fibre be found, 5 grm. of a dry preparation, 8 to 10 grm. of an extract, or 20 to 25 grm. of a fluid preparation should be treated with cold water, the insoluble matter collected on a filter, washed with cold water, dried at 100°, and weighed. The weight obtained represents the *meat-fibre* and *insoluble matter* of the preparation. An alternative and in some respects preferable plan is to treat the moist residue by the Kjeldahl-Gunning process, using mercury (see page 297). The nitrogen found multiplied by 6.25 will give the

insoluble meat-proteins as distinguished from the crude meat-fibre, etc., obtained by weighing the insoluble matter. If there is a large amount of insoluble matter, a larger quantity can be weighed out, made up to volume in a graduated flask and the nitrogen estimated in an aliquot after filtering through a folded filter and removing nitrates if present. The percentage of soluble organic nitrogen deducted from the percentage of total nitrogen gives the percentage of nitrogen in insoluble proteins.

Coagulable Albumin can be estimated in the filtrate from the insoluble matter by rendering the liquid slightly acid with acetic acid or sodium hydroxide, boiling for 5 minutes, filtering, and estimating the nitrogen in the coagulum.[1] Only insignificant amounts of albumin are usually present in meat-extracts, but in certain preparations which have received an addition of scale-albumin the amount may be considerable.

Syntonin.—An aliquot portion of the liquid filtered from the coagulable albumin should be further acidified with acetic acid and tested with potassium ferrocyanide. If any precipitate be formed the liquid should be heated, and if re-solution does not ensue the presence of *acid-albumin* is certain. If found, the remainder of the liquid should be rendered exactly neutral to litmus, the precipitate filtered off, and the contained nitrogen estimated. Syntonin is stated by Denaeyer to be present in considerable proportion in "Kemmerich's meat peptone," while "Somatose" consists largely of alkali-albumin, which will be determined as syntonin by the above process.

Albumoses (and Gelatin).—The filtrate from the precipitate of syntonin, or, in the absence of syntonin, the liquid filtered from the coagulable albumin, is evaporated to small volume and saturated with zinc sulphate, using as little excess as possible. About 35 grm. of the salt are required per 50 c.c. of solution at room temperature. Allow the precipitate to stand several hours or overnight, filter and wash the precipitate with saturated zinc sulphate acidified with sulphuric acid. In case the precipitate is voluminous and unwieldy, make up to volume with saturated zinc sulphate solution, and estimate nitrogen in an aliquot after filtering through a folded filter. The precipitate produced contains all the albumose of the extract, together with any gelatin which may be present and any coagulable or insoluble proteins not previously removed. Peptones, meat-bases, amino-compounds, and ammoniacal salts are not precipitated.

Peptones (and Meat-bases).—The most satisfactory method of esti-

[1] The filter paper used for the separation of these albuminous precipitates must be as free as possible from nitrogen, or a correction must be made for the amount present. Stutzer recommends the filters of Schleicher and Schüll.

mating peptones is the tannin-salt method of Bigelow and Cook. Although it leaves much to be desired it offers the best method of separation at the present time, and it will be still better whenever a nitrogen-free tannic acid is obtainable. The original instructions of Bigelow and Cook[1] for the conduct of the tannin-salt method are as follows:

"1 grm. of meat powders, 2 grm. of preparations of pasty consistency and from 10 to 20 c.c. of liquid or semi-liquid extracts, should be employed. Solid and pasty preparations are dissolved in a little cold water in a 100 c.c. graduated flask, keeping the volume within 20 c.c.

"Then 50 c.c. of a solution containing 30 grm. of sodium chloride per 100 c.c. are added and the flask agitated to insure the thorough mixing of its contents and the solution of the sample. The flask is now placed in the ice-box at approximately 12°. After the solution has reached the ice-box temperature (this requires and hour usually) 30 c.c. of a 24% solution of tannin (which must be at ice-box temperature) are added. The total volume is now diluted to 100 c.c. The contents of the flask are thoroughly mixed and the flask returned to the ice-box, where it remains overnight. In the morning the solution is filtered at ice-box temperature into a 50 c.c. graduated flask. The nitrogen is estimated in this filtrate and also in an aliquot portion of the filtrate from a blank, in which the reagents alone are employed. The nitrogen found in the 50 c.c. portion multiplied by 2 (after correction for the nitrogen in the blank) gives the total nitrogen in the filtrate, and includes the nitrogen present as ammonia and all of the nitrogen of the meat-bases except that portion of the creatine precipitated by the tannin-salt reagent.

"In the examination of products that contain insoluble or coagulable proteins, 20 c.c. of the filtrate from the coagulable proteins are used for this estimation. The volumes of the solution of the meat-extract employed and of the two reagents will then make 100 c.c. and require no further dilution. The nitrogen thus precipitated by the tannin-salt reagent (calculated by difference) consists of that present in the form of proteoses and peptones, and the peptone nitrogen may of course be found by deducting the proteose nitrogen, obtained by precipitation with zinc sulphate, from the total amount of nitrogen precipitated by the tannin-salt reagent." This method gives the meat base and the ammonium nitrogen directly. For calculating the meat-bases, the factor 3.12 is used.

[1] *J. Am. Chem. Soc.*, 1906, 28, 1497.

The peptones are calculated by subtracting the sum of the various other forms of nitrogen from the total nitrogen.

Creatinine and Creatine.—Of the meat-bases present in base extracts it is at present possible to estimate individually only creatinine and creatine by a simple, practical, method; and the method in this case, cannot be considered an accurate one. In 1904 Folin[1] proposed a method for estimating creatinine in urine based on the orange-yellow colour produced by the action of creatinine on picric acid in alkaline solution (Jaffé reaction). As applied to meat-extracts and the cold-water-extract of meat, the details of the method as given by Grindley[2] are as follows:

Dissolve about 5 grm. of the meat-extract in water and make the resulting solution up to a volume of 250 c.c. Thoroughly mix. Filtration is not necessary. For the performed creatinine, transfer aliquot portions of the sample solution to 500 c.c. measuring flasks, add 15 c.c. of a 1.2% picric acid solution, mix thoroughly, add 10 c.c. of a 10% sodium hydroxide solution, shake thoroughly, and allow the mixture to stand 5 minutes and then dilute to the mark at once and after thoroughly mixing compare the depth of the colour of the solutions with that of a half normal dichromate solution (24.54 grm. per litre) set at 8 mm. upon the Duboscq colourimeter. If the sample solutions first taken do not give a reading between 7 to 9 mm., then repeat the above operations taking such a quantity of the solution under examination as will give a reading between these limits.

For the estimation of the creatine measured portions of the sample solution are transferred to beakers if the quantity taken is more than 10 c.c. or to 100 c.c. measuring flasks, if the quantity is 10 c.c. or less. In the former instance, evaporate the solution on the water bath to 10 c.c. In either case make the volume of the liquid up to 10 c.c., if necessary, and add 10 c.c. of $N/1$ hydrochloric acid. Rotate the vessels to thoroughly mix the liquids. Transfer the acid solutions to an autoclave and heat then at a temperature of 117 to 119°, for 30 minutes. After removal, cool and dilute to the mark. If beakers were used, transfer the contents to 100 c.c. measuring flasks and dilute. To aliquot portions of this converted creatinine solution contained in 500 c.c. measuring flasks, add 30 c.c. of 1.2% picric acid, shake, and then add 10 c.c. of the 10% sodium hydroxide. Mix thoroughly and after standing exactly 5 minutes dilute, and read the depth of the colour of the solution as directed above. If the solutions thus first treated do

[1] *Z. physiol. Chem.*, 1904, 41, 223; *Am. J. Physiol.*, 1905, 13, 48. Compare Vol. 7, p. 31.6
[2] *J. Biol. Chem.*, 1907, 3, 491-516.

not give a reading between 7 to 9 mm., then take such a quantity of the converted creatine solution under examination as will give a reading between these limits.

According to Folin, the correct reading in millimetres of the colourimeter divided into 81 gives the number of milligrams of creatinine contained in the portion of the solution taken for the treatment with picric acid and sodium hydroxide. In other words, 10 mg. of pure creatinine after the addition of the picric acid and the alkali and dilution to 500 c.c., gives a reading of 8.1 mm. when compared with 8 mm. of $N/2$ potassium dichromate solution (24.54 grm. per litre).

In order to convert milligrams of creatinine into creatine multiply by the factor 1.16.

The method as originally applied by Grindley (*J. Biol. Chem.*, 1907, **2**, 309–314) and others was criticised by Hehner (*Pharm. J.*, 1907, **78**, 683) on several grounds. The conversion of creatine to creatinine in the autoclave is due to Benedict and Myers (*Am. J. Physiol.*, 1907, **18**, 397). In the writer's laboratory several chemists have been unable to obtain consistent results by the method. Time and temperature appear to affect the results. The colour first increases in intensity and later fades away. Cooperative work in 1907 by the Association of Official Agricultural Chemists on the method gave results, which varied in the extreme and on the whole were of no value, except to demonstrate the necessity of much more work on the method to make it usable. Chapman (*Proc. 7th Internat. Cong. App. Chemistry*, London, 1909) has shown that the red colour in Jaffé's reaction is not due to the formation of creatinine picrate but is due to the reduction of picric acid in alkaline solution to a mixture of amino-dinitrophenol (picramic acid) and diamino-nitrophenol, the alkaline salts of which are deeply coloured. The same colouration is produced by numerous reducing agents such as nascent hydrogen, hydroxylamine, acetone, aldehyde, ammonium sulphide, etc. The colour is due to both monamino and diaminophenol and solutions of the sodium salt of picramic acid could not be used for matching purposes. The factors of temperature, time and presence of dextrose are shown to influence the result.

Grindley and others maintain that excellent results are obtainable by the method, but the author's experience does not confirm this.

Grindley and Woods publish the following figures (*J. Biol. Chem.*, 1907, **2**, 313–14):

CREATININE IN COMMERCIAL BEEF EXTRACTS.

No. of sample	Weight of sample	Reading of colourimeter	Creatinine	
			Weight	%
	Grm.	Mm.	Mgrm.	
1	0.3008	7.8	10.38	3.45
2	0.2545	8.6	9.42	3.70
3	0.5800	8.1	10.00	1.72
4	0.7221	8.0	10.13	1.40
5	0.1972	7.8	10.38	5.27
6	0.7234	8.2	9.88	1.37
7	0.8206	8.2	9.88	1.20
8	0.5458	7.7	10.52	1.93
9	1.1391	8.6	9.42	0.83
10	0.4720	8.1	10.00	2.12
11	0.6786	8.5	9.47	1.39
12	0.7540	7 5	10.08	1.30
13	0.2774	7.3	11.10	4.00

CREATINE IN COMMERCIAL BEEF EXTRACTS.

No. of sample	Weight of sample	Reading of colourimeter	Weight of original creatinine plus creatinine due to creatine	Weight of original creatinine	Weight of creatinine due to creatine (A)	Weight of creatine A×1.16	Creatine
	Grm.	Mm.	Mgrm.	Mgrm.	Mgrm.	Mgrm.	%
2	0.1833	7.9	10.25	6.78	3.47	4.03	2.18
3	0.2636	7.7	10.52	4.55	5.97	6.93	2.63
4	0.1850	7.9	10.25	2.60	7.65	8.74	4.79
5	0.1512	8.4	9.64	7.96	1.68	1.95	1.29
6	0.1841	8.6	9.42	2.38	7.04	8.17	4.35
7	0.1641	8.0	10.13	1.98	8.15	9.45	1.20
8	0.2593	8.0	10.13	5.00	5.13	5.95	2.29
9	0.7088	8.8	9.20	5.86	3.34	3.87	0.55
11	0.3016	5.0	16.20	4.19	12.01	13.93	4.62
12	0.2262	6.85	11.82	2.94	8.88	10.30	4.55
13	0.2080	7.00	11.57	8.32	3.25	3.77	1.81

COMBINED CREATININE AND CREATINE IN COMMERCIAL BEEF EXTRACTS.

No of sample	Creatinine	Creatine	Sum of creatinine and creatine
	%	%	%
2	3.70	2.18	5.88
3	1.72	2.63	4.35
4	1.40	4.79	6.19
5	5.27	1.29	6.56
6	1.37	4.35	5.72
7	1.20	1.20	2.40
8	1.93	2.29	4.22
9	0.83	0.55	1.38
11	1.39	4.62	6.01
12	1.30	4.55	5.85
13	4.00	1.81	5.81

Xanthine Bases.—Bigelow and Cook (see tables, page 402) have also estimated the xanthine bases in their work on meat-extracts. Their remarks under this head and their method follow:

"In addition to creatine and creatinine, a true meat-extract or meat-

juice should contain small amounts of xanthine bases, including xanthine, hypo-xanthine, guanine, and adenine. These substances are derived from the nuclei of the cells, and, consequently, in an extract that is prepared from fresh, unaltered beef a certain amount of these substances should be obtained together with the salts and other extractive matter. The estimation of the xanthine bases is, therefore, of value in estimating the origin of an alleged extract of meat.

"The xanthine base figures in the tables show a variety of results, which is explained by the fact that in the preparation of the extract under certain conditions of heat and pressure some of these substances are destroyed. The following modification of Schittenhelm's method was employed for their estimation:

"Use an amount of the standard solution equivalent to 5 grm. of the original extract. Place in a large evaporating dish and add 500 c.c. of 1% sulphuric acid. Evaporate to 100 c.c. within 4 to 5 hours. Cool and neutralise with sodium hydroxide. Add 10 c.c. of 15% sodium disulphate, and 15 c.c. of 20% copper sulphate; allow this to stand overnight, filter, and wash. The precipitate suspended in water is treated with sodium sulphide and warmed on the steam bath. Add acetic acid to acidify and filter hot. To the filtrate add 10 c.c. of 10% hydrochloric acid and evaporate to a volume of about 10 c.c. Filter, make ammoniacal, and add ammoniacal silver nitrate of 3% strength. After standing several hours the solution is filtered and washed with distilled water until no longer alkaline. The nitrogen in the precipitate is that of the xanthine bases."

Other Methods for Meat Bases.—Various methods for the separation and estimation of meat bases have been proposed, but have not come into general use because of their length and complexity. One of the older methods in brief consists in first boiling the solution and filtering off the albumin, concentrating the filtrate to small bulk and adding lead acetate to remove sulphates, phosphates and chlorides. The filtrate is freed from lead by adding hydrogen sulphide and filtering and the latter removed by concentrating to a syrup. After long cooling creatin crystallises out. The crystals are filtered off, washed with 88% alcohol and weighed. After removing alcohol from the filtrate and digesting for 3 hours with dilute sulphuric acid (1 : 3) the xanthine bases are precipitated by the copper bisulphite method of K. Micko (*Z. Nahr. Genussm.*, 1902, **5**, 193; 1903, **6**, 781. See also König (*Nahrungs und Genussmittel*, **3** (i), 314–17). From the filtrate from the xanthine bases creatinine is obtained as zinc chloride compound.

Kutscher's Method (*Z. Nahr. Genussm.*, 1905, **10**, 528; 1906, **11**, 582).—Kutscher has developed a new method for the separation and estimation of the meat bases and by means of it has discovered several new bases in meat extract. Some of the bases he has named Ignotine, Carnomuscarine, Neosine, Novaine, and Oblitine, respectively. Kutscher worked on about 450 grm. of Liebig's meat extract and this he treated with 500–600 grm. tannin in solution. Purification of the filtrate from the tannin precipitate was effected by means of barium hydroxide, filtration, dilute sulphuric acid and lead oxide and filtration. The filtrate was then rapidly concentrated to a syrup. Lead compounds separated at first, then creatine crystals. Upon standing for 24 to 48 hours more crystals separated consisting chiefly of creatine and creatinine. The crystals were filtered quickly and washed with a small quantity of ice-cold water. The somewhat alkaline filtrate (from the lead oxide) was acidified with sulphuric acid, filtered from the lead sulphate and the new filtrate precipitated with 20% silver nitrate. The precipitate consisted chiefly of silver chloride, alloxan bases and others, and was filtered after 24 hours. The filtrate was again treated with 20% silver nitrate until upon testing, no longer a white but a brown precipitate was formed in saturated baryta water. Baryta water was then added in slight excess. The silver precipitate was treated first with sulphuric acid then with hydrogen sulphide and the filtrate concentrated to a syrup. From this syrupy solution the new bases were separated. For complete directions the analyst should consult Kutscher's original papers.

Ammonium Salts.—There is no satisfactory method for the estimation of ammoniacal nitrogen in meat-extracts. An approximation can be obtained by distillation with magnesium oxide or barium carbonate, but the results are too high, and there is no definite limit to the evolution of ammonia. The air aspiration method (page 316) may be used, but it is extremely slow and the results are of doubtful accuracy, unless the method is most carefully handled and checked.

Gelatin is present in notable quantity in some meat-extracts, and its estimation presents considerable difficulty. Gelatin is precipitated more or less completely by most of the reagents for proteins, including tannin, phosphotungstic acid, bromine-water, and ammonium and zinc sulphates. Unchanged gelatin is said not to be precipitated by Stützer's copper hydroxide reagent but the commercial article is largely thrown down. The same is the case when a solution of commercial gelatin (Nelson's) is treated with mercuric chloride or with potassium-mercuric iodide, although these reagents are stated by

Denaeyer not to precipitate gelatin; in fact the first reagent is employed by him to separate gelatin from peptone, and the second to differentiate gelatin from albumose.

Unchanged gelatin is precipitated by alcohol of very moderate strength (50 to 60%), but the non-jellifiable modification produced by the prolonged action of hot water or of weak acids on gelatin is only precipitated by very strong alcohol (95%). This modified gelatin, often called *gelatin-peptone* or *gelatone*, has been but imperfectly examined, but differs materially from colloidal gelatin in its chemical reactions.[1]

On the whole the gelatin of meat-extracts is best determined by the following modification by Stützer of a method devised by Denaeyer, but the process cannot be regarded as wholly satisfactory:

The tin-foil capsule containing the dry residue resulting from the estimation of the total solids of the sample (page 401), together with the sand, is cut into small strips and exhausted in a beaker four times with absolute alcohol. The alcohol is passed through an asbestos filter, taking care to leave the insoluble matter as far as possible in the beaker. After removal of the adherent alcohol, the mixture of tin-foil, sand, and gelatin is treated with ice-water, to which 10% of alcohol has been added, the temperature being kept below + 5° by the gradual addition of small pieces of ice. After being shaken for 2 minutes in a suitable apparatus, the extraction with ice-water is repeated.[2] The insoluble portion (together with the tin-foil and sand) is washed with alcoholised ice-water until the filtrate is colourless. The residue is then boiled with water, the solution of gelatin filtered, and the contained nitrogen estimated by Kjeldahl's method. This may be

[1] Various experiments have been conducted in Allen's laboratory by A. B. Searle with a view of finding a reliable chemical method of separating gelatin from proteins. So far, none of the reagents generally credited with effecting a separation of gelatin from albumoses have been found to behave in accordance with published statements, and no method of distinguishing sharply between gelatin and gelatin-peptone has been devised up to the time of writing.

[2] The following is Stützer's most recent method of operating (*Zeit. Anal. Chem.* 1895, 34, 568): The beaker (marked *a*) together with four others (marked *b*, *c*, *d*, *e*) and a flask containing a mixture of 100 c.c. of alcohol, 300 grm. of ice, and cold water to 1 kgrm., are then immersed in a bath containing crushed ice. About 100 c.c. of the mixture in the flask, the temperature of which must not exceed 5°, is poured on the sand, stirred with a glass rod for 2 minutes, and decanted into beaker *b*, a piece of ice being added to keep down the temperature. The extraction in the beaker is repeated with a second quantity of alcoholised water which is poured into *c*, and the treatment repeated until the last washing is colourless, a fragment of ice being added to each quantity of extraction liquid as soon as it is poured off. Three funnels, of about 7 cm. diameter, are then arranged with filter-beds of long-fibred asbestos supported by perforated porcelain plates about 4 cm. in diameter, and connected with a pump by which gentle and gradually-increasing suction can be applied. The contents of beaker *a* are filtered into the first, *b* is poured into the second, and *c*, *d*, and *e* into the third. The filters, as well as that through which the absolute alcohol extract has been filtered, are then thoroughly washed with the ice-cold alcoholised water, transferred to a porcelain basin, and repeatedly extracted by boiling with water. The aqueous extract is filtered, concentrated, and treated by Kjeldahl's process.

Stützer finds that when the process is conducted exactly in the manner prescribed above, that from 95 to 98% of the total gelatin present is obtained, and that the small quantities of gelatin-peptone present in meat-extracts are precipitated by alcohol together with the gelatin proper, and may be suitably estimated therewith.

applied to the liquid itself, or the gelatin may be thrown down by bromine-water, and the resultant precipitate treated. The nitrogen found, multiplied by 5.55, gives the gelatin of the sample.

Qualitative Test for Yeast Extracts.—A product made from waste yeast and used as a substitute or an adulterant of meat-extract came on the market some years ago, especially in Germany. Bigelow and Cook[1] report as follows on these preparations:[2]

"Searl (*Pharm. J.*, 1903, **71**, 516 and 704; 1904, **72**, 86) suggests as a method for detecting yeast products added to meat preparations, that a solution of the extract be boiled 1 or 2 minutes with a modified Fehling's solution. In the presence of yeast extract a bluish-white precipitate is obtained. Arnold and Mentzel (*Pharm. Ztg.*, 1904, **49**, 176) claim that a slight bluish-white precipitate is given even with pure meat-extracts, but by experience an analyst learns to detect by this method the presence of about 20% of yeast extract in meat preparations. Micko (*Zts. Nahr. Genussm.*, 1902, **5**, 193; 1903, **6**, 781) suggests the estimation of creatine and xanthine substances as a means of determining the nature of the extract. Wintgen (*Arch. Pharm.*, 1904, **242**, 537) states that the filtrate from the zinc sulphate precipitate obtained in the estimation of albumoses is entirely clear in the case of meat-extracts, but somewhat turbid with yeast extracts. This he finds to be true even when the best S. & S. filter paper is employed. By this method the authors could detect from 20 to 30% of added yeast extract.

"E. Baur and H. Barschall (*Arb. kaiserl. Gesundheitsamte*, 1906, **24**, 562) have applied the colourimetric test, as outlined by Folin, for creatinine to meat and yeast extracts. They find no creatine or creatinine in yeast extracts and base a distinction between the two on this test. Salkowski (*Ber. d. chem. Ges.*, 1894, **27**, 499) has studied the various carbohydrates of yeast and gives several tests for yeast gum.

"The most reliable test is unquestionably the estimation of creatine. A yeast extract contains no creatine and in a typical meat-extract there is found from 10 to 20% of the total nitrogen in the form of creatine and creatinine. The distribution of the various xanthine bases also is different in the two kinds of extracts; in meat-extracts, according to Micko (*loc. cit.*), xanthine and hypo-xanthine predominate, while in yeast extracts adenine and guanine predominate.

"A test for yeast extracts consisting in boiling the samples for 1 or

[1] *U. S. Dept. of Agriculture Bur. of Chem. Bull.*, 114.
[2] See also F. C. Cook, A Comparison of Beef and Yeast Extracts of Known Origin. *U. S. Dept. Agric. Bur. Chem. Circular* 62, 1910.

2 minutes with an unmodified Fehling's solution was tried. Four samples were tested with the following results:

	Colour of precipitate.
A. Meat-extract	Very deep violet colour.
B. Yeast extract	Very deep green colour.
C. 50% yeast and 50% meat-extract	Intermediate colour.
D. 25 % yeast and 75% meat-extract	Violet colour, not as strong as A.

This test is of value as a qualitative and a confirmatory test for yeast extracts in the presence of meat-extracts.

"The method of Searl for the detection of yeast extract by the use of a modified Fehling's solution is as follows:

Prepare a modified Fehling's solution by dissolving 12.96 grm. of copper sulphate and 16.20 grm. of neutral tartrate of sodium in 113.4 c.c. of water. Add to this 16.20 grm. of sodium hydroxide dissolved in 113.4 c.c. of water. Dissolve 0.6481 grm. of the sample to be examined in 42.5 c.c. of water, add to this 1/2 volume of the above solution and boil for 1 or 2 minutes. With genuine meat-extract no precipitate is given. When yeast extract is present a curdy, bluish-white precipitate is formed.

"This method was tested on a sample of meat-extract, a yeast extract, a 50% solution of yeast and meat-extract, and a solution containing 20% of yeast and 80% of meat-extract. In the case of the meat-extract a very fine precipitate was obtained. In the three cases where yeast extract was present a flocky, bluish-white precipitate was formed. It is evident from these results that the presence of 20% of yeast extract in meat mixtures may be detected by this method.

"Another test is described by Wintgen (*Arch. Pharm.*, 1904, **242**, 537), who claims that the zinc sulphate filtrate in the case of meat-extracts is clear, but with yeast extracts it is turbid. This was found to be the case, as the following results show:

	Zinc sulphate filtrate.
A. Meat-extract	Clear.
B. Yeast-extract	Cloudy.
C. 50 % yeast and 50 % meat-extract	Cloudy.
D. 25 % yeast and 75 % meat-extract	

The solutions of these extracts, or mixtures, were saturated with chemically pure zinc sulphate after adding 2 drops of strong sulphuric acid. The solutions stood overnight and the filtrates were examined in the morning. The only clear filtrate obtained was that from meat-extract alone.

The most important test for determining the nature of an extract, whether meat or yeast, is the determination of creatine and creatinine. This test, which has been used in the Bureau of Chemistry for 2 or 3 years and found to be of great value, was perhaps first applied by Micko (*loc. cit.*). As before stated, yeast extracts contain no creatine

or creatinine, while in meat-extracts these two bodies are present in considerable amounts.

"Some experiments on meat-extract, yeast extract, and mixtures of the two were tried with satisfactory results, using the Folin (*Zts. physiol. Chem.*, 1904, **41**, 223) colourimetric method. In estimating the creatinine by this method in the presence of yeast extract, slightly higher results are obtained than when yeast extract is not present. When the creatine and creatinine are determined together (after dehydrolysis) in a sample of meat-extract the presence of yeast extract does not seem to affect the results. In the case of the yeast extract no creatine or creatinine was found.

CREATININE IN MEAT AND YEAST EXTRACTS.

No.	Description of sample	Weight of sample	Creatinine		Creatinine calculated to meat-extract used	Increase of creatinine due to presence of yeast extract
			Weight	%		
		Grm.	Mg.		%	%
1	Meat-extract	0.2660	8.804	3.39	3.39	0
		.2663	8.804	3.31	3.31	
	Average			3.35	3.35	
2	Yeast extract	.4800	0	0	0	0
3	Mixture—50% meat and 50% yeast extract	.4154	7.788	1.87	3.75	
		.3756	7.013	1.87	3.75	0.40
	Average			1.87	3.75	
4	Mixture—75% meat and 25% yeast extract	.2638	8.437	3.19	4.25	
		.3030	7.941	2.62	3.49	.52
	Average			2.91	3.87	

TOTAL CREATININE (INCLUDING CREATINE CONVERTED TO CREATININE) IN MEAT AND YEAST EXTRACTS.

No.	Description of sample	Weight of sample	Creatinine		Creatinine calculated to meat-extract used	Increase of creatinine due to presence of yeast extract	Creatine calculated as creatinine (by difference)
			Weight	%			
		Grm.	Mg.		%	%	%
1	Meat-extract	0.2210	11.571	5.24	5.24	0	4.92—3.35 =1.57
		.2144	9.870	4.60	4.60		
	Average			4.92	4.92		
2	Yeast extract	.5250	0	0	0	0	0
3	Mixture—50% meat and 50% yeast extract	.4378	11.571	2.64	5.29		5.05—3.75 =1.30
		.4020	9.691	2.41	4.82	0.13	
	Average			2.53	5.05		
4	Mixture—75% meat and 25% yeast extract	.3554	15.577	4.38	5.85		5.05—3.87 =1.18
		.3255	10.385	3.19	4.25	.13	
	Average			3.79	5.05		

Cook reports the following figures showing the comparative composition of meat and yeast extracts of known origin.[1]

COMPARATIVE ANALYSIS OF BEEF AND YEAST EXTRACTS.
ON BASIS OF ORIGINAL SAMPLE.

Determinations	Yeast extract		Beef extract			
	A. P. 724	A. P. 725	A. P. 794	A. P. 795	A. P. 796	A. P. 797
	%	%	%	%	%	%
Solids	73.73	72.34	84.78	86.23	79.73	86.66
Total ash	22.18	19.48	19.57	16.23	14.63	14.49
Chlorine of ash as sodium chloride.	2.38	1.72	1.67	2.07	2.05	1.83
Phosphoric acid:						
Of ash	2.71	2.76	2.30	2.63	1.96	2.22
Total	2.73	2.78	2.50	3.00	2.04	2.27
Organic	.24	.29	.25	.27	.20	.25
Ether extract	.83	1.01	6.33	7.85	6.34	6.95
Nitrogen:						
Total	5.40	5.31	9.43	9.43	8.63	9.13
Proteose and peptone[2]	2.15	2.18	3.40	3.04	1.89	2.39
Amino	3.25	3.13	6.03	6.39	6.74	6.74
Purine base	.58	.64	.....	.65	.77	.64
Creatine	0	0	4.00	2.90	1.51	1.95
Creatinine	0	0	2.22	2.60	2.35	2.62
Acidity[3]	9.86	10.27	11.23	10.54	10.14	12.33

CALCULATED TO A MOISTURE-FREE AND FAT-FREE BASIS.

Total ash	30.42	27.32	24.94	20.71	19.93	18.18
Chlorine of ash as sodium chloride.	3.27	2.41	2.13	2.64	2.79	2.30
Phosphoric acid:						
Of ash	3.72	3.87	2.93	3.36	2.64	2.79
Total	3.74	3.89	3.19	3.83	2.78	2.85
Organic	.33	.41	.32	.35	.27	.31
Nitrogen:						
Total	7.41	7.45	12.02	12.03	11.76	11.46
Proteose and peptone[2]	2.95	3.06	4.33	3.88	2.58	3.00
Amino	4.46	4.39	7.70	8.15	9.16	8.46
Purine base	.80	.90	.....	.83	1.05	.80
Creatine	0	0	5.10	3.70	2.06	2.44
Creatinine	0	0	2.83	3.32	3.20	3.28
Acidity[3]	13.52	14.40	14.31	13.45	13.82	15.47

Beef-extract Cubes.—A number of preparations are now on the market known by such names as "Oxo," "Steero," "Beef Cubes," "Bouillon Cubes," etc., which consist mainly of nearly dry meat extract, salt, dried ground beef and such flavouring matters as finely ground celery and tomato seed, tomato pulp, etc. These preparations are sold in the form of small cubes, rather less than 1/2 inch on an edge, which are separately wrapped in metal foil, and packed to the number of 1 or 2 dozen in small tinned boxes. It is stated that 1 cube will make a cup of broth by the simple addition of hot water.

The methods of analysis for meat extracts apply to these products.

[1] *Circular 62, U. S. Dept. Agric. Bur. Chem.*, 1910.
[2] This figure includes about 25% of the total creatine and creatinine nitrogen.
[3] Expressed as c.c. of *N*/10 sodium hydroxide per grm.

Various Methods Applicable to Meat-extracts.

Assay of Meat-extracts by Alcohol Precipitation.—A simple means of roughly differentiating between the soluble forms of nitrogen-containing matters in meat-extracts has been in use for many years in the laboratories of manufacturers of such preparations. It consists in treating the sample with alcohol of such strength as to precipitate as much as possible of the protein and gelatinoid constituents of the extract, and as little as possible of the meat-extractives and salts. For this purpose, O. Hehner recommends (*Analyst*, 1885, **10**, 221) that 2 grm. of the sample should be dissolved in 25 c.c. of water, and 50 c.c. of strong methylated spirit added to the solution. The precipitate is allowed to settle overnight, and the clear liquid then decanted as completely as possible. The (unwashed) precipitate is dissolved in a little hot water, the solution evaporated in a weighed basin, and the residue dried at 100° and weighed.

O. Hehner (*Analyst*, 1885, **10**, 221) gives the following results of analyses of some well-known preparations which he examined by the above method. The results yielded by two samples of "Essence of beef" of South African manufacture, analysed in Allen's laboratory by the same process, are added for the purpose of comparison:

Description	Water	Total solids	Alcohol precipitate	Ash	Phosphoric acid	Nitrogen	Authority
Liebig's extract....	18.70	81.30	5.16	23.38	6.07	7.94	O. Hehner.
Nelson's gelatin....	.....	.....	93.19	3.25[1]	None.	.....	O. Hehner.
Concentrated Beef Tea:							
English.....	36.96	63.04	27.40	4.36	1.16	8.25	O. Hehner.
English.....	31.00	69.00	30.30	4.13	1.00	8.36	O. Hehner
English..... ..	41.93	58.07	25.50	4.92	1.10	7.52	O. Hehner
Russian.....	24.56	75.44	35.40	6.72[2]	0.95	9.89	O. Hehner.
X.....	54.31	45.69	32.30	7.57	2.11	6.79	O. Hehner.
Commercial Essence of Beef:							
English.....	89.25	10.75	3.07	1.17	0.34	1.36	O. Hehner.
English	89.61	10.39	3.74	1.00	0.26	1.36	O. Hehner.
English.....	92.32	7.68	1.99	1.30	0.38	0.79	O Hehner.
South African.....	90.50	9.50	2.98	1.74	0.50	1.22	A. H. Allen.
South African.....	87.55	12.45	2.88	2.36	0.17	1.41	A. H. Allen.

J. Bruylants (*J. Pharm. Chim.*, 1897, **5**, 515) has described a method of analysing meat-extracts, based on fractional precipitation by alcohol of different strengths.[3] Thus, gelatin is thrown down by

[1] This ash was insoluble in water, and consisted chiefly of calcium carbonate.

[2] This ash was only partly soluble in water, and was almost entirely composed of calcium carbonate. The ashes of the other samples, with the exception of Nelson's gelatin, were completely soluble in water, and practically devoid of lime.

The phosphoric acid was determined in the samples by precipitation with molybdate solution, resolution of the precipitate in ammonia, the solution evaporated at 100°, and calculated to P_2O_5.

[3] K. Micko has found Bruylants' method to work well (*Zeits. des allgem. österreich Apotheker-Vereins*, 30, 1).

alcohol of 40%, albumoses by 80%, and peptones by 93 to 94% alcohol. The following results were obtained by the analysis of typical preparations:

	Liebig's extract		Solid Bovril		Bovril for invalids		Liquid Bovril[1]	
Water	16.75		19.20		2.35		43.25	
Sodium chloride	2.95		4.50		4.00		9.75	
Other mineral salts	18.24		16.20		17.05		6.25	
Insoluble in water (meat fibre)	.....		1		7.10		8.19	
Organic matters	62.06		60.10		54.50		32.06	
Total nitrogen	9.30		8.85		9.12		4.85	
Nitrogen in part insoluble in water.	.....		1		1.09		1.19	
Nitrogen, ammoniacal, uric acid, etc.	0.60		0.50		0.45		0.30	
Nitrogen, from lead precipitate (non-protein matters)	0.65	5.09	0.57	4.56	0.45	4.48	0.27	2.67
Nitrogen, non-protein from 80% alcohol.	0.15		0.20		0.18		0.05	
Nitrogen, soluble in strong alcohol.	3.69		3.29		3.40		1.05	
Nitrogen, from gelatin	0.19		0.25		0.12		0.05	
Nitrogen, from albumoses	0.80	3.93	0.95	3.78	0.75	3.75	0.45	1.83
Nitrogen, from peptones	2.94		2.58		2.70		1.33	
Total soluble proteins	24.56		23.62		22.40		11.43	
Insoluble albumin (meat fibrin).	.....		1		6.81		7.43	

All methods of examining meat-extracts based on precipitation of various proteins by alcohol of certain strengths, though useful in practice, are open to objection as deficient in accuracy. The method has been subjected to criticism by König and Bömer (*Zeit. anal. Chem.*, 1895, **5**, 548; abst. *Analyst*, 1896, **21**, 17), who point out that in meat-extracts prepared at low temperatures, and which are only concentrated to the required consistency after filtration, the amount of gelatin present must be excessively small.

Assay of Meat-extract by Bromine Precipitation.—The fact that aqueous solutions of the proteins and of gelatin are precipitated by chlorine has been utilised by Rideal and Stewart (*Analyst*, 1897, **22**, 228) as the basis of a process of assaying meat-extracts, etc. As employed by these chemists, the method consists in passing a current of chlorine-gas through the solution to be tested, filtering off and washing the resultant precipitate, and weighing it after drying at a temperature not exceeding 70° or preferably *in vacuo* over sulphuric acid. The precipitate is stated to be remarkably stable at ordinary temperatures but to be readily decomposed on heating, becomnig nearly black and rotting the filter paper.

In order to avoid the inconveniences attaching to the use of chlorine-

[1] The meat-fibre in this sample does not appear to have been separately determined.

gas, and the drying and weighing of an unstable precipitate, Allen devised a modified process (*Analyst*, 1897, **22**, 258), which is rapid, easily worked, and gives concordant results. In this method, bromine-water is employed as the precipitant, in place of chlorine. The precipitate is filtered through asbestos, treated with strong sulphuric acid while still moist, and the contained nitrogen determined by the Kjeldahl-Gunning process (see page 297). The following are the details of the operation:

A quantity of the solution containing about 1 grm. of the albuminoid matter is diluted with cold water to a volume of about 100 c.c., and treated in a conical beaker with sufficient hydrochloric acid to render the liquid distinctly acid to litmus. Bromine-water is then added in considerable excess, and the liquid stirred vigorously for some time. The yellowish precipitate which separates is at first flocculent, but becomes more viscous on stirring, and finally adheres in great part to the sides of the beaker. When this occurs the liquid is allowed to stand at rest for about 1/2 hour, or until the precipitate has settled. It is then decanted through an asbestos filter.[1]

The precipitate adhering to the sides of the beaker is washed several times with cold distilled water, the washings being poured through the filter. Occasionally, when the greater part of the bromine has been washed out of the precipitate, the liquid does not filter clear. It is therefore advisable to keep the washings separate from the filtrate, and, if necessary, to add bromine or sodium sulphate to the wash-water.

The contents of the filter-tube (including the asbestos, and, if necessary, the glass-wool) are returned to the beaker used for the precipitation, 20 c.c. of strong sulphuric acid added, and the beaker covered with a watch-glass and heated over wire-gauze. The substance chars and bromine-vapour is evolved. When frothing has ceased, about 10 grm. of powdered potassium sulphate should be added, and the liquid boiled vigorously until colourless. It is then allowed to cool, diluted with water, an excess of sodium hydroxide added, the ammonia distilled off, and the distillate titrated with standard acid. From the nitrogen found the amount of protein or gelatinoid body present is deduced by a suitable factor.

As the results of experiments by the foregoing process, it was found (Allen and Searle, *Analyst*, 1897, **22**, 259) that practically the whole of the nitrogen of gelatin, gelatin-peptone, egg-albumin, syntonin, and of

[1] The filter is made by placing a plug of glass-wool in a cylindrical funnel (constructed of a vertical glass tube drawn out at the lower end), and covering it with a pad of pulped asbestos. If the filter is properly constructed, no water-pump will be required, and a perfectly clear filtrate will be obtained.

the mixed products of the acid-pepsin digestion of egg-albumin was thrown down in the precipitate produced by bromine.[1] In the case of syntonin, fairly good results were obtained whether the acid used for conversion of the egg-albumin was left unneutralised or was exactly saturated by sodium hydroxide; but if the solution was made alkaline and bromine then added, the protein could not be completely precipitated by subsequent free acidification of the liquid.[2] The mixed peptones formed by the acid-pepsin digestion of the white of hard-boiled eggs were also completely precipitated by bromine.

On the other hand, bromine produced no precipitate in acidified solutions of creatine, creatinine, asparagine, or aspartic acid. A meat-extract prepared by soaking raw beef in 10 parts of cold water, straining, boiling, and filtering from the coagulated albumin, gave only a trifling precipitate on addition of bromine-water. In the liquid concentrated to one-tenth a larger precipitate was obtained, the greater part of which dissolved on diluting the liquid with an equal volume of water, and almost the whole on addition of a few drops of hydrochloric acid. On the other hand, the complete precipitation of albumin and gelatin by bromine seemed to be quite unaffected by dilution or the presence of free hydrochloric acid.

On applying the bromine-method to commercial meat-extracts the

[1] The following is a tabular statement of the chief results obtained:

Substance	Nitrogen %		Nitrogen multiplied by factor		Factor employed
	Total in original substance	Precipitated by bromine	Total in original substance	Precipitated by bromine	
Commercial gelatin	14.10	14.00	76.42	76.14	5.42
Gelatin-peptone	14.10	13.90	76.42	75.44	
Commercial scale-albumin	8.80	8.72	55.8	55.2	
Syntonin from scale-albumin	9.86	9.76	62.41	61.78	
Digested scale-albumin	8.89	8.81	56.3	55.8	6.33
Fresh white of egg	1.89	1.88	11.96	11.90	
Syntonin from white of egg	1.89	1.89	11.96	11.96	
Peptone from white of egg	0.70	0.69	4.43	4.37	
Beef-extractives	0.33	0.004	2.11	0.03	

[2] A sample of commercial scale-albumin was converted into acid-albumin (syntonin) by heating it in 1% solution with hydrochloric acid for 6 hours. The nitrogen was then estimated directly in the resultant liquid and in the bromine precipitate produced in different ways, as shown below. The figures are calculated to 100 parts of the original albumin, which was found to contain 9.88% of nitrogen and to yield 8.32% of ash on ignition. Hence the sample was far from pure.

	Nitrogen
A. By direct Gunning-Kjeldahl process on syntonin solution	9.86%
B. By precipitate from unneutralised syntonin solution	9.76%
C. By precipitate from nearly neutralised syntonin solution	9.69%
D. By precipitate from syntonin solution, rendered strongly alkaline and then re-acidified	9.60%
E. Precipitate from syntonin solution, made strongly alkaline by soda, bromine added, and the liquid acidified after 1/2 hour	6.69%
F. Precipitate from syntonin solution, made strongly alkaline by soda, bromine added, and the liquid acidified after 24 hours	3.52%

following results were obtained. The solutions were not previously filtered, and therefore the figures include the nitrogen of any meat-fibre present in the preparations:

	Nitrogen in precipitate by bromine	×6.3 = proteins
	%	%
Liebig Company's Extract	1.41	8.88
Seasoned Bovril	1.94	12.22
Bovril for Invalids	2.64	16.63
Brand's Beef Bouillon	1.52	9.58
Vimbos	1.83	11.53

Another sample of Liebig Company's extract analysed in Allen's laboratory by entirely different methods gave 9.37% of total proteins.[1]

In another experiment, 5 grm. of the Liebig Company's extract was dissolved in 100 c.c. of water, and the solution saturated with zinc sulphate. On adding bromine to the filtered liquid, a precipitate was produced which redissolved on diluting with water and adding hydrochloric acid. When 50 c.c. of the filtrate from the zinc sulphate was diluted with water to 250 c.c., and freely acidified with hydrochloric acid, no precipitate was produced on subsequently adding bromine. This result appears to negative the presence of considerable quantities of real peptones in Liebig's extract, and confirms the conclusion of König and Bömer on this point.

A parallel experiment with Bovril gave a precisely similar result.

Various other reagents for separating the nitrogenous substances of meat-extracts have been proposed and used at various times, such as phosphotungstic acid, formaldehyde, chromic acid, potassio-mercuric iodide, etc. (See page 407 and the second edition of this work, pages 326–330.)

Most of these methods have been abandoned in favor of those detailed on previous pages.

Non-nitrogenous Extractive Matters.—These consist of lactic acid, succinic acid, glycogen, added glycerol, sugar, starch, etc.

Of these constituents of meat-extracts very little is known quantitatively. *Lactic acid* and *lactates* probably predominate, but their actual amount does not appear to have been ascertained. *Glycogen* is present in sensible quantity, and is determined by dissolving the sample in a little water and precipitating the solution with alcohol of 60%. The resultant precipitate is treated with a dilute solution of

[1] König and Bömer have shown that the protein nitrogen in meat-extracts has generally been much overestimated. They found a total of 1.17% of protein nitrogen in Liebig Company's extract, which is equivalent to 7.41 of total proteins (mostly albumose).

potassium hydroxide and the solution obtained treated by Pflüger's method (page 284).

Kemmerich states that meat-extract is free from dextrin, sugar, and similar substances, and contains no substance which is converted into glucose by boiling with dilute sulphuric acid. Cured meat-extracts from sweet pickle meats naturally contain sugar.

The salts of meat-extracts have already been considered. They consist chiefly of *earthy phosphates* and *potassium chloride* and *acid phosphate.* Lactates and other organic salts of potassium are also present, and on ignition of the residue obtained by evaporating the extract are of course converted into carbonates. The acid potassium ortho-phosphate is also decomposed with formation of metaphosphate ($KH_2PO_4 = KPO_3 + H_2O$). These reactions necessarily affect the amount and composition of the ash, and should be borne in mind.

Taking the proportion of lactic acid in fresh meat at 0.06%, and assuming that 34 parts of meat are required for the production of 1 part of extract, the proportion of lactic acid in the latter would be 2.04%.

Cured meat-extracts contain besides the salts enumerated, sodium chloride and potassium nitrate. The latter may be estimated by the Schloesing-Wagner method, after a qualitative test using diphenylamine in sulphuric acid.

Extraneous Matters.—In addition to *meat-fibre*, the detection and estimation of which has already been described (page 407), other foreign matters are present in certain commercial preparations classed broadly as meat-extracts. In the table on page 398 there are several instances of preparations containing *glycerin*, and one in which a *vegetable extract* was present. *Albumin* and *gelatin* are sometimes added as such. *Glucose* and *milk-sugar* are sometimes present.

Alcohol is an occasional constituent of "meat-juices."

It is not practicable to draw a sharp distinction between meat-extracts and the so-called "peptones" of commerce.

Boric acid was formerly added to meat-extracts as a preservative, and, some years since, was found by Allen in notable quantity in a widely-used preparation. A process proposed by C. Fresenius and Popp (abst. *Analyst*, 1897, **22**, 282) and applied by them to the examination of sausages, etc., may be employed for the estimation of boric acid in meat-extracts. An amount of the extract corresponding to about 3 grm. of dry substance should be concentrated to a syrup, if necessary, and mixed in a mortar with from 40 to 80 grm. of recently ignited sodium sulphate. The mixture is heated in the water-oven for about an hour, and as soon as the mass is dry some more sodium

sulphate is added, and the whole reduced to a fine powder. This is digested with 100 c.c. of cold methyl alcohol for 12 hours, with frequent shaking, after which the alcohol is distilled off. As a rule the boric acid passes over completely in one distillation, but it is desirable to extract the residue a second time, using 50 c.c. of methyl alcohol. The distillate is made up to 150 c.c., and 50 c.c. treated with 75 c.c. of water and 25 c.c. of pure glycerol. The mixture is titrated with $N/10$ solution of sodium hydroxide (free from carbonate), using phenolphthaleïn as an indicator. A pale-rose colour indicates the end of the titration. When it appears, some more glycerin should be added, and if the colour is not permanent the titration is continued till that point is attained. The volume of alkali used (in c.c.) multiplied by 0.0031 gives the boric acid, H_3BO_3 (in grams), in the volume of the distillate titrated. Borates will be dissolved out of the organic matters by the methyl alcohol, but will not pass over with the free boric acid. They may be determined in the usual manner in the methyl alcoholic extract, after evaporation, ignition, etc.

Poultry.

The method of estimating boric acid given under Preservatives in Meat, page 371, may also be used for its estimation in meat-extract.

The flesh of the common fowl is of two kinds; the light meat is found principally about the breast and the dark meat chiefly in the leg and wing muscles. The colour of the dark flesh is of course due to the presence of a larger quantity of blood pigment than is present in the lighter flesh, but from an analytical standpoint the chief differences lie in the amounts of fat and cold-water extractives present. The average of a large number of analyses shows that the light meat contains 1.5% fat and the dark meat 6%, and that the light meat contains more extractives than the dark, in part conditioned on the lower fat content. The tables which follow give more information on this subject.

Analysis of Poultry.—The general methods of analysis already given for fresh meats (pages 295 *et seq.*) apply equally well to poultry. In sampling it is advisable to separate the white from the dark meat and to analyse each separately, since otherwise irregular results will be obtained owing to the varying proportion of the two kinds of meat in different individuals and also to the varying percentages of fat in the two kinds of meat. The sampling must be done carefully by hand, the visible fat, skin and tendons being removed and analysed apart from the muscular tissue if this is desired.

Composition of Poultry.—The following table shows the composition of fresh killed poultry, the analyses being conducted according to

COMPOSITION OF LIGHT AND DARK MEAT OF FRESH-KILLED POULTRY.

Sample No.	Dressed weight	Kind of meat	Moisture %	Ash %	Fat %	Total N. %	Ammoniacal N.		Free acid in fat %	Cold-water extract						
							Method 1, %	Method 2, %		Total solids %	Ash %	Total N. %	Coagulable N. %	Albumose N. %	Meat base N. %	Acid as lactic ac. %
1.	1531 grm.	Light	73.56	1.32	0.72	3.82	0.037	0.013	Avg. 0.42	8.25	1.20	1.155	0.620	0.030	0.506	0.97
	1224 grm.															
	1262 grm.	Dark	73.86	1.26	2.08	3.23	0.038	0.010		5.49	1.20	0.661	0.306	0.028	0.344	0.54
2.	1791 grm.	Light	73.69	1.35	1.87	3.85	0.037	0.013	0.27	7.59	1.27	1.080	0.538	0.020	0.533	0.95
	1495 grm.															
	2081 grm.	Dark	74.35	1.29	4.14	3.38	0.037	0.011		5.14	1.20	0.673	0.293	0.023	0.356	0.55
3.	1825 grm.	Light	72.63	1.32	2.24	3.88	0.038	0.013	0.27	8.06	1.21	1.166	0.613	0.026	0.534	0.95
	2343 grm.															
	1718 grm.	Dark	72.91	1.24	5.32	3.35	0.037	0.011		5.19	1.16	0.659	0.302	0.027	0.339	0.53
4.	1815 grm.	Light	73.10	1.31	2.51	4.02	0.040	0.012	0.50	8.10	1.16	1.200	0.624	0.026	0.548	0.90
	2047 grm.															
	2182 grm.	Dark	74.08	1.26	3.87	3.48	0.041	0.010		5.21	1.16	0.683	0.311	0.025	0.350	0.53
Light	Max.	.....	73.69	1.35	2.51	4.02	0.040	0.013	.....	8.25	1.27	1.200	0.624	0.030	0.548	0.97
	Min.	.....	72.63	1.31	0.72	3.82	0.037	0.012	.....	7.59	1.16	1.080	0.538	0.020	0.506	0.90
	Av.	.....	73.24	1.32	1.83	3.89	0.038	0.013	.....	8.00	1.21	1.150	0.599	0.025	0.530	0.94
Dark	Max.	.....	74.35	1.31	5.32	3.82	0.041	0.011	.....	5.49	1.20	0.683	0.311	0.028	0.356	0.55
	Min.	.....	72.91	1.24	2.08	3.23	0.037	0.010	.....	5.14	1.16	0.659	0.293	0.023	0.339	0.53
	Av.	.....	73.89	1.26	3.85	3.36	0.038	0.011	.....	5.25	1.18	0.669	0.303	0.025	0.347	0.54

the methods given on pages 293 *et seq.* Each analysis was made on a sample derived from three Plymouth Rock chickens the dressed weight of each being given in the first column. The free fatty acid estimations were made on the abdominal fat which was taken out, hashed and rendered at a low temperature and filtered.

Cold Storage of Poultry.—It is customary in various parts of the world, especially in North America and Russia, to store poultry in freezers at a temperature below $-9°$ from the season of plenty to the season of scarcity. The sanitary and dietetic aspects of this practice have been carefully investigated during the last few years and the conclusion reached that under proper conditions of storage poultry does not deteriorate when solidly frozen during the usual time of storage. Pennington's experiments lead her to the conclusion that poultry may be kept in good condition for a year (Pennington, Hearings before U. S. Senate Committee on Manufactures relative to Foods Held in Cold Storage, 1911) and the writer's experiments indicate that the time may be extended to at least three years and probably longer. More decomposition is developed in a fowl stored at room temperature (26°) for 24 to 48 hours than when stored below $-9°$ for 5 years. In the latter case decomposition is practically nil. It was formerly considered the best practice to store poultry in the eviscerated condition but it has been shown that if the viscera are left in place and the fowls chilled and frozen at once they will reach the consumer in better condition than when eviscerated.[1] The following tables show the composition of poultry stored in frozen condition at $-9°$ for various periods of time. The chickens were Plymouth Rocks of uniform weight killed at the same time and stored in the same place. The figures present some variations but indicate no progressive change of any kind and in general agree closely with the similar figures for fresh-killed chickens.

The abdominal fat of chickens appears to be more susceptible to hydrolysis than fats from some other sources and it has been thought by some investigators that the percentage of free fatty acids in chicken fat would afford an indication of the length of time the fowl had been held in cold storage. On the contrary the percentage of free fatty acids in chicken fat affords a sure indication of the length of time the bird has been held out of cold storage, either before or after the storage period, since the free acid does not increase appreciably while the bird is solidly frozen during the ordinary period of storage. High fatty acid content indicates storage at the higher temperatures for too long a

[1] See Pennington, The Comparative Rate of Decomposition in Drawn and Undrawn Market Poultry, *Circular* 70, *Bur. Chem. U. S. Dept. Agri.*

COMPOSITION OF FROZEN POULTRY, AT VARIOUS PERIODS OF STORAGE.

Age	Dressed weight	Kind of meat	Moisture %	Ash %	Fat %	Total N. %	Ammoniacal N. Method 1, %	Ammoniacal N. Method 2, %	Free acid in fat %	Cold-water extract: Total solids %	Ash %	Total N. %	Coagulable N. %	Albumose N. %	Meat base N. %	Acid as lactic ac. %
88 days.	.....	Light	72.93	1.41	0.78	4.13	0.036	0.014	.....	8.69	1.21	1.285	0.671	0.025	0.571	1.00
	.....	Dark	71.95	1.25	5.25	3.63	0.032	0.012	.....	5.58	1.16	0.689	0.320	0.028	0.372	0.67
129 days.	1600 grm.	Light	74.60	1.28	2.05	3.78	0.034	0.011	0.45	7.70	1.13	1.126	0.588	0.028	0.519	0.81
	2040 grm.															
	1810 grm.	Dark	73.21	1.24	5.97	3.34	0.031	0.010		5.27	1.16	0.679	0.329	0.023	0.334	0.50
228 days.	1680 grm.	Light	73.88	1.28	1.20	3.76	0.039	0.012	0.36	7.68	1.13	1.160	0.560	0.030	0.554	0.91
	1445 grm.															
	1873 grm.	Dark	72.34	1.22	5.33	3.45	0.036	0.012		5.61	1.16	0.759	0.380	0.026	0.360	0.52
238 days.	1650 grm.	Light	73.34	1.31	1.08	4.08	0.038	0.012	0.50	7.89	1.15	1.152	0.555	0.022	0.575	0.94
	1705 grm.															
	1500 grm.	Dark	73.81	1.22	4.23	3.45	0.035	0.012		5.81	1.11	0.761	0.369	0.024	0.368	0.66
268 days	1882 grm.	Light	72.81	1.27	1.45	4.00	0.040	0.013	0.48	8.10	1.16	1.190	0.606	0.031	0.574	0.97
	1655 grm.															
	1765 grm.	Dark	72.61	1.22	4.77	3.48	0.039	0.012		5.49	1.11	0.732	0.358	0.033	0.359	0.54
700 days	2290 grm.	Light	71.97	1.27	2.64	3.96	0.039	0.017	0.62	8.13	1.13	1.158	0.542	0.021	0.589	0.95
	2157 grm. (2 only).	Dark	69.85	1.18	8.22	3.41	0.036	0.015		5.43	1.06	0.697	0.300	0.031	0.373	0.50
706 days	2245 grm.	Light	73.08	1.24	0.72	3.99	0.038	0.016	0.60	8.18	1.12	1.197	0.602	0.028	0.582	0.96
	2065 grm.															
	1785 grm.	Dark	72.99	1.23	4.99	3.43	0.036	0.015		5.52	1.07	0.715	0.332	0.028	0.368	0.56
740 days	1935 grm.	Light	72.23	1.23	1.65	3.94	0.040	0.015	0.55	8.19	1.11	1.185	0.600	0.025	0.564	0.92
	2210 grm.															
	2230 grm.	Dark	71.23	1.19	6.39	3.37	0.036	0.015		5.38	1.04	0.691	0.313	0.032	0.349	0.53
760 days	1915 grm.	Light	73.13	1.24	1.39	3.95	0.041	0.014	0.60	7.91	1.16	1.162	0.577	0.028	0.560	0.90
	1970 grm.															
	1945 grm.	Dark	73.14	1.21	4.82	3.45	0.040	0.014		5.44	1.14	0.710	0.324	0.031	0.361	0.62
Light	Max.	.....	74.60	1.41	2.64	4.13	0.041	0.017	.....	8.69	1.21	1.285	0.671	0.031	0.589	1.00
	Min.	.....	71.97	1.23	0.72	3.76	0.034	0.011	.....	7.68	1.11	1.126	0.542	0.022	0.519	0.81
	Av.	.....	73.11	1.28	1.44	3.95	0.038	0.013	.....	8.05	1.14	1.179	0.589	0.026	0.565	0.92
Dark	Max.	.....	73.81	1.25	8.22	3.63	0.040	0.015	.....	5.81	1.16	0.759	0.380	0.033	0.373	0.67
	Min.	.....	69.85	1.18	4.23	3.34	0.032	0.010	.....	5.27	1.04	0.679	0.300	0.023	0.334	0.50
	Av.	.....	70.10	1.21	5.55	3.44	0.035	0.013	.....	5.50	1.10	0.714	0.336	0.028	0.360	0.56
Average of fresh chickens for comparson.		Light	73.24	1.32	1.83	3.89	0.038	0.013	.....	8.00	1.21	1.150	0.599	0.025	0.530	0.94
		Dark	73.80	1.26	3.85	3.36	0.038	0.011	.....	5.25	1.18	0.669	0.303	0.025	0.347	0.54

period, and is, therefore, an excellent indication of improper handling. While no absolute limit can be set it is safe to say that a free fatty acid content below 1% in the abdominal fat of poultry is indicative of good handling and above 3% in evidence of bad handling.

(*A*) PERCENTAGE COMPOSITION OF FRESH CHICKEN MUSCLE.

Sample	Kind of meat	Water	Fat	Ash	Protein (N × 6.25)	Creatine (N × 3.11)	Total solids	Sum of constituents determined
No. 66. Plymouth Rock broiler.	Light.	75.50	0.49	1.17	23.46	1.10	24.50	97.92
	Dark..	71.75	2.40	1.21	21.40	0.827	28.25	101.33
No. 68. Plymouth Rock young roaster or broiler.	Light.	75.73	0.17	1.33	21.84	1.01	24.27	100.08
	Dark..	75.86	1.38	1.49	21.07	0.64	24.14	100.44
No. 73. Plymouth Rock roaster.	Light.	73.30	0.51	1.24	22.52	0.920	24.70	100.92
	Dark..	74.48	2.88	1.18	20.69	0.743	25.52	99.97
No. 78 Rhode Island Red roaster.	Light.	73.56	0.98	1.26	23.50	1.01	26.44	100.31
	Dark..	73.02	2.99	1.35	23.13	0.64	26.99	101.13
No. 86. Rhode Island Red large broiler.	Light.	75.01	0.53	1.21	21.95	1.02	24.99	99.13
	Dark..	75.94	2.15	1.13	19.77	0.796	24.06	99.78
White, Max.		75.73	0.98	1.33	23.50			
Min.		73.30	0.17	1.17	21.84			
Dark, Max.		75.94	2.99	1.49	23.13			
Min.		71.75	1.38	1.13	19.77			

(*B*) ANALYSIS OF FAT OF FRESH CHICKEN.

Determinations	Plymouth Rock broiler, No. 66	Plymouth Rock broiler, No. 68	Plymouth Rock broiler, No. 73	Rhode Island Red roaster, No. 78	Average	Max.	Min.
Iodine number	62.6	71.2	62.7	61.6	64.4	71	61
Saponification number.	188.2	176.7	190.2	176.8	181.4	190	177
Acid value	0.5	1.5	0.8	0.5	0.7	1.5	0.50
Ester value	181.7	175.2	189.4	176.3	180.6	189	175
% of free acid calculated as oleic.	0.25	0.76	0.40	0.25	0.41	0.76	0.25
Hehner number	86.8	84.25	88.7	85.79	86.36	89	84
Index of refraction at 35°.	.....	.....	.....	1.4566	.....	.....	.....

(*C*) PERCENTAGE OF NITROGEN IN MUSCLE OF FRESH CHICKEN.

Light Meat.

Determinations	No. 66.	No. 68	No. 73	No. 78	No. 86	Average	Max.	Min.
Total nitrogen	4.11	3.82	3.90	4.0	3.84	3.94	4.11	3.82
Total nitrogen in aqueous extract.	0.920	0.954	0.995	0.923	0.923	23.91	0.995	0.920
	22.40	25.00	25.5	22.65	24.03			
Coagulable nitrogen in aqueous extract.	0.338	0.408	0.534	0.402	0.371	10.42	0.534	0.338
	8.23	10.7	13.7	9.90	9.66			
Albumose nitrogen	0.0269	0.0290	0.0181	0.0188	0.0392	0.675	0.3920	0.011
	0.655	0.775	0.465	0.462	1.02			
Amino acid nitrogen	0.355	.....	0.296	0.325	0.328	8.27	0.355	0.296
	8.65	.....	7.90	8.00	8.55			
Peptone nitrogen (by difference).	0.200	.....	0.147	0.177	0.185	4.19	0.200	0.147
	4.87	.....	3.76	4.35	4.81			
Nitrogen insoluble in water (by difference).	3.19	2.86	2.90	3.15	2.917	76.09	3.19	2.86
	77.60	74.8	74.5	77.5	76.00			

Dark Meat.

Determinations	No. 66	No. 68	No. 73	No. 78	No. 86	Average.	Max.	Min.
Total nitrogen.....	3.69	3.58	3.55	3.91	3.42	3.63	3.91	3.42
Total nitrogen in aqueous extract.	0.659	0.704	0.639	0.569	0.645	17.73	0.704	0.569
	a 17.85	19.68	18.00	14.30	18.85			
Coagulable nitrogen in aqueous extract.	0.291	0.320	0.331	0.252	0.262	8.05	0.331	0.252
	7.89	8.93	9.34	6.45	7.66			
Albumose nitrogen.....	0.0235	0.0205	0.0112	0.0103	0.0215	0.438	0.0235	0.0103
	0.637	0.573	0.316	0.263	0.628			
Amino acid nitrogen.....	0.266	.....	0.239	0.208	0.256	6.69	0.266	0.208
	7.21	.....	6.75	5.33	7.48			
Peptone nitrogen (by difference).	0.087	.....	0.057	0.099	0.106	2.39	0.106	0.057
	2.36	.....	1.60	2.56	3.1			
Nitrogen insoluble in water (by difference).	3.03	2.88	2.91	3.34	2.77	82.27	3.34	2.77
	82.00	80.5	82.1	85.5	81.3			

a All figures in bold-faced type are percentages based on total nitrogen.

(*D*) NITROGENOUS CONSTITUENTS OF FRESH CHICKENS (CALCULATED ON WATER-ASH-FAT-FREE BASIS).

Light Meat.

Determinations	No. 66	No. 68	No. 73	No. 78	No. 86	Average
	%	%	%	%	%	%
Total nitrogen.....	19.74	16.77	16.95	16.80	16.38	17.32
Total nitrogen in aqueous extract....	4.43	4.19	4.33	3.81	3.94	4.14
Coagulable nitrogen in aqueous extract.	1.626	1.79	2.32	1.66	1.58	1.77
Albumose nitrogen.....	0.110	0.130	0.0778	0.0788	0.167	0.112
Amino acid nitrogen.....	1.46	.....	1.29	1.34	1.40	1.37
Peptone nitrogen (by difference).....	0.962	.....	0.639	0.409	0.790	0.7
Nitrogen insoluble in water (by difference).	15.31	13.97	12.62	12.99	12.42	13.46

Dark Meat.

Determinations	No. 66	No. 68	No. 73	No. 78	No. 86	Average
Total nitrogen.....	13.90	16.65	16.50	17.25	16.42	16.14
Total nitrogen in aqueous extract....	2.49	3.27	2.97	2.51	3.10	2.86
Coagulable nitrogen in aqueous extract.	1.10	1.49	1.62	1.11	1.26	1.31
Albumose nitrogen.....	0.0890	0.0953	0.0472	0.0455	0.101	0.075
Amino acid nitrogen.....	1.008	.....	0.968	0.916	1.23	1.030
Peptone nitrogen (by difference).....	0.328	.....	0.265	0.437	0.509	0.384
Nitrogen insoluble in water (by difference).	11.42	13.38	13.53	14.74	13.32	13.27

Analyses of Cold-stored Chickens.—Pennington (*Bull.* 115, *Bur. Chem. U. S. Dept. Agric.*) reports several analyses of fresh chickens, shown in the above tables and draws particular attention to the comparatively low acid values shown in Table B. These are as they should be in fresh fatty tissue, the figure 0.76%, calculated as oleic

acid, from the Plymouth Rock broiler No. 68, being high rather than low. Attention is also directed, in Table C, to the relatively large amount of protein in the light meat as compared with that in the dark and its greater solubility in water.

Pennington determined moisture by drying 10 grm. at 100° in a tared lead bottle-cap to practically constant weight; fat by extracting the dried residue in Knorr's apparatus for 16 hours with Squibb's ether; ash by charring 2 to 3 grm. in platinum crucible in a muffle, extracting several times with hot water, filtering, igniting the char and filter and after evaporating the filtrate combining the residues and again igniting in the muffle; total nitrogen by the Kjeldahl-Gunning method; cold-water extract by a complicated modification of the process which seems to have no advantage over that described on page 297; amino acids by the uncertain tannin-salt method; and creatine by the Folin picric acid method.

Eggs.

Proteins of Eggs.—According to W. D. Halliburton, the egg of the hen contains, on an average, in 1,000 parts: shell, 106.9; white, 604.2; and yolk, 288.9. (See page 439.)

Egg shells consist of a keratinoid substance infiltrated with calcium carbonate and traces of calcium phosphate and magnesium carbonate.

The colouring matter is a bile pigment.

White of Egg.—The white of eggs consists of a semi-fluid material of alkaline reaction, contained in a double skin or net-work of firmer fibrous substance, which latter is insoluble in hot or cold water, dilute acetie acid or solution of common salt. According to Lehmann, the interstitial semi-fluid substance, or white of egg proper has sp. gr. 1.045 and contains from 85 to 88% of water and an average of 13.3% of solids. Of this, 12.2% consists of proteins, 0.5% of dextrose, 0.66 of ash, with traces of cholesterol, lecithin, fat, alkaline soaps, etc.[1] Hencc white of egg is a nearly pure solution of proteins, the principle of which is that commonly called egg-albumin.[2] A variety of globulin is also

[1] Poleck and Weber (*Poggendorff's Annalen*, 79, 155; 81, 91) found the ash of white of egg to contain: K_2O, 27 to 28%; Na_2O, 23 to 32; CaO, 1.7 to 2.9; MgO, 1.6 to 3.7; Fe_2O_3, 0.4 to 0.5; Cl, 25 to 28; P_2O_5, 3.7 to 4.8; SO_3, 1.3 to 2.6; SiO_2, 0.2 to 2.0; and CO_2, 7 to 9%. Nicklés found a trace of fluorine.

[2] The term "albumen" should be limited to its original signification, namely, the white of egg; the word "albumin" being applied to the most characteristic constituent thereof, and extended to other analogous substances contained in blood-serum, etc. The terms albumen and albumin will then have the same relation to each other as benzol and benzene. In pronouncing the word albumin, it is correct to accentuate the ponultimate, but of late years extensive custom has justified the accentuation of the first syllable.

present in small quantity and may be separated from the albumin by treating the solution with dilute acetic acid or carbon dioxide, or by saturating it with common salt or magnesium sulphate. The globulins from about 6.7% of the total proteins of egg white. (Compare page 33.) Peptones and albumoses appear to be absent from fresh eggs, but make their appearance in increasing amount as the egg becomes stale. According to E. Salkowski (abst. *J. Chem. Soc.*, 1894, **1**, 214), if a solution of hen's egg albumin be carefully neutralised by dilute acetic acid, and the liquid precipitated by boiling, a hitherto unobserved albumose is found in the filtrate, and may be precipitated by concentrating the liquid and adding absolute alcohol.

Ovalbumin is the chief protein constituent of egg-white. It is obtained in the filtrate from the precipitation of the globulins by saturating the solution with ammonium sulphate and allowing the solution to evaporate at a relatively low temperature.

The percentage composition of ovalbumin is C, 52.75; H, 7.10; N, 15.51; S, 1.62; P, 0.12; O, 22.90.

When purified ovalbumin crystallises in well-formed needles that have been obtained to the extent of 50% of the proteins of the egg-white. In a 2.5% aqueous solution coagulation takes place at 64°. With more dilute solutions a higher temperature is required, at 0.5% solution coagulation does not take place at all. With saline solutions the temperatures of coagulation are raised. The specific rotation of ovalbumin is $[a]_D = -30°$.

According to Osborne and Campbell (*J. Amer. Chem. Soc.*, 1900, **22**, 422–450) besides ovalbumin and the globulins, other protein constituents of egg-white are *conalbumin* and *ovomucin*.

The non-crystallisable fractions after obtaining ovalbumin have a higher rotation and sulphur content. By heating to 65° *conalbumin* is obtained. Its specific rotation is $[\alpha]_D = (\text{about}) - 36°$. *Ovomucin* is a glycoprotein, which forms about 10% of the protein constituents of the egg-white. It is found in the residue after all other proteins have been removed by heat coagulation.

White of egg, when evaporated to dryness at 60°, yields from 12 to 13% of solid albuminous residue, having a density of about 1.314 and losing 4% of water on further heating to 140°, without thereby becoming insoluble. The residue yields about 7% of ash on ignition, consisting chiefly of sodium chloride and carbonate, with calcium phosphate.

Commercial Egg Albumin.—A method of examining this preparation has been published by P. Carles (*J. Pharm. Chem.*, 1897, **6**, 102; abst. *J. Chem. Soc. Ind.*, 1897, p. 767).

Egg-albumin.—When white of egg is beaten up thoroughly with water, the albumin and salts pass into solution, while the insoluble membranous matter may be strained off. The albumin may be partially separated from the soluble salts by dialysis,[1] or by precipitating the liquid with basic lead acetate, decomposing the precipitate by carbonic acid, and removing the last traces of lead by hydrogen sulphide. On very cautiously warming the liquid to 60° incipient coagulation occurs, and the first flakes of albumin carry down with them every trace of lead sulphide, leaving the liquid perfectly colourless. On evaporating the solution at a temperature below 40°, and completing the desiccation in shallow trays, the albumin is obtained in the form of transparent, pale yellow, horny scales, which may be reduced to a yellowish-white powder. In the solid state it may be kept without change, but the solution readily putrefies.

Albumin so obtained has a sp. gr. of 1.262, and is tasteless, odourless, and neutral in reaction. It dissolves slowly in pure water, but more readily in presence of a little neutral salt or a trace of free alkali. The solution is glairy.

The albumin of blood-serum closely resembles egg-albumin, but the two substances are not identical. The differences between them are detailed on page 20.

The coagulation of albumin is fully described on page 54.

If very dilute and added in small quantity only, the majority of mineral acids do not precipitate cold albumin solutions; but larger proportions of acid precipitate the albumin completely. Nitric acid acts most strongly, while the precipitate produced by hydrochloric acid is soluble in excess, and on diluting the resultant solution with water a precipitate is produced which, when separated and freed from the mother-liquor, dissolves in water and exhibits the reactions of acid-albumin.

Cold solutions of egg-albumin are not precipitated under ordinary circumstances by carbonic, acetic, tartaric, or orthophosphoric acid, but in presence of a certain proportion of chloride of sodium, or other neutral salt, precipitation ensues. Hence common salt will precipitate a solution of albumin in acetic acid, the precipitate being soluble in pure water if heating has been avoided.

According to F. Blenn (*Zeit. Anal. Chem.*, 1896, **22**, 127; abst. *J. Chem. Soc.*, 1896, 1, 658), if white of egg be diluted with water, the

[1] Albumin obtained by dialysing white of egg or blood serum always retains about 1% of mineral matter. An ash-free product has been obtained from white of egg by Hofmeister, but from its method of preparation it cannot be regarded as unchanged albumin (see E. Harnack, *Ber.*, 1890, 23, 3745).

precipitated globulins filtered off, and a little formaldehyde added to the filtrate, the albumin will be found to have lost its power of coagulating, and to have undergone conversion into a substance exhibiting reactions distinct from those of any known protein.

Egg Oil.—A very complete analysis of the oil of egg-yolk has been published by M. Kitt (*Chem. Zeit.*, 1897, **21**, 303; abst. *J. Soc. Chem. Ind.*, 1897, p. 448).

Yolk of Egg.

The solids of egg-yolk consist chiefly of a mixture of proteins, together with a considerable proportion of fat. Other constituents are small quantities of colouring matter, dextrose, cholesterol, and a very considerable proportion of lecithin (Vol. 7). According to Gobley, yolk of egg has the following composition:

	%		%
Vitellin	15.8	Cholesterol	0.4
Nuclein	1.5	Fats	20.3
Cerebrin	0.3	Coloring matters	0.5
Lecithin	7.2	Salts	1.0
Glycero-phosphoric acid	1.2	Water	51.8

Paladino and Toso (abst. *Analyst*, 1896, 161) state that egg-fat is used in ointments. They find an iodine-number of 81.2 to 81.6. The saponification number is 18.6. Crystals of cholesterol often separate from the fat.

E. Spaeth (abst. *Analyst*, 1896, 233) gives the following as the analytical characters of the fat of egg-yolk:

Sp. gr. at 100° (water at 15° being 1)	0.881
Iodine-number of fat	68.48
Reichert-Meissl value	0.66
Refractive-index at 25° (on Zeiss' scale)	68.5
Melting-point of fatty acids	36°
Iodine-number of fatty acids	72.6

Phosphorus content = 0.6% (equivalent to 15.04% lecithin).

Spaeth proposes to employ these data for ascertaining whether pastry and other flour-products have been colored by yolk of egg, or by saffron, picric acid, etc. It would be a simpler and more certain plan to examine the material directly for such colouring matters.

The ash of the yolk of eggs has the following composition:

Na_2O	5.12 to 6.57%
K_2O	8.05 to 8.93%
CaO	12.2 to 13.2 %
MgO	2.07 to 2.11%
Fe_2O_3	1.19 to 1.45%
P_2O_5	63.8 to 66.7 %
SiO_2	0.55 to 1.40%

Lecithin is present both free and in combination with vitellin and may be obtained by repeated extraction of the egg yolks with alcohol, distilling off the alcohol under reduced pressure. Then after adding ether to the syrupy residue acetone is used as a precipitant of the lecithin.

Of these constituents, the fats, chloresterol and glycero-phosphoric acid are extracted by ether.

F. Jean (*Monit. Scient.*, 1892, 561) gives the following as the average composition of three specimens of egg-yolk:

	%
Water (loss at 110°)	52.6
Fatty matter (extract by petroleum-spirit)	28.0
Vitellin (including aqueous extract)	18.0
Ash	1.4

The predominating protein of egg-yolk is a globulin called vitellin. Albumin is also present in small quantities, together with nuclein, with which last substance the iron of egg-yolk is in combination. The proteins found by Valenciennes and Fremy in the yolk of the eggs of fishes, and called by them ichthin, ichthulin, and emydin, probably consisted of mixtures of vitellin with nuclein and lecithin.

Vitellin is insoluble in water, and is obtained as a white granular residue on extracting egg-yolk with large quantities of ether. It closely resembles myosin, the globulin of muscle, and may be purified by similar means. It differs, however, from other globulins in being soluble in a saturated solution of common salt. Vitellin may be purified by repeatedly dissolving it in a 10% solution of common salt and precipitating by excess of water. The neutral solution of vitellin in very dilute brine coagulates when heated to 70° to 75°. Vitellin appears to exist in egg-yolk in combination or intimate association with lecithin and nuclein.

Nuclein closely resembles mucin (page 685) in its physical characters, but contains a notable proportion (1.89 to 2.28%) of phosphorus, and no sulphur. It is probable that numerous varieties of nuclein exist, all being compounds of simple proteins with nucleic acid. (See page 73.)

Plant-vitellin, extracted by dilute solution of common salt from the seeds of oats, maize, peas, white mustard, etc., agrees in all its characters with egg-vitellin.

Analysis of Commercial Yolk of Egg.—Salted yolks of eggs, either alone or mixed with borax, are largely employed for dressing hides in the tanning process.

For the analysis of such products, F. Jean (*Monit. Scient.*, 1892, 561;

abst. *J. Soc. Chem. Ind.*, 1892, **11**, 941) treats 10 grm. of the sample with a few drops of acetic acid, and evaporates the mixtures slowly, with occasional stirring, at a temperature of 50° to 60°. The drying is completed at 110°, and the residue weighed as *dry extract*, the loss being taken as *water*. The solids are powdered and extracted in a Soxhlet-tube with hot petroleum-spirit, the solution being evaporated and the residual fatty matter weighed. The residue insoluble in petroleum-spirit is freed from the solvent by a current of air and then extracted with boiling distilled water, and the solution thus obtained evaporated. The total ash may be determined in the usual manner, but in presence of borax it is difficult to avoid loss of chlorine. Hence, for the determination of the chlorides Jean recommends that the aqueous extract of the sample should be treated with tannin, and an aliquot part of the filtered liquid concentrated, acidified with nitric acid, and precipitated with silver nitrate. In another portion of the clarified aqueous extract the sulphates, borates, etc., may be determined. Jean gives the following examples of analyses made by the above method:

Analytical data	A	B	C	D	E
Water	58.54	48.910	52.694	46.60	50.76
Ash	18.50	17.468	18.740	16.91	15.13
Oil (petroleum ether extract)	14.23	18.840	15.550	18.52	19.78
Vitellin (insoluble)	14.23	13.840	11.460	13.78	12.87
Aqueous extract	10.34	0.942	1.556	1.24	1.46
	100.00	100.000	100.000	100.00	100.00

Saline matters	A	B	C	D	E
Normal ash	1.10	1.112	1.07	1.112	1.112
Sodium chloride	16.71	14.850	17.80	14.420	13.080
Boric acid, etc.	0.78	1.500	17.80	1.478	0.938

Jean calculates the proportion of *pure yolk* in the samples from the fat, on the assumption that the normal proportion of this is constant at 28%. The *added salts* are deduced from the excess over the normal ash corresponding to the pure yolk present. The *added water* is the excess over the quantity corresponding to the fat (that is, $\frac{52.6}{28}$ of the petroleum ether extract). The "*albumin in excess*" is the difference between the "vitellin" found and that corresponding to the fat present, on the assumption that the ratio between them is constant. In this manner, Jean deduced the following as the composition of the samples of egg-yolk in question.

	A	B	C	D	E
	%	%	%	%	%
Pure yolk	50.80	67.000	55.00	65.00	70.00
Albumin in excess	1.19	0.890	3.01	3.13	1.63
Added salts	17.40	14.782	18.74	15.80	14.01
Added water	30.52	17.328	23.25	15.98	13.76

In the opinion of Allen, the results of Jean can only be regarded as rough approximations. Phosphorus is a constituent of egg-yolk that can be given fairly accurately as a constant; according to Bein (*Ber*, **23**. 423) 1.129 grm. of phosphorus is equivalent to 100 grm. of egg-yolk.

Estimation of Phosphorus.—This is made by igniting with a little pure sodium carbonate and sodium nitrate and precipitating by means of ammonium molybdate after acidifying with nitric acid in the usual way.

Eggs in Commerce.—Eggs, particularly those of the common fowl have always been an important article of diet, but recently they have assumed a new importance in commerce owing to the fact that they are marketed in enormous numbers and also because they are held in cold storage from the season of plenty to the season of scarcity. In England eggs are received in large numbers from Russia, New Zealand, Australia, and Canada. They are usually packed in one or more layers in large flat boxes between layers of straw or excelsior. In the United States of America it is customary to pack eggs in wooden cases 30 dozen to the case. Each egg stands on end in a pasteboard cell and is separated from its neighbors by pasteboard partitions. The case is made up of 35 layers or tiers in two divisions of "fillers" 6 dozen eggs to a layer, or 3 dozen to the filler. 400 of these cases or 120,000 dozen eggs make one carload. The eggs are placed in the cold storage rooms in the cases, and the rooms are held at a carefully regulated temperature and relative humidity. The temperature is kept as low as possible without freezing the egg, and the humidity as high as possible without causing a growth of mould on the surface of the egg shells. If the humidity is too low there will be considerable evaporation of moisture from the interior through the shell and the egg will lose in weight and its appearance (when broken) will be altered. On the other hand if the humidity is too high a growth of mould will rapidly appear on the shells. It is considered good practice to hold the temperature at about 1° to −2° and the humidity at about 80% of saturation at this temperature as determined by a sensitive sling psychrometer graduated in 1/10 degrees Fahrenheit. The humidity maintained differs in different establishments.

The cold storage of eggs in the unfrozen condition is very different from that of meats in the frozen condition. In the former case the growth and activity of bacteria and the activity of enzymes are retarded but not stopped. When good eggs (April eggs are considered best in North America) are promptly placed in cold storage, they can be held in excellent condition for about one year. However, cold storage eggs decompose more rapidly than fresh eggs when they are removed from storage to higher temperatures.

Composition of Eggs.—Cook (*Bull.* 115, *Bur. Chem., U. S. Dept. Agr.*, 1908) has determined the weights of eggs in lots of 1 dozen each as follows:

Lot No.	*Grams*	*Lot No.*	*Grams*
Dozen No. 2	669	Dozen No. 7	647
Dozen No. 3	639	Dozen No. 8	647
Dozen No. 4	648	Dozen No. 9	612
Dozen No. 5	670	Dozen No. 10	611
Dozen No. 6	641	Dozen No. 11	623

The average weight per dozen calculated from the above table is 640.7 grm. and the average weight per egg 53.4 grm.

Atwater and Bryant (*Bull.* 28, *Revised, Office Exp. Sta. U. S. Dept. Agric.*, 1899) report the following analyses of eggs, uncooked and boiled:

Food material	Number of analyses %	Refuse, %	Water, %	Protein		Fat, %	Total carbohydrates, %	Ash, %	Fuel value per pound %
				N×6.25 %	By difference, %				
EGGS									
Hens', uncooked: *a*									
Edible portion—									
Mininmum	60	.....	67.2	11.6	11.4	8.6	....	0.6	660
Maximum	60	.....	75.8	16.0	17.4	15.1	....	1.6	910
Average	60	.....	73.7	13.4	14.8	10.5	....	1.0	720
As purchased	.....	*b*11.2	65.5	11.9	13.1	9.3	....	0.9	635
Hens', boiled:									
Edible portion—									
Minimum	19	.....	68.6	10.0	10.3	9.1	....	0.6	575
Maximum	19	.....	79.9	15.6	16.8	14.7	....	1.1	880
Average	19	.....	73.2	13.2	14.0	12.0	....	0.8	765
As purchased	.....	*b*11.2	65.0	11.7	12.4	10.7	....	0.7	680
Hens', boiled whites:									
Edible portion—*c*									
Minimum	11	.....	83.1	11.6	12.3	....	....	0.4	235
Maximum	11	.....	87.1	14.8	15.4	0.3	....	1.0	295
Average	11	.....	86.2	12.3	13.0	0.2	....	0.6	250
Hens', boiled yolks:									
Edible portion—*d*									
Minimum	11	.....	48.4	15.3	15.5	32.2	....	1.0	1,685
Maximum	11	.....	50.2	16.8	18.0	34.4	....	1.4	1,745
Average	11	.....	49.5	15.7	16.1	33.3	....	1.1	1,705

a Eggs are difficult of analysis and the discrepancy between the protein by factor and by difference may be due in part to incomplete determination of nitrogen and fat. It is also probable that the factor 6.25 is not correct for eggs. The value of protein by difference is perhaps the more nearly correct and has been used in the computation of the fuel value per pound.

b Average percentage refuse (shell) in 34 samples.

c The ash of the whites of 73 eggs contained 3.3 % phosphoric anhydride.

d The ash of the yolks of 73 eggs contained 57.2 % phosphoric anhydride.

In the uncooked eggs the yolks and whites were mixed and analysed together whereas in the boiled eggs yolks and whites were analysed separately. Therefore no direct comparison can be made between the two sets of analyses.

Langworthy (Eggs and their uses as food. *Farmers Bulletin* 128, *U. S. Dept. Agric.*) has compiled the following figures from various sources, showing the composition of eggs of several sorts of domesticated and wild birds, and two varieties of turtles.

AVERAGE COMPOSITION OF EGGS.

	Refuse	Water	Protein	Fat	Carbohydrates	Ash	Fuel value per pound
	%	%	%	%	%	%	Calories
Hen:							
Whole egg as purchased	11.2	65.5	11.9	9.3	.....	0.9	635
Whole egg, edible portion	.....	73.7	13.4	10.5	.....	1.0	720
White	.....	86.2	12.3	0.2	.....	0.6	250
Yolk	.....	49.5	15.7	33.3	.....	1.1	1,705
Whole egg boiled, edible portion.	.....	73.3	13.2	12.0	.....	0.8	765
White-shelled eggs as purchased.	10.7	65.6	11.8	10.8	.....	.6	675
Brown-shelled eggs as purchased.	10.9	64.8	11.9	11.2	.....	0.7	695
Duck:							
Whole egg as purchased	13.7	60.8	12.1	12.5	.....	0.8	750
Whole egg, edible portion	.....	70.5	13.3	14.5	.....	1.0	860
White	.....	87.0	11.1	0.03	.....	0.8	210
Yolk	.....	45.8	16.8	36.2	.....	1.2	1,840
Goose:							
Whole egg as purchased	14.2	59.7	12.9	12.3	.....	0.9	760
Whole egg, edible portion	.....	69.5	13.8	14.4	.....	1.0	865
White	.....	86.3	11.6	0.02	.....	0.8	215
Yolk	.....	44.1	17.3	36.2	.....	1.3	1,850
Turkey:							
Whole egg as purchased	13.8	63.5	12.2	9.7	.....	0.8	635
Whole egg, edible portion	.....	73.7	13.4	11.2	.....	0.9	720
White	.....	86.7	11.5	0.03	.....	0.8	215
Yolk	.....	48.3	17.4	32.9	.....	1.2	1,710
Guinea fowl:							
Whole egg as purchased	16.9	60.5	11.9	9.9	.....	0.8	640
Whole egg, edible portion	.....	72.8	13.5	12.0	.....	0.9	755
White	.....	86.6	11.6	0.03	.....	0.8	215
Yolk	.....	49.7	16.7	31.8	.....	1.2	1,655
Plover:							
Whole egg as purchased	9.6	67.3	9.7	10.6	.....	0.9	625
Whole egg, edible portion	.....	74.4	10.7	11.7	.....	1.0	695
Fresh-water turtle eggs	.....	65.0	18.1	11.1	.....	2.9	778
Sea-turtle eggs	.....	76.4	18.8	9.8	.....	0.4	738

Greenlee (*Circular* 83, *Bur. Chem., U. S. Dept. Agric.*) gives the following figures showing the percentage of shell, yolk and white in fresh and stale eggs.

The figures for the stale egg plainly show the drying effect which occurs, chiefly in the white, when kept for some time.

PERCENTAGE OF SHELL, WHITE AND YOLK IN FRESH AND STALE EGGS.

Fresh: Experiment number and condition	Weight of eggs	Shell	Yolk	White
Fresh:	Grm.	%	%	%
I	54.3153	10.13	31.35	58.52
II	58.5077	9.57	30.81	59.62
III	54.1700	10.83	30.00	59.17
IV	61.9017	10.53	29.40	60.07
Average	57.2237	10.26	30.39	59.35
Stale:				
I	51.3885	11.06	33.12	55.82

Changes in Eggs during Cold Storage.—The most noticeable change in the composition of eggs during storage is a loss of moisture, the principal loss being sustained by the whites. If held too long the flavour is impaired although under good conditions this is not noticeable in one year's time by careful comparison with perfectly fresh eggs. Cook (*Bull.* 115, *Bur. Chem., U. S. Dept. Agric.*, 1908) reports the following losses in weight in eggs stored for different periods at 0.5°—a rather high temperature for storage.

LOSS OF WEIGHT PER DOZEN EGGS PLACED IN COLD STORAGE MAY 24, 1906, TEMP. 0.5° (33° F.).

Time of storage	Original weight	Final weight	Loss in weight	
	Grm.	Grm.	Grm.	%
1906				
September 7, 3.5 months	623	602	21	3.4
1907				
January 7, 7.5 months	611	578	33	5.4
June 17: 12.6 months	612	565	47	7.7
October 16, 16.6 months	647	582	65	10.0
1908				
January 14, 19.6 months	647	582	65	10.0

Under good conditions of storage the loss of weight should not be greater than 5% in twelve months although in many cases the shrinkage in different warehouses amounts to as much as 10%.

The difference in moisture content between fresh and stored eggs shows itself in another way. When fresh eggs are boiled a loss in weight occurs, owing probably to the contraction of albumin during coagulation, whereas stored eggs which have lost weight, gain weight when boiled.

Greenlee (*Circular* 83, *Bur. Chem., U. S. Dept. Agric.*, 1911) has

PERCENTAGE CHANGE OF THE MOISTURE CONTENT OF WHITE AND YOLK ON HOLDING AT DIFFERENT TEMPERATURES FOR VARYING PERIODS.

Experiment 196 (0°)			Experiment 197 (0°)			Experiment 356 (0°)			Experiment 357 (0°)		
Age in days	White	Yolk	Age in days	White	Yolk	Age in days	White	Yolk	Age in days	White	Yolk
41	87.42	49.15	43	87.54	48.80	0	88.25	47.35	14	88.75	47.17
76	87.15	49.77	83	86.42	49.25	14	87.59	47.87	21	88.06	47.54
166	86.65	50.25	197	86.30	50.54	35	87.55	48.05	35	87.94	48.08
201	86.19	49.73	268	85.96	50.81	49	87.10	49.35	49	88.16	48.37
266	85.35	50.60	.....	.....	.....	.....	.....	.....	.....	.....	.....

Experiment 363 (11°)			Experiment 355 (18°)			Experiment 334 (21°)			Experiment 336 (26°)		
Age in days	White	Yolk	Age in days	White	Yolk	Age in days	White	Yolk	Age in days	White	Yolk
0	88.24	46.63	0	87.66	47.64	0	87.60	47.17	0	87.75	47.97
14	87.57	47.80	7	86.71	48.96	6	87.09	48.53	7	86.98	49.00
35	87.44	49.39	18	86.11	50.28	13	86.63	49.13	11	86.51	49.72
.....	.....	.....	34	85.99	50.56	19	86.45	49.26	15	86.77	49.78
.....	.....	.....	.....	.....	.....	26	85.16	50.27	19	85.89	50.44
.....	.....	.....	.....	.....	.....	33	85.09	50.19	.....	.....	.....
.....	.....	.....	.....	.....	.....	40	84.74	50.60	.....	.....	.....

studied the moisture losses of eggs stored at different temperatures. He used white Leghorn eggs obtained from a poultry farm, and held them no longer than 24 hours before commencing the experiments. Those held at 0° (32° F.)—a rather high temperature for storage—were kept in the standard 30-dozen egg crate while those held at higher temperatures were in pasteboard cartons each holding one dozen. After analysing the fresh eggs, samples from the lots held at 0° were analysed at intervals of from one to three months and from the lots stored at higher temperatures at intervals of 3 to 10 days. Two dozen eggs were usually taken for a sample.

Greenlee's comments on the figures given in the table are as follows:

"The decrease in moisture in the white appears to be easily accounted for by evaporation to the external atmosphere, but the loss in weight as shown by means of the balance is not sufficient to account for the entire decrease in the percentage of moisture. This fact, together with the increased moisture in the yolk, suggests a transfer of water from white to yolk.

"As far as the results given in the table indicate, it cannot be definitely proved that water passes from the white to the yolk. The same results would be obtained if the white took up solids from the yolk, and the yolk would apparently increase in water if, during the process of desiccation in the determination of solids in the oven at 100°, some of the solids were volatilised. The two chief constituents in the yolk, aside from water, are fat and protein. A determination of the Reichert-Meissl number of the extracted fat indicates that no increase of volatile fatty acids, from which it may be reasonably assumed that there is no volatilisation of fatty substance. Furthermore, an analysis of the dried sample gives the same percentage of nitrogen as the percentage of nitrogen in the fresh sample calculated on the water-free basis, which would show that there is no loss of nitrogenous or protein matter. By a process of mathematical calculation it can easily be shown that solids have not passed from the yolk to the white. Although the yolk has decreased in the percentage of its solids by about the same number of points that the white has increased, nevertheless, since the percentage of solids in the yolk greatly exceeds that in the white, the amount which it would be necessary for the yolk to lose in order to account for the experimental data would be more than sufficient to raise the percentage of solids in the white to the experimental figures, regardless of the fact that there is almost twice as much white as yolk, and even if there were no loss of moisture to the external atmosphere.

"This phenomenon of a transfer of water from the white to the yolk

may easily be explained by the simple process of osmosis. The yolk, which contains a very high percentage of solids, is surrounded by a membranous tissue called the vitellin membrane, which in turn is surrounded by the egg white, a liquid much more dilute than the yolk. By osmosis the water passes through the membrane from the more dilute to the more concentrated solution until a constant equilibrium is obtained. In the egg this process continues until the vitellin membrane becomes so weak that it breaks, when the white and yolk begin to lose their identity. This action proceeds with such definiteness that by a process of calculation, knowing the original weight of the egg, the loss in moisture to the external atmosphere can be calculated with surprising closeness to the actual loss as shown by the balance."

The following examples are given:

Example I. No. 334 1–7 (40 days at room temperature).
(Fresh eggs = 59.35 % white, 30.39 % yolk, 10.26 % shell.)
561 grm. = original weight of 10 fresh eggs.
561 × 59.35 % = 332.95 grm., original weight of white in 10 fresh eggs.
561 × 30.39 % = 170.49 grm., original weight of yolk in 10 fresh eggs.
332.95 × 12.40 % = 41.28 = solids in white of 10 eggs.
170.49 × 52.83 % = 90.07 = solids in yolks of 10 eggs.
41.28 ÷ 15.26 % = 270.52 = final weight of white.
90.07 ÷ 49.40 % = 182.33 = final weight of yolk.
62.43 grm. = total loss to white (calculated).
11.84 grm. = total gain to yolk (182.33 − 170.49).
50.29 grm. = loss to external atmosphere (calculated).
(Experimental loss to 10 eggs as taken from No. 344–7 shows an average of 49.53 grm.)

Example II. No. 336 1–5 (19 days at room temperature).
556.6 grm. = original weight of 10 fresh eggs.
556.6 × 59.35 % = 330.34 grm., original weight of white in 10 eggs.
556.6 × 30.39 % = 169.15 grm., original weight of yolk in 10 eggs.
330.34 × 12.25 % = 40.46 grm., solids in white of 10 eggs.
169.15 × 53.02 % = 88.01 grm., solids in yolk of 10 eggs.
40.46 ÷ 14.11 % = 286.8 grm., final weight of white.
88.01 ÷ 49.56 % = 177.6 grm., final weight of yolk.
43.54 grm. = total loss to white (calculated).
8.40 grm. = total grain to yolk (calculated).
35.14 = loss to external atmosphere (calculated).
35.83 = average loss to 10 eggs as shown by experiment No. 336–5.

Cook (*loc. cit.*) has made numerous analyses of fresh and cold-storage eggs with the results given in the following tables. Estimations were made on whites and yolks, separately, of both boiled and unboiled eggs, but since the separation of whites and yolks of the unboiled eggs was unsatisfactory, the results were calculated to the whole egg. Moisture, ash ether extract, total sulphur and total phosphorus were determined by the methods of the A. O. A. C. (*Bull.* 107, *Revised, Bur. Chem., U. S. Dept. Agric.*) Lecithin phosphorus and ether extract were estimated in the same portion of sample. The portion was extracted with absolute alcohol for 8 hours directly into the ether extract flask and the total phosphorus estimated in the ether-alcohol extract, and calculated as lecithin phosphorus. Total nitrogen was estimated by the A. O. A. C. Kjeldahl-Gunning method and the amino compounds by the tannin-salt method. (*J. Amer. Chem. Soc.*, 1906,

28, 1485. See also pp. 301 and 409.) Proteose and peptone figures were found by difference.

ANALYSIS OF ENTIRE EGG, UNBOILED, FRESH, AND COLD STORAGE.[1]

Wet Basis.

Descriptions of sample	Solids	Ash	Ether extract	Total sulphur	Total P_2O_5	Total nitrogen
	%	%	%	%	%	%
Fresh eggs	25.28	0.69	.....	.....	.....	1.90
Fresh eggs	27.70	0.95	12.89	0.24	0.67	2.01
Storage 3 1/2 mos	27.29	0.97	.....	0.16	0.39	1.68
Storage 7 1/2 mos	33.56	0.91	.....	0.13	1.00	2.10
Storage 12 2/3 mos	24.53	0.91	.....	0.18	0.58	2.09
Storage 16 2/3 mos	31.62	1.08	12.69	0.20	0.74	2.23
Storage 19 2/3 mos	29.78	1.00	12.15	0.27	0.70	2.26
Maximum	33.56	1.08	12.89	0.27	1.00	2.26
Minimum	24.53	0.69	12.15	0.13	0.39	1.68

Calculated to a Water-free Basis.

Description of sample	Ash	Ether extract	Total sulphur	Total P_2O_5	Total nitrogen
	%	%	%	%	%
Fresh eggs	2.73	.....	.....	.....	7.51
Fresh eggs	3.42	46.54	0.87	2.42	7.26
Storage 3 1/2 mos	3.55	.....	0.59	1.43	6.16
Storage 7 1/2 mos	2.71	.....	0.39	2.98	6.26
Storage 12 2/3 mos	3.71	.....	0.73	2.36	8.52
Storage 16 2/3 mos	3.42	40.13	0.63	2.34	7.05
Storage 19 2/3 mos	3.36	40.80	0.91	2.35	7.59
Maximum	3.55	46.54	0.91	2.98	8.52
Minimum	2.71	40.13	0.39	1.43	6.16

Cook's figures indicate a decrease in coagulable protein and an increase in the proteoses and peptones in the boiled samples. There are marked irregularities in the figures and confirmation should be had from analyses of uncooked eggs held at $-1.5°$ in comparison with eggs held at room temperature (about 21°).

Pennington and Robertson (*Circular* 104, *Bur. Chem., U. S. Dept. Agric.*) have studied the enzymes present in hens' eggs, as influenced by storage and report lipase and catalase present. The presence of pepsin, trypsin, reductase, and oxidase, is doubtful.

Commercial Examination and Classification of Eggs.—The commercial grading of eggs is based upon a number of points, such as colour, size, form, cleanliness of the shell, and freedom from checks and cracks. The common method of ascertaining the quality of the interior of the egg is known as "candling" from the light originally used for the purpose. According to this method a source of light

[1] Whites and yokes analyzed separately and results calculated for total egg.

ANALYSIS OF BOILED YOLKS OF FRESH AND COLD-STORAGE EGGS.

Wet Basis.

Description of sample	Moisture	Ash	Ether extract	Total sulphur	Total P_2O_5	Lecithin P_2O_5	Nitrogen of egg present as—				
							Total	Coagulable	Uncoagulable	Proteoses and peptones	Amino
	%	%	%	%	%	%	%	%	%	%	%
Fresh eggs	48.74	1.08	30.43	0.21	1.31	0.90	2.61	2.412	0.198	0.090	0.108
Fresh eggs	45.39	1.28	33.89	0.23	1.47	1.025	2.63	2.546	0.084	0.008	0.076
Storage 3 1/2 mos	49.02	1.54	31.38	0.20	1.10	0.55	2.50	2.37	0.130	0.064	0.066
Storage 7 1/2 mos	50.49	1.34	36.40	0.16	1.90	.....	2.56	2.424	0.136	0.069	0.067
Storage 12 2/3 mos	52.09	1.41	29.66	0.173	1.293	0.831	2.39	2.199	0.191	0.112	0.079
Storage 16 2/3 mos	64.06	1.92	24.82	0.20	1.35	0.84	2.315	1.117	1.198	1.144	0.054
Storage eggs 19 2/3 mos	50.82	1.66	29.19	0.22	1.28	0.83	2.545	0.865	1.680	1.654	0.026

Calculated to a Water-free Basis.

Description of sample	Ash	Ether extract	Total sulphur	Total P_2O_5	Lecithin P_2O_5	Total P_2O_5 as lecithin P_2O_5	Nitrogen of egg present as—					Nitrogen results in per cent. of total nitrogen			
							Total	Coagulable	Uncoagulable	Proteoses and peptones	Amino	Coagulable	Uncoagulable	Proteoses and peptones	Amino
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Fresh	2.1069	59.3640	0.4097	2.5560	1.7558	68.69	5.0917	4.7054	0.3863	0.1756	0.2107	92.41	7.59	3.45	4.14
Fresh	2.3439	62.0582	0.4212	2.6918	1.8769	69.73	4.8160	4.6621	0.1531	0.0142	0.1389	96.81	3.17	0.29	2.88
Storage 3 1/2 mos	3.0208	61.5535	0.3923	2.5177	1.0789	50.00	4.9039	4.6489	0.2550	0.1255	0.1295	94.79	5.21	2.57	2.64
Storage 7 1/2 mos	2.7065	72.5204	0.3232	3.8377	.....	.....	5.1707	4.8960	0.2747	0.1394	0.1353	94.69	5.31	2.70	2.61
Storage 12 2/3 mos	2.9430	61.9077	0.3611	2.6988	1.7345	64.26	4.9885	4.5899	0.3987	0.2338	0.1649	92.01	7.99	4.68	3.31
Storage 16 2/3 mos	5.3422	69.0595	0.5565	3.7563	2.3372	62.22	6.4413	3.1080	3.3333	3.1830	0.1503	48.25	51.76	49.42	2.34
Storage 19 2/3 mos	3.3754	59.3534	0.4473	2.6027	1.6877	64.84	5.1749	1.7588	3.4160	3.3631	0.0529	33.99	66.01	64.99	1.02

ANALYSIS OF BOILED WHITES OF FRESH AND COLD-STORAGE EGGS.

Wet Basis.

Description of sample	Moisture	Ash	Ether extract	Total sulphur	Total P_2O_5	Nitrogen of egg present as—				
						Total	Coagulable	Uncoagulable	Proteoses and peptones	Amino
	%	%	%	%	%	%	%	%	%	%
Fresh eggs	86.15	0.17	0.07	0.21	0.19	1.815	1.51	0.305	0.233	0.072
Fresh eggs	86.08	0.55	0.16	0.28	.....	1.71	1.417	0.293	0.293	0.000
Storage 3 1/2 mos	87.41	0.95	Trace	0.16	0.057	1.79	1.57	0.22	0.195	0.025
Storage 7 1/2 mos	84.24	0.82	.....	0.19	0.350	2.18	1.789	0.391	0.391	0.000
Storage 12 2/3 mos	85.02	0.76	.....	0.219	0.098	1.98	1.677	0.303	0.191	0.112
Storage 16 2/3 mos	84.53	0.76	0.14	0.21	0.20	1.99	1.63	0.360	0.306	0.054
Storage 19 2/3 mos	83.14	0.48	0.25	0.29	0.13	2.21	1.68	0.530	0.521	0.009

Calculated to a Water-free Basis.

Description of sample	Ash	Ether extract	Total sulphur	Total P_2O_5	Nitrogen of eggs found as—				Nitrogen results in per cent. of total nitrogen				
					Total	Coagulable	Uncoagulable	Proteoses and peptones	Amino	Coagulable	Uncoagulable	Proteoses and peptones	Amino
	%	%	%	%	%	%	%	%	%	%	%	%	%
Fresh eggs	5.1264	0.5054	1.5162	0.7942	13.1047	10.9025	2.2022	1.6823	0.5199	83.20	16.80	12.84	3.97
Fresh eggs	3.9511	1.1494	2.0115	.....	12.2845	10.1795	2.1049	2.1049	.....	82.86	17.14	17.14	0.00
Storage 3 1/2 mos	7.3457	Trace	1.2708	0.4527	14.2176	12.4702	1.7474	1.5487	0.1987	87.77	12.23	10.87	1.36
Storage 7 1/2 mos	4.2030	.....	1.2056	2.2801	13.8325	11.3515	2.4810	2.4810	.....	82.07	17.93	17.93	0.00
Storage 12 2/3 mos	5.0734	.....	1.4619	0.6542	13.2176	11.1950	2.0226	1.2750	0.7476	84.68	15.32	9.63	5.69
Storage 16 2/3 mos	4.9127	0.9050	1.3575	1.9392	12.8636	10.5365	2.3271	1.9780	0.3491	81.91	18.09	15.38	2.71
Storage 19 2/3 mos	2.8470	1.4858	1.7200	0.7711	13.1079	9.9644	3.1435	3.0902	0.0534	76.02	23.98	23.56	0.41

such as an incandescent lamp is placed behind an aperture of slightly less diameter than an egg in a dimly lighted room. The examiner holds the egg against the aperture at the same time turning the egg rapidly back and forth so that the light shines through it and to a certain extent illuminates the interior of the shell. According to the appearance presented an expert can usually tell at once whether the eggs are of good or poor quality and can separate them into a number of classes which are well known in the egg trade. A few inferior eggs usually escape the candler. The following grades of eggs are generally recognised in the United States:

(1) *Extras* and *firsts* are fresh-laid, sound eggs with clean shell of medium or large size. Extras are specially selected and graded according to the colour of the shell, some localities preferring a brown and some a white shell.

(2) *Seconds*, includes all grades not included under (1) and which are not *spots* or *rots*. The following are the principal grades.

(*a*) *Undersized*, are eggs which would be classed as *firsts* except for their small size.

(*b*) *Checks* and *cracks* are eggs whose shells are broken but whose shell membranes are intact.

(*c*) *Leakers*, are eggs whose shells and shell membranes are both broken.

(*d*) *Dirties* are eggs whose shells are soiled.

(*e*) *Weak eggs* have watery whites.

(3) *Spots* are eggs which show a spot in the interior when candled. This may be due to various causes such as mould, blood ring, developing embryo, etc. These eggs are used for manufacturing purposes, chiefly in the leather industry.

(4) *Rots*.—The name is sufficiently descriptive. They are used for the same purposes as spots.

Other types of eggs, also, are met with by the large handler such as eggs with brown yolks (these are perfectly sound), "green" eggs, having a greenish-white, "musty" eggs and "sour" eggs. It is the last three kinds which are most apt to escape the candler.

Methods of Analysis of Eggs.—The chemist who is called on to examine eggs or pass judgment on their quality must of necessity be an egg expert, since their examination presents rather greater difficulties than that of other food products. He must know the commercial classification and grades and have practice in candling. In many cases the bacteriological examination is a necessity, not so much in the case of shell eggs, as of frozen or dried eggs. The general methods

of analysis already given covering, moisture, ash, fat, nitrogen, etc. (page 295), are applicable to eggs. The chemical methods of detecting decomposition such as Eber's sulphide test and estimation of ammoniacal nitrogen are also applicable, but it is generally quicker and equally certain to make use of the sense of smell. *Lecithin* may be estimated by following the ether extraction with absolute alcohol extraction and estimating phosphorus in the residue, which is dissolved in a mixture of 6 parts nitric acid and 1 part hydrochloric acid for the purpose. The final weighing is made as magnesium pyrophosphate after first precipitating as ammonium phosphomolybdate.

Frozen Eggs.—Many eggs arrive at cold-storage warehouses in a cracked, broken, or otherwise damaged condition and in order to avoid the economic waste which would otherwise ensue, the contents of those that are suitable of these eggs are separated and frozen in tinned containers and thus preserved. The demand for frozen eggs has greatly increased during recent years so that at the present time many whole eggs are broken and the contents frozen to supply this trade. There is much to be said in favor of preserving eggs by freezing rather than by storage at temperatures above their freezing points, provided good eggs are used for the purpose.

Pennington (*Circular* 98, *Bur. Chem., U. S. Dept. Agric.*) has studied the preparation of frozen eggs and devised a working system for handling the manufacture in a sanitary manner. She finds that fresh eggs less than one day old, analysed by the Folin aeration method (page 316) show 0.0012% N. "White rots" contain 0.0030% N or more; whereas "market seconds" in the producing section in summer time contain on an average from 0.0017 to 0.0022% N. Cracked eggs or "checks" as they are called in the trade, dirty-shell eggs and other seconds are commonly used by egg breakers. No "spot" eggs of any sort are used by reputable breakers. The conclusions are reached that "properly conducted, the freezing and drying of eggs is an industry which is economically desirable, as long as the centres of egg production and egg consumption are so widely separated, and as long as the poor handling methods bring to the concentrators of the producing sections such enormous numbers of eggs that are wholesome but not available for long hauls."

Desiccated Eggs.—There are several preparations of dried eggs on the market. Some of these are made from perfectly fresh sound eggs and are adapted to household use. Others are made from the lower grades of eggs, but still belong in the edible class, although of inferior quality.

Bacteriological Examination of Eggs.—G. W. Stiles, Jr., has made a bacteriological examination of 10 fresh and 8 cold-storage eggs in connection with the work of Cook (*loc. cit.*) using the following method. After washing the eggs for a few minutes in a 1 to 1,000 mercuric chloride solution or 5% carbolic acid, they were dried with sterile absorbent cotton and placed, large end uppermost, in a small beaker. The air space was then scorched for a few seconds with a gas flame, and an opening immediately made into the cavity with sterile forceps, after removing a sufficient amount of shell without rupturing the membrane below. The membrane was then broken with a hot platinum spatula and 0.5 c.c. of the white quickly removed with a sterile pipette and placed in the usual Petri dishes. The remaining white was then decanted leaving in the shell the unbroken yolk. A small portion of the yolk was then removed for examination with another sterile pipette. Difficulty was experienced in breaking up the respective layers of the egg after the samples were taken. The eggs were held in 0.5° (33° F.) storage, and naturally all the changes including increase in bacteria occurred more rapidly than would be the case at a lower temperature. The tables follow:

BACTERIOLOGICAL EXAMINATION OF FRESH EGGS.

No. of sample	Received for examination	Portion of egg	Number of colonies per c.c. developing on lactose agar at 35° to 37°	Number of colonies per c.c. developing on lactose gelatin at 12° to 15°	Species of bacteria isolated
4067.....	5/24/1906	White....	1	0	Mic. cinnabareus, Flügge.
		Yolk.....	190	3	Myobacterium avium, Streptothrix farcinica, Sarcina lutea.
4068.....	5/24/1906	White....	0	0	
		Yolk.....	0	0	
4069.....	5/24/1906	White....	1	1	Mic. cumulatus?
		Yolk.....	0	0	
4070.....	5/24/1906	White....	1	1	Bact. hæmatoides.
		Yolk.....	240	3	Micrococcus, not classified.
4071.....	5/24/1906	White....	0	0	
		Yolk.....	5	0	B. mesentericus.
4072.....	5/24/1906	White....	0	0	
		Yolk.....	6	0	Saccharomyces, B. Pammelii
B. C. 551....	10/17/1907	White....	1	0	1 fungus.
		Yolk.....	2	1	1 fungoid colony.
B. C. 552....	10/17/1907	White....	0	0	
		Yolk.....	1	0	1 gray fungus.
B. C. 669*a*...	1/16/1908	White....	0	0	
		Yolk.....	1	0	Actinomyces.
B. C. 670....	1/16/1908	White....	0	0	
		Yolk.....	0	0	

a Gelatin plates incubated at 15° to 20°.

BACTERIOLOGICAL EXAMINATION OF COLD-STORAGE EGGS.

No. of sample	Duration of cold storage	Portion of egg	Number of colonies per c.c. developing at room temperatures
4193	3 mos.	White	6,250
		Yolk	3,370
4195	3 mos.	White	1,280
		Yolk	3,260
4282	7.5 mos.	Total egg	0
4283	7.5 mos.	Total egg	1
B. C. 55	11.8 mos.	Total egg	1 fungus.
550	11.8 mos.	Total egg	White fungus at ice-box temperature.
B. C. 667	1 yr. 7 mos. 22 days	White	1 colony.
		Yolk	1 colony.
B. C. 668	1 yr. 7 mos. 22 days	White	0
		Yolk	0

Stiles in collaboration with Bates has also made other and more extensive bacteriological studies of eggs (A Bacteriological Study of Shell, Frozen and Desiccated Eggs; Made under Laboratory Conditions at Washington, D. C., *Bull.* 158 *Bur. Chem. U. S. Dept. Agric.*, 1912). The following tables give the results of these studies:

STRICTLY FRESH SHELL EGGS APRIL TO OCTOBER, 1911.

Number of organisms per c.c., and month	Number of samples of—		Percentage of samples of—	
	Yolk	Albumin	Yolk	Albumin
April, 1911:				
0	37	58	39.78	62.36
1–10	44	27	47.31	29.03
10–25	6	4	6.45	4.30
25–50	2	3	2.15	3.22
50–100	2	0	2.15	0.00
100–250	2	1	2.15	1.07
250–500	0	0	0.00	0.00
500 and higher	0	0	0.00	0.00
May, 1911:				
0	5	16	14.28	45.71
1–10	9	13	25.71	37.14
10–25	5	3	14.28	8.57
25–50	5	1	14.28	2.85
50–100	2	1	5.71	2.85
100–250	3	1	8.57	2.85
250–500	0	0	0.00	0.00
500 and higher	6	0	17.14	0.00
June, 1911:				
0	5	25	6.25	30.12
1–10	16	45	20.00	54.21
10–25	23	8	28.75	9.63
25–50	14	4	17.50	4.82
50–100	14	0	17.50	0.00
100–250	2	1	2.50	1.20
250–500	2	0	2.50	0.00
500 and higher	4	0	5.00	0.00

STRICTLY FRESH SHELL EGGS APRIL TO OCTOBER, 1911. (*Con.*)

Number of organisms per c.c., and month	Number of samples of—		Percentage of samples of—	
	Yolk	Albumin	Yolk	Albumin
July, 1911:				
0	9	38	6.52	27.53
1–10	24	60	17.39	43.47
10–25	24	20	17.39	14.49
25–50	17	11	12.32	7.97
50–100	26	3	18.84	2.17
100–250	15	1	10.87	0.72
250–500	8	2	5.79	1.44
500 and higher	15	3	10.87	2.17
August, 1911:				
0	7	20	5.65	16.12
1–10	22	67	17.74	54.04
10–25	3	10	2.42	8.07
25–50	10	12	8.07	9.67
50–100	16	10	12.90	8.07
100–250	31	5	25.00	4.03
250–500	17	0	13.71	0.00
500 and higher	18	0	14.51	0.00
September, 1911:				
0	5	8	16.66	26.66
1–10	3	18	10.00	60.00
10–25	5	4	16.66	13.33
25–50	8	0	26.66	0.00
50–100	3	0	10.00	0.00
100–250	3	0	10.00	0.00
250–500	2	0	6.66	0.00
500 and higher	1	0	3.33	0.00
October, 1911:				
0	10	19	8.85	16.81
1–10	15	75	13.28	66.39
10–25	19	10	16.81	8.85
25–50	16	4	14.16	3.53
50–100	19	4	16.81	3.53
100–250	18	1	15.95	0.88
250–500	6	0	5.31	0.00
500 and higher	10	0	8.85	0.00

SUMMARY OF RESULTS.

Month	Number of samples	Minimum count		Maximum count		Average count		Percentage of sterile samples	
		Yolk	Albumin	Yolk	Albumin	Yolk	Albumin	Yolk	Albumin
April	93	0	0	180	140	6.2	3.6	39.78	62.36
May	35	0	0	2,750	120	318.6	10.1	14.28	45.71
June	83	0	0	17,500	220	335.1	6.74	6.25	30.12
July	83[1]	0	0	18,600[2]	900	473.2	36.5	6.52	27.53
August	124	0	0	9,300	200	344.2	18.9	5.65	16.12
September	30	0	0	1,400	20	99.66	3.6	16.66	26.66
October	113	0	0	6,000	328	240.2	9.3	8.85	16.81
Total and average.	616	.....	.....	.....	.....	271.7	15.9	.....	.....

[1] Three samples of yolk were broken.
[2] This is the individual yolk containing B. coli.

STRICTLY FRESH WHOLE EGGS, FROZEN AND EXAMINED AT INTERVALS FROM FEB. 14, 1910, TO MAR. 29, 1911.

(Six dozen eggs used, none over 4 days old. B. coli not present in 1 c.c. quantities.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2167	300	300	0	0
2176	400	400	0	1
2213	1,200	1,400	.....	5
2234	1,000	1,000	.....	11
2250	900	700	.....	19
2299	1,000	800	.....	30
2308	500	400	0	37
2376	800	800	.....	45
2396	600	600	.....	50
2429	1,400	1,300	.....	56
2435	1,600	1,200	.....	63
2455	1,000	900	0	71
2556	900	900	.....	80
2583	1,400	700	0	87
2592	1,200	1,200	.....	92
2621	1,700	2,000	.....	100
2643	200	100	.....	109
2659	300	300	.....	115
2769	600	400	0	185
2826	100	100	.....	197
2906	600	200	.....	210
2931	80	50	.....	219
2962	700	700	.....	253
3038	300	400	.....	276
3112	400	200	0	286
3215	100	100	.....	323
3366	200	200	0	407
Maximum average count	.....	.....	.....	1,850
Minimum average count	.....	.....	.....	56

FROZEN ALBUMEN FROM STRICTLY FRESH EGGS, EXAMINED AT INTERVALS FROM FEB. 15, 1910, TO MAR. 29, 1911.

(Four dozen eggs, none over 4 days old.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2168	0	0	0	0
2177	20	10	.....	1
2214	100	100	.....	4
2251	200	100	.....	14
2300	300	200	0	29
2308	400	400	.....	36
2377	160	150	.....	44
2397	200	100	.....	49
2557	600	600	.....	79
2622	500	500	.....	99
2644	100	100	.....	106
2770	200	100	0	182
2826	100	100	.....	194
2907	100	100	.....	206
2932	70	90	.....	216
2963	180	100	.....	240
3040	200	100	.....	262
3113	60	60	0	272
3224	100	100	.....	323
3373	8	7	.....	410
Maximum average count	.....	.....	.....	600
Minimum average count	.....	.....	.....	0
B. coli not present in 1 c.c. quantities.				

FROZEN ALBUMEN FROM COMMERCIAL EGGS, EXAMINED AT INTERVALS FROM FEB. 16, 1910, TO MAR. 29, 1911.
(Four dozen eggs bought on the market as fresh.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2189	60	40	0	0
2253	400	300	.....	14
2302	700	500	0	29
2311	600	500	.....	36
2379	700	500	.....	44
2772	400	200	0	182
2827	100	100	.....	194
2934	300	300	0	216
2965	100	100	.....	240
3037	100	100	.....	262
3115	180	100	0	272
3225	100	100	.....	323
3374	20	10	.....	410

Maximum average count 600
Minimum average count 15
B. coli not present in 1 c.c. quantities.

COMMERCIAL WHOLE EGGS, FROZEN AND EXAMINED AT INTERVALS FROM FEB. 16, 1910, TO MAR. 29, 1911.
(Eight dozen eggs said to have been in cold storage for 8 months.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2188	800	700	(1)	0
2215	900	900	.....	3
2252	1,400	1,000	.....	14
2301	3,600	2,700	0	29
2311	600	500	.....	36
2378	1,200	1,000	.....	44
2398	2,500	1,300	.....	49
2431	4,400	3,000	.....	55
2436	2,700	2,400	.....	62
2457	2,800	1,000	(1)	70
2558	12,000	7,000	.....	79
2585	1,500	1,400	.....	86
2593	2,200	2,000	.....	91
2623	3,000	1,000	.....	99
2645	7,000	7,000	.....	106
2661	2,000	2,000	.....	112
2771	900	800	(1)	182
2831	1,400	400	.....	194
2908	500	300	.....	206
2933	200	100	.....	216
2964	800	800	.....	240
3036	100	.....	.....	262
3114	800	400	(1)	272
3223	900	1,100	.....	321
3367	900	800	.....	406

(1) *B. coli* absent in 0.1 c.c.
Maximum average count 9,500
Minimum average count 100

EGGS CALLED CHECKS AND CRACKS FROZEN AND EXAMINED AT INTERVALS FROM MAY 12, 1910, TO MAR. 29, 1911.
(Six dozen eggs as received by a commercial egg house among current receipts.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2578	17,000	13,000	(1)	0
2626	200,000	20,000	.....	13
2663	140,000	120,000	.....	27
2777	7,000	4,000	(1)	96
2833	30,000	40,000	.....	108
2935	90,000	60,000	.....	120
2966	34,000	31,000	.....	164
3043	70,000	22,000	.....	186
3116	30,000	21,000	(1)	196
3220	10,000	4,000	.....	262
3369	16,000	15,000	.....	317

(1) *B. coli* not present in 0.1 c.c.
Maximum average count 130,000
Minimum average count 5,500

EGGS CALLED "DIRTIES" FROZEN AND EXAMINED AT INTERVALS FROM MAY 12, 1910, TO MAR. 29, 1911.
(Six dozen eggs as received in current receipts by commercial trade.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2579	200,000	140,000	(1)	0
2627	170,000	100,000	.....	13
2664	320,000	.....	.....	27
2778	80,000	20,000	(2)	96
2834	100,000	3,200	.....	108
2912	160,000	80,000	.....	120
2936	90,000	30,000	.....	130
2967	100,000	40,000	.....	164
3042	18,000	.....	.....	186
3221	57,000	14,000	.....	262
3370	50,000	40,000	.....	317

(1) *B. coli* not present in 0.1 c.c.
(2) *B. coli* not present in 0.01 c.c.
Maximum average count 320,000
Minimum average count 18,000

"HOT WEATHER" EGGS[1] FROZEN AND EXAMINED AT INTERVALS FROM AUG. 18, 1910, TO MAR. 29, 1911.
(Four dozen eggs received by a commercial house from current receipts.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2779	700	200	(2)	0
2913	1,000	1,000	.....	25
2937	1,400	1,000	.....	36
2968	5,000	5,000	.....	68
3039	600	900	.....	90
3222	700	1,000	.....	137
3368	400	300	.....	221

[1] By this commercial term was meant rollers or floaters and eggs with watery albumen.
(2) *B. coli* not present in 0.01 c.c.
Maximum average count 5,000
Minimum average count 350

"LIGHT SPOT" EGGS[1] FROZEN AND EXAMINED AT INTERVALS FROM MAY 12, 1910, TO JAN. 4, 1911.

(Four dozen eggs received by a commercial firm.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2580	6,700,000	2,000,000	100,000	0
2647	28,000,000	20,000,000	.....	22
2774	2,500,000	3,000,000	100,000	98
2835	10,000,000	4,400,000	.....	110
2969	8,000,000	6,000,000	.....	166
3041	4,300,000	2,900,000	.....	189
3216	5,000,000	4,000,000	.....	236

"HEAVY SPOT" EGGS[2] FROZEN AND EXAMINED AT INTERVALS FROM MAY 12, 1910, TO MAR. 29, 1911.

(Seven dozen eggs received from the trade as discards.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2581	22,000,000	6,000,000	1,000,000	0
2595	90,000,000	40,000,000	.....	7
2628	150,000,000	150,000,000	.....	13
2648	150,000,000	110,000,000	.....	21
2775	80,000,000	50,000,000	1,000,000	96
2832	78,000,000	70,000,000	.....	108
2914	90,000,000	70,000,000	.....	120
2938	90,000,000	67,000,000	.....	130
2970	120,000,000	34,000,000	.....	164
3177	180,000,000	100,000,000	.....	262
3371	120,000,000	120,000,000	.....	317

EGGS CALLED BLOOD "RINGS"[3] FROZEN AND EXAMINED AT INTERVALS FROM MAY 12, 1910, TO JAN. 4, 1911.

(Four dozen eggs received from the trade as discards from candling.)

Sample No.	Organisms per c.c.		B. coli per c.c.	Days in storage
	At 25°	At 37°		
2582	500,000,000	300,000,000	.....	0
2649	2,160,000,000	2,000,000,000	.....	22
2776	800,000,000	500,000,000	.....	96
2829	310,000,000	380,000,000	.....	108
2971	800,000,000	800,000,000	.....	164
3044	675,000,000	600,000,000	.....	180
3219	900,000,000	1,000,000,000	.....	262

[1] In this grade were included all of the eggs that before the candle showed the beginning of spot development which may have been a stuck spot, an embryo spot, blood ring, or a mold spot.

Maximum average count..... 24,000,000
Minimum average count..... 2,750,000

[2] In this grade is included all eggs showing a marked development of stuck, mold, embryo spots or blood rings.

Maximum average count..... 150,000,000
Minimum average count..... 14,000,000

[3] These eggs showed the development of at least 3-day embryos; in no case were the embryos living; therefore designated as blood rings.

Maximum average count..... 2,080,000,000
Minimum average count..... 350,000,000

"ROTTEN EGGS"[1] FROZEN AND EXAMINED AT INTERVALS FROM MAR. 16, 1910, to MAR. 29, 1911.
(Four dozen eggs received by the trade, discards from candling.)

Sample No.	Organisms per c.c.		*B. coli* per c.c.	Days in storage
	At 25°	At 37°		
2303	720,000,000	1,400,000,000	100,000,000	0
2312	8,000,000,000	3,000,000,000	.....	7
2380	1,200,000,000	1,000,000,000	.....	15
2399	600,000,000	300,000,000	.....	21
2458	900,000,000	300,000,000	.....	46
2559	600,000,000	400,000,000	.....	49
2586	800,000,000	900,000,000	.....	56
2624	700,000,000	600,000,000	.....	69
2646	400,000,000	200,000,000	.....	79
2773	900,000,000	800,000,000	.....	155
2910	500,000,000	300,000,000	.....	176
2972	500,000,000	400,000,000	.....	220
3118	500,000,000	200,000,000	10,000,000	253
3218	600,000,000	300,000,000	.....	291
3372	600,000,000	600,000,000	.....	378

Maximum average count..... 5,500,000,000
Minimum average count..... 300,000,000

Stiles and Bates as a result of their investigations arrive at the following tentative conclusions. Under normal conditions, strictly fresh eggs contain few if any bacteria. Strictly fresh and commercially fresh frozen eggs held in storage for more than one year showed little variation in their bacterial content during this period. *B. coli* was absent in the strictly fresh and commercially fresh samples.

Fish.

Fish contain in general the same constituents as the higher vertebrates, but differ from them in containing more collagen and less extractives. The flesh of fish is for most species white but some, as the salmon family, contain distinctive red colouring matters belonging to the lipochromes (fat pigments). The fat of fish is very liable to oxidation, and this accounts, to a large extent, for the fact that fish develops a strong and disagreeable flavour, unless consumed soon after death or unless preserved by sufficiently low temperatures (freezing). Great quantities of fish are held in frozen condition, but the temperature required is lower than for mammalian meat in consequence of the ease of oxidation of the fat.

Composition of the Flesh of Fish.—W. O. Atwater (*Amer. Chem. J.*, 1887, **9**, 421) has published the results of his analyses of a large number of fish caught in American waters. Of these, the following may be quoted:

[1] Includes all classes of "rots" except "black rots" not used for tanning.

Fish	No. of samples contributing to average	Water	Nitrogenous matters	Fat	Ash
Herring	2	74.6	14.5	9.0	1.78
Mackerel	8	71.2	19.4	8.0	1.36
Halibut	3	75.2	18.5	5.2	1.06
Conger-eel	2	71.4	18.5	9.1	1.00
Salmon	8	64.3	21.6	12.7	1.39
Cod	6	82.2	16.2	0.33	1.36
Plaice	2	78.3	18.7	1.9	1.01
Sole	1	86.1	11.9	0.25	1.22
Carp	1	76.9	21.86	1.1	1.33

In a subsequent paper (*ibid.*, **10**, 1) Atwater gives the following data respecting the nature and proportions of nitrogenised matters, etc., in the water-free *flesh of various fishes:*

Fish	Coagulable albumin in cold-water extract	Non-coagulable matter in cold-water extract	Gelatinoïds extracted by hot water	Insoluble proteins	Phosphoric acid
Herring	5.23	4.51	9.46	.....	1.77
Mackerel	7.27	8.61	5.74	47.37	2.11
Halibut	0.42	7.04	12.89	28.14	1.81
Pike	6.95	9.55	10.20	56 71	2.21
Haddock	7.89	6.18	16.36	65.06	2.49

Atwater has also compared the composition of the *ash* yielded by the ignition of the flesh of the haddock and the pike, as typical of salt-water and fresh-water fishes respectively:

Fish	Ash in dry flesh	Percentage composition of ash						
		K_2O	Na_2O	CaO	MgO	P_2O_5	SO_3	Cl
Haddock	11.26	13.84	36.51	3.39	1.90	13.70	0.31	38.11
Pike	6.13	23.92	20.45	7.38	3.81	38.16	2.50	4.74

From these figures the presence is apparent of a considerable proportion of sodium chloride in the flesh of salt-water fish; but the flesh of the pike also shows a much larger proportion of sodium salt than is found in the flesh of ruminants.

The following analyses of *cooked fish* are selected from a number published by Miss K. I. Williams (*J. Chem. Soc.*, 1897, **71**, 649).[1]

[1] The fish was prepared just as it would be served at table, being first cleaned and then boiled in water of 26 degrees of hardness (chiefly due to calcium carbonate). The salt cod and herrings were previously soaked in cold water for several hours, while the sardines were well washed in both boiling and cold distilled water, to remove as much of the surface-oil as possible.' When cold, all the bones, head, and such portion of the skin as would not be eaten were removed and carefully weighed, crushed in a mortar, boiled in distilled water, and the liquid siphoned off and evaporated over a water-bath. The residue, when constant in weight, was taken as gelatin.

Name of fish	Date	Portion analysed	As served at table			
			Waste bones, etc.	Gelatin	Water	Nutrients
Herrings	February	Whole	11.74	0.63	52.99	34.54
Salt herrings	January	Flesh	.....	.....	46.03	53.97
Sprats	November	Whole	17.90	0.90	61.50	19 70
Sardines	March	Whole	4.91	.....	42.17	52.92
Salmon	July	Section	5.99	0.53	61.06	32.02
Trout	May	Whole	8.23	0.55	67.12	24.10
Eels	October	Heads removed	11.66	1.09	53.29	33.96
Mackerel	April	Whole	10.51	0.25	65.21	24.03
Cod	January	Section	15.99	0.43	63.78	19.79
Salt cod	February	Section	6.13	0.33	67.68	25.86
Haddock	January	Whole	35.10	0.80	46.46	17.64
Whiting	January	Whole	25.10	0.86	61.29	16.35
Turbot	February	Anterior and head	31.20	0.59	53.09	15.12
Halibut	May	Section	6.84	0.03	69.35	23.78
Plaice	December	Flesh	.....	.....	79.86	20.14
Soles	March	Whole	22.02	0 74	61.18	16.06
Lemon soles	January	Whole	26.17	1.42	56.56	15.85
Oysters	March	Shell contents	.....	.....	77.71	22.29

The following additional data were obtained by the analysis of the same samples of *cooked fish:*

Name of fish	Water in flesh of fish	Analysis of dried substance						
		Ash	Nitrogen	Phosphorus	Fat; or ether extract	Proteins	Reducing substances reckoned as glucose	Nitrogen pentoxide
Herrings	60.54	5.56	11.11	0.91	25.25	67.07	.....	0.66
Salt herrings	46.03	19.69	7.12	0.89	21.90	38.88	17.59	1.64
Sprats	75.77	6.42	9.26	1.17	27.37	57.94	9.88	.....
Sardines	44.35	12.03	8.54	0.97	33.49	55.44	.....	.....
Salmon	65.32	4.94	10.70	0.51	29.43	56.65	14.89	0.46
Trout	73.58	6.60	11.96	1.13	8.84	80.00	4.68	.....
Eels	61.08	2.11	7.36	0.42	44.68	42.88	8.91	.....
Mackarel	73.13	4.07	10.46	0.85	25.73	62.32	13.93	0.33
Cod	76.32	3.31	15.30	0.62	1.15	91.55	6.67	0.63
Salt cod	72.35	14.26	12.41	0.29	0.94	76.06	7.14	0.31
Haddock	72.37	3.28	13.11	0.53	1.29	79.57	13.15	0.43
Whiting	78.78	1.92	13.28	0.73	1.86	79.55	17.54	.....
Turbot	77.84	2.41	13.76	0.57	4.75	84.71	11.81	.....
Halibut	74.46	4.11	13.32	0.67	15.81	79.67	.....	.....
Plaice	76.86	4.06	13.02	0.71	9.84	75.16	11.56	2.78
Soles	79.20	3.47	14.00	0.52	1.71	86.71	11.87	.....
Lemon soles	78.11	4.42	11.04	0.54	12.96	69.88	14.80	.....
Oysters	77.71	12.16	11.85	0.49	7.77	65.42	18.32	.....

The proteins in these analyses were estimated by multiplying the nitrogen determined by the soda-lime method by 6.25.

For the estimation of the reducing substances, the fat was first removed by benzene, and the residue digested with 100 c.c. of water and 10 c.c. of hydrochloric acid (sp. gr. 1.125) in a flask connected with a reflux-condenser. The whole was heated as strongly as possible over a water-bath for 3 hours, the liquid filtered, treated with basic lead

acetate, and sulphur dioxide passed through the filtered liquid. The solution was again filtered, concentrated at 100°, and a little washed alumina added until it was no longer dissolved. The filtered liquid was then evaporated to dryness at 100°, the residue treated with boiling alcohol, the liquid filtered, and the alcohol distilled off. The residue was dissolved in water, the solution boiled with animal charcoal and a few drops of milk of lime, filtered, and the filtrate titrated with Fehling's solution.

The proportion of reducing substances shown in the foregoing analyses by Miss Williams of the flesh of fish is, in most cases, remarkably large. As the method of estimation involved treatment with hydrochloric acid for some hours at the boiling-point of water, it seems probable that the reducing substances did not pre-exist as such, but were the products of the hydrolysis of bodies of the gluco-protein class. These compounds have been observed by Hammarsten (*Zeit. physiol. Chem.*, **19**, 19; abst. *J. Chem. Soc.*, 1894, 1, 310), Pavy (*Physiology of the Carbohydrates*, 1894), and others to result from action of hot dilute acids on protein matters. The conjecture receives support from the fact that the sum of the ash, fat, proteins, reducing substances, and nitrogen pentoxide is in some cases materially in excess of 100.00.

The presence of notable quantities of nitrates in the flesh of fish is remarkable. The analyses are not in accordance with the popular belief that the proportion of phosphorus is materially in excess of that present in the (dry) flesh of terrestrial animals.

Fish-roe and Caviare.—J. König gives the following analyses illustrating the percentage composition of caviare:

	No of samples	Water	Nitrogenous substances	Fat	Nitrogen free extract	Ash	Common salt
Caviare.....	5	43.89	30.79	15.66	1.67	8.09	6.02
Paionsnàja....	1	30.89	40.33	18.90	.....	9.88	.....
Fish-roe cheese	1	19.38	34.81	28.87	(6.33)	10.61	.....

Gobley gives the following as the composition of the *eggs* of the *carp* (compare hens' eggs, page 440): water, 64.08; paravitellin, 14.06; fat, 2.57; cholesterol, 0.27; lecithin, 3.04; cerebrin, 0.21; membranous substance, 14.53; extractive matters, 0.39; colouring matters, 0.03; and salts, 0.82%.

Food materials	Number of analyses	Refuse	Water	Protein		Fat	Total carbohydrates	Ash	Fuel value per pound
				N×6.25	By difference				
FISH, FRESH.		%	%	%	%	%	%	%	Cals.
Bass, striped, whole:									
Edible portion—									
Average	6	.....	77.7	18.6	18.3	2.8	.....	1.2	465
As purchased—									
Average	5	55.0	35.1	8.4	8.3	1.1	.....	.5	200
Bass, striped, entrails removed, as purchased.	1	51.2	37.4	8.8	8.7	2.2	.....	.5	255
Blackfish, whole:									
Edible portion—									
Average	4	.....	79.1	18.7	18.5	1.3	.....	1.1	405
As purchased—									
Average	2	60.2	31.4	7.4	7.3	.7	.....	.4	165
Blackfish, entrails removed, as purchased:									
Average	2	55.7	35.0	8.4	8.3	.5	.....	.5	175
Bluefish, entrails removed:									
Edible portion	1	.....	78.5	19.4	19.0	1.2	.....	1.3	410
As purchased	1	48.6	40.3	10.0	9.8	.6	.....	.7	210
Buffalo fish, entrails removed:									
Edible portion	1	.....	78.6	18.0	17.9	2.3	.....	1.2	430
As purchased	1	52.5	37.3	8.5	8.5	1.1	.....	.6	205
Butter-fish, whole:									
Edible portion	1	.....	70.0	18.0	17.8	11.0	.....	1.2	800
As purchased	1	42.8	40.1	10.3	10.2	6.3	.....	.6	460
Catfish:									
Edible portion	1	.....	64.1	14.4	14.4	20.6	.....	.9	1,135
As purchased	1	19.4	51.7	11.6	11.6	16.6	.....	.7	915
Ciscoe, whole:									
Edible portion—									
Average	3	.....	74.0	18.5	18.1	6.8	.....	1.1	630
As purchased	1	42.7	43.6	11.1	11 0	2.0	.....	.7	290
Ciscoe, entrails removed, as purchased:									
Average	2	10.1	65.6	16.3	15.9	7.5	.....	.9	620
Cod, whole:									
Edible portion—									
Average	5	.....	82.6	16.5	15.8	.4	.....	1.1	325
As purchased—									
Average	2	52.5	38.7	8.4	8.0	.2	.....	.6	165
Cod, dressed, as purchased:									
Average	3	29.9	58.5	11.1	10.6	.2	.....	.8	215
Cod, sections, edible portion:									
Average	3	.....	82.5	16.7	16.3	.3	.....	.9	325
Cod, steaks:									
Edible portion	1	.....	79.7	18.7	18.6	.5	.....	1.2	370
As purchased	1	9.2	72.4	17.0	16.9	.5	.....	1.0	335
Cusk, entrails removed:									
Edible portion	1	.....	82.0	17.0	16.9	.2	.....	.9	325
As purchased	1	40.3	49.0	10.1	10.1	.1	.....	.5	190
Eels, salt water, head, skin, and entrails removed:									
Edible portion—									
Average	2	.....	71.6	18.6	18.3	9.1	.....	1.0	730
As purchased—									
Average	2	20.2	57.2	14.8	14.6	7.2	.....	.8	580
Flounder, whole:									
Edible portion—									
Average	3	.....	84.2	14.2	13.9	.6	.....	1.3	290
As purchased—									
Average	2	61.5	32.6	5.4	5.1	.3	.....	.5	115
Flounder, entrails removed, as purchased:	1	57.0	35.8	6.4	6.3	.3	.....	.6	130
Haddock, entrails removed:									
Edible portion—									
Average	4	.....	81.7	17.2	16.8	.3	.....	1.2	335
As purchased—									
Average	4	51.0	40.0	8.4	8.2	.2	.....	.6	165

Food materials	Number of analyses	Refuse	Water	Protein		Fat	Total carbohydrates	Ash	Fuel value per pound
				N×6.25	By difference				
FISH, FRESH—Continued.		%	%	%	%	%	%	%	Cals.
Hake, entrails removed:									
Edible portion	1	.....	83.1	15.4	15.2	.7	.....	1.0	315
As purchased	1	52.5	39.5	7.3	7.2	.3	.....	.5	150
Halibut, steaks or sections:									
Edible portion—									
Average	3	.....	75.4	18.6	18.4	5.2	.....	1.0	565
As purchased—									
Average	3	17.7	61.9	15.3	15.1	4.4	.....	.9	470
Herring, whole:									
Edible portion—									
Average	2	.....	72.5	19.5	18.9	7.1	.....	1.5	660
As purchased—									
Average	2	42.6	41.7	11.2	10.9	3.9	.....	.9	375
Kingfish, whole:									
Edible portion	1	.....	79.2	18.9	18.7	.9	.....	1.2	390
As purchased	1	56.6	34.4	8.2	8.1	.4	.....	.5	170
Lamprey, whole:									
Edible portion	1	.....	71.1	15.0	14.9	13.3	.....	.7	840
As purchased	1	45.8	38.5	8.1	8.1	7.2	.....	.4	455
Mackerel, whole:									
Edible portion—									
Average	6	.....	73.4	18.7	18.3	7.1	.....	1.2	645
As purchased—									
Average	5	44.7	40.4	10.2	10.0	4.2	.....	.7	365
Mackerel, entrails removed, as purchased.	1	40.7	43.7	11.6	11.4	3.5	.....	.7	365
Mullet, whole:									
Edible portion	1	.....	74.9	19.5	19.3	4.6	.....	1.2	555
As purchased	1	57.9	31.5	8.2	8.1	2.0	.....	.5	235
Muskellunge, whole:									
Edible portion	1	.....	76.3	20.2	19.6	2.5	.....	1.6	480
As purchased	1	49.2	38.7	10.2	10.0	1.3	.....	.8	245
Perch, white, whole:									
Edible portion—									
Average	2	.....	75.7	19.3	19.1	4.0	.....	1.2	530
As purchased—									
Average	2	62.5	28.4	7.3	7.2	1.5	.....	.4	200
Perch, pike (wall-eyed pike):									
Edible portion	1	.....	79.7	18.6	18.4	.5	.....	1.4	365
As purchased	1	57.3	34.0	7.9	7.9	.2	.....	.6	155
Perch, yellow, whole:									
Edible portion—									
Average	2	....	79.3	18.7	18.7	.8	.....	1.2	380
As purchased	1	62.7	30.0	6.6	6.7	.2	.....	.4	130
Perch, yellow, dressed, as purchased.	1	35.1	50.7	12.8	12.6	.7	.....	.9	265
Pickerel, pike, whole:									
Edible portion—									
Average	3	.....	79.8	18.7	18.6	.5	.....	1.1	370
As purchased—									
Average	2	47.1	42.2	9.9	9.9	.2	.....	.6	190
Pickerel, pike, entrails removed, as purchased.	1	42.7	45.7	10.7	10.7	.3	.....	.6	210
Pike, gray, whole:									
Edible portion	1	.....	80.8	17.9	17.3	.8	.....	1.1	365
As purchased	1	63.2	29.7	6.6	6.4	.3	.....	.4	135
Pollock, dressed:									
Edible portion	1	.....	76.0	21.6	21.7	.8	.....	1.5	435
As purchased	1	28.5	54.3	15.4	15.5	.6	.....	1.1	310
Pompano, whole:									
Edible portion—									
Average	2	.....	72.8	18.8	18.7	7.5	.....	1.0	665
As purchased—									
Average	2	45.5	39.5	10.3	10.2	4.3	.....	.5	375

Food materials	Number of analyses	Refuse	Water	Protein		Fat	Total carbohydrates	Ash	Fuel value per pound
				N×6.25	By difference				
FISH, FRESH—Continued.		%	%	%	%	%	%	%	Cals.
Porgy, whole:									
Edible portion—									
Average	3	.....	75.0	18.6	18.5	5.1	.....	1.4	560
As purchased—									
Average	3	60.0	29.9	7.4	7.4	2.1	.....	.6	225
Red grouper, entrails removed:									
Edible portion—									
Average	2	.....	79.5	19.3	18.8	.6	.....	1.1	385
As purchased—									
Average	2	55.9	35.0	8.5	8.4	.2	.....	.5	165
Red snapper, whole:									
Edible portion—									
Average	3	.....	78.5	19.7	19.2	1.0	.....	1.3	410
As purchased—									
Average	2	46.1	42.0	10.8	10.6	.6	.....	.7	225
Red snapper, entrails and gills removed, as purchased.	1	45.3	43.7	10.6	10.0	.3	.....	.7	210
Salmon, whole:	6	.....	64.6	22.0	21.2	12.8	.....	1.4	950
Edible portion—									
Average									
As purchased—									
Average	4	34.9	40.9	15.3	14.4	8.9	.....	.9	660
Salmon, entrails removed, as purchased.									
Average	2	29.5	48.1	13.8	13.5	8.1	.....	.8	600
Salmon, landlocked, whole, spent:									
Edible portion—									
Average	4	.....	77.7	17.8	17.8	3.3	.....	1.2	470
As purchased—									
Average	4	45.5	42.3	9.7	9.8	1.8	.....	.6	255
Salmon, California, anterior sections:									
Edible portion—									
Average	2	.....	63.6	17.8	17.5	17.8	.....	1.1	1,080
As purchased	1	10.3	57.9	16.7	16.1	14.8	.....	.9	935
Shad, whole:									
Edible portion—									
Average	7	.....	70.6	18.8	18.6	9.5	.....	1.3	750
As purchased—									
Average	7	50.1	35.2	9.4	9.2	4.8	.....	.7	380
Shad, roe, as purchased	1	.....	71.2	20.9	.....	3.8	2.6	1.5	600
Sheepshead, whole.									
Edible portion—									
Average	2	.....	75.6	20.1	19.5	3.7	.....	1.2	530
As purchased	1	66.0	26.9	6.6	6.4	.2	.....	.5	130
Sheepshead, entrails removed, as purchased.	1	56.6	31.2	9.0	8.8	2.9	.....	.5	290
Skate, lobe of body:									
Edible portion	1	.....	82.2	18.2	15.3	1.4	.....	1.1	400
As purchased	1	51.0	40.2	8.9	7.5	.7	.....	.6	195
Smelt, whole:									
Edible portion—									
Average	2	.....	79.2	17.6	17.3	1.8	.....	1.7	405
As purchased—									
Average	2	41.9	46.1	10.1	10.0	1.0	.....	1.0	230
Spanish mackerel, whole:									
Edible portion	1	.....	68.1	21.5	21.0	9.4	.....	1.5	795
As purchased	1	34.6	44.5	14.1	13.7	6.2	.....	1.0	525
Sturgeon, anterior sections:									
Edible portion	1	.....	78.7	18.1	18.0	1.9	.....	1.4	415
As purchased	1	14.4	67.4	15.1	15.4	1.6	.....	1.2	350
Tomcod, whole:									
Edible portion	1	.....	81.5	17.2	17.1	.4	.....	1.0	335

Food materials	Number of analyses	Refuse	Water	Protein		Fat	Total carbohydrates	Ash	Fuel value per pound
				N×6.25	By difference				
FISH, FRESH—Continued.		%	%	%	%	%	%	%	Cals.
Tomcod, whole:									
As purchased	1	59.9	32.7	6.9	6.8	.2	....	.4	135
Trout, brook, whole:									
Edible portion—									
Average	3	.....	77.8	19.2	18.9	2.1	....	1.2	445
As purchased—									
Average	3	48.1	40.4	9.9	9.8	1.1	....	.6	230
Trout, salmon or lake:									
Edible portion—									
Average	2	.....	70.8	17.8	17.7	10.3	....	1.2	765
As purchased—									
Average	2	43.5	36.6	9.1	9.2	5.1	....	.6	385
Turbot:									
Edible portion	1	.....	71.4	14.8	12.9	14.4	....	1.3	885
As purchased	1	47.7	37.3	7.7	6.8	7.5	....	.7	460
Weakfish, whole:									
Edible portion	1	.....	79.0	17.8	17.4	2.4	....	1.2	430
As purchased	1	51.9	38.0	8.6	8.4	1.1	....	.6	205
Whitefish, whole:									
Edible portion	1	.....	69.8	22.9	22.1	6.5	....	1.6	700
As purchased	1	53.5	32.5	10.6	10.3	3.0	....	.7	325
FISH, COOKED.									
Bluefish, cooked, edible portion	1	.....	68.2	25.9	26.1	4.5	....	1.2	670
Spanish mackerel, broiled:									
Edible portion	1	.....	68.9	23.7	23.2	6.5	....	1.4	715
As purchased	1	7.9	63.5	21.8	21.4	5.9	....	1.3	655
FISH, PRESERVED AND CANNED[1]									
Cod, salt:[2]									
Edible portion—									
Average	2	.....	53.5	25.4	21.5	.3	....	[3]24.7	410
As purchased—									
Average	2	24.9	40.2	19.0	16.0	.4	....	18.5	315

PHOSPHORIC ANHYDRIDE, SULPHURIC ANHYDRIDE, AND CHLORINE IN SAMPLES OF PRESERVED AND CANNED FISH.[1]

Kind of fish	Phosphoric anhydride		Sulphuric anhydride		Chlorine	
	Number of estimations	Average	Number of estimations	Average	Number of estimations	Average
		%		%		%
Cod, salt.[2]	2	.25	2	.74	2	11.92
Cod, salt, boneless	1	.36	1	.68	1	11.19
Halibut, smoked	1	.47	1	.44	1	8.66
Herring, smoked	1	.84	1	1.24	1	7.21
Mackerel, salt[3]	1	.35	1	.61	.....	.....
Salmon, canned	1	.61	1	.44	.....	.....

[1] A considerable number of determinations of phosphorus, sulphur, and chlorine have been made in the flesh of preserved and canned fish. These are recorded in the following table in terms of phosphoric anhydride (P_2O_5), sulphuric anhydride (SO_3), and chlorine (Cl), and in percentages of the total weight of "edible portion" or flesh.

[2] It is observable that in salt cod the proportion of protein by difference is much smaller than by factor. The former value is apparently more nearly correct, and has been used in estimating the fuel value per pound.

[3] Two samples averaged 23% common salt.

Cured Fish.—Large quantities of fish are cured by salting, especially cod, haddock, cusk, hake and pollock (U. S. A.) and mackerel. Sardines and other small fat fish are packed in oil. Enormous quantities of fish especially salmon are canned by Appert's process. (See *Bull.* 133, *Bur. Chem. U. S. Dept. Agric.* for preparation of salt fish.)

Analysis of Fish.—The methods already described for meat, are entirely satisfactory for the analysis of fish. The vacuum oven is especially necessary for making moisture estimations and for drying extracted oil.

THE DIGESTION PRODUCTS OF THE PROTEINS.

By S. B. SCHRYVER, D.Sc., Ph.D.

THE GENERAL CHARACTER OF THE DIGESTION PRODUCTS.

The proteins, as has been already stated (page 17), are essentially polypeptides, which, on boiling with mineral acids undergo hydrolysis, yielding as the ultimate products the amino-acids. If they be treated, however, by less drastic methods, as, for example, with cold, or only slightly warmed, mineral acids, or by certain ferments, hydrolysis will take place without the production of the ultimate products; in such cases substances still containing the peptide linkage will be produced, which themselves, on treatment by more drastic methods, will ultimately yield the simple amino-acids. In some respects the degradation of the proteins is analogous to that of the polysaccharides, such as starch, which by partial hydrolysis yields first the less complex polysaccharides such as the dextrins, which themselves can be gradually degraded, until only the ultimate hydrolysis products, the monosaccharides are obtained. In one respect, however, the digestion process in the case of the proteins is far more complex than that in the case of the polysaccharides, for whereas the latter yield as ultimate hydrolysis products sometimes one and at other times two or three monosaccharides, the former give rise to a large number of amino-acids after complete hydrolytic scission. As a consequence, the degradation products of the proteins are far more complex than are those of the starches and other polysaccharides.

As already stated, the intermediary degradation products of the proteins can be obtained by hydrolysis with acids at lower (room or incubator) temperatures or more usually, by treatment with certain (proteoclastic) enzymes. These latter substances, which act catalytically, are widely distributed in the animal and plant world, and those derived from the alimentary tract of animals have been most widely studied. By the mucous membrane of the stomach of mammals is excreted pepsin,

by the pancreas, trypsin, while more recently from the mucous membrane of the small intestine has been extracted a third enzyme to which the discoverer, Cohnheim, has given the name of erepsin. All these enzymes play an important part in the digestion of food. Furthermore, proteoclastic enzymes have been isolated from almost all organs of the body, as well as from pathological growths, which during the last few years have formed the subject of exhaustive studies. (See numerous papers by Abderdalden and his pupils in the recent volumes of the *Zeitsch. physiol. Chem.*)

As an example of a proteoclastic enzyme from plants may be cited the so-called papayotin from the papaw tree (*carica papaya*), preparations of which are obtainable commercially (papain). Many other plants contain proteoclastic ferments.

None of these enzymes has so far been obtained in a chemically pure condition, and they act, as already mentioned catalytically, a small quantity of enzyme being capable of hydrolysing relatively large amounts of proteins (or partially hydrolysed proteins). Their essential chemical action is to accelerate the hydrolytic scission of a peptide linking according to the equation

$$\begin{array}{l} \qquad\quad R \qquad\qquad\quad R' \qquad\qquad\qquad\qquad\quad R \\ -NH-CH-CO-NH-CH-CO- + H_2O = -NH-CH- \\ \qquad\qquad\qquad\qquad\qquad\quad R' \\ \qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad COOH + NH_2-CH-CO- \end{array}$$

Although the enzymes have not been isolated as chemical entities they can be readily distinguished from one another by various criteria of action. Thus, for example, some proteoclastic enzymes, such as pepsin, act only in an acid medium, whereas others, such as trypsin (pancreatin) act best in an alkaline or neutral medium. The conditions as regards acidity or alkalinity vary with the individual enzymes, each having its own characteristic condition for the exertion of its optimal activity. The enzymes, furthermore, are extremely specific with regard to their action; they will promote the peptide scission only when the hydrolysis takes place between certain definite groups. The synthesis of the polypeptides by Emil Fischer and his pupils has given considerable stimulus to the study of this important aspect of digestion. The action of a ferment on a peptide depends on several factors of which the chief are the following.: (*a*) *The nature of the amino-acid;* certain groups, such as tyrosine are specially susceptible to the action of trypsin.

(*b*) *The stereo-chemical configuration of the molecule.* (*c*) *The general configuration of the whole molecule.* As an example of this specificity may be cited the action of the pancreatic juice of the dog on various synthetic polypeptides. (Fischer and Abderhalden, *Zeitsch. physiol. Chem.*, 1907, **51**, 264). Thus, *d*-alanyl-*d*-alanine ($d-[(CH_3)CH(NH_2)CO]-d[NH.CH(CH_3).COOH]$) and *l*-leucyl-*l*-leucine undergo scission when treated with pancreatic juice yielding *d*-alanine and *l*-leucine respectively, whereas the optical isomers, *d*-alanyl-*l*-alanine ($d[-(CH_3)CH.(NH_2).CO-l(NH.CH(CH_3).COOH]$) and *l*-leucyl-*d*-leucine remain unacted upon. The peptide grouping appears to be capable of undergoing hydrolytic scission on treatment with enzymes, only when it is formed from such amino-acids as exist in nature. If a racemic peptide, which can be digested by an enzyme, be submitted to such digestion, it will undergo asymmetric scission; only those amino-acids, being set free which occur in nature. Thus, if the racemic alanyl-leucines be digested with pancreatic juice, only the one containing the combination (*d*-alanyl-*l*-leucine + *l*-alanyl-*d*-leucine) undergoes hydrolysis, whereas the other (*d*-alanyl-*d*-leucine + *l*-alanyl-*l*-leucine) remains unchanged.

From these facts, it seems extremely unlikely that a complete hydrolytic scission of all the peptide groups contained in so complex a substance as a protein should take place, and as the individuals are formed by the conjugation of varying numbers of the numerous amino-acids, the amount of hydrolysis produced by a given enzyme will vary very considerably with the different proteins. (*Cf.* London and Solowjew, *Zeitsch. physiol. Chem.*, 1911, **74**, 309). Furthermore, the enzymes differ among themselves in their predilection for attacking the peptide linkages formed by different amino-acids.

From the foregoing remarks it is obvious that the study of digestion of proteins by enzymes is an extremely complex one, and that in actual practice the examination of a digestion mixture entails the investigation of material containing a large number of different substances.

The Preparation of Pure Digestion Products.—Although a protein digest contains as a rule many substances, various attempts have been made to isolate individual polypeptides in a chemically pure state.

Systematic investigations in this direction have been undertaken by Siegfried and his pupils, who employed as a precipitating reagent ferric ammonium sulphate dissolved in concentrated ammonium sulphate solution. Ammonium sulphate solution by itself precipitates the more complex products and another series, less complex, is precipitated by

Siegfried's reagent from the filtrate. By this means several amorphous substances appearing to have a constant composition and specific rotation have been obtained, such as pepsin gelatin peptone (*Zeitsch. physiol. Chem.*, 1904, **41**, 68), trypsin gelatin peptone (*Ibid.*, 1903, **38**, 320) pepsin fibrin peptone (*Ibid.*, 1903, **38**, 289) and two trypsin fibrin peptones (*Ibid.*, 1903, **38**, 265). All the above acid substances were obtained by the action of enzymes on proteins. In addition to these, Siegfried has prepared by the action of acids on proteins a series of digestion products which he has designated *kyrines*, which he has precipitated from the digestion mixture by phosphotungstic acid. The hydrolysis was carried out by about 12% hydrochloric acid at incubator temperature. The kyrines were all isolated in the form of the sulphates, and possess a markedly basic character. (*Zeitsch. physiol. Chem.*, 1904, **43**, 47; 1906, **48**, 54; 1906, **50**, 163, 1908, **58**, 215.)

Attempts have also been made to prepare still simpler digestion products, after separation of the precipitates obtained by ammonium sulphate solution, and ferric ammonium sulphate in saturated ammonium sulphate solution by precipitation with copper sulphate and potassium mercuric iodide, the separation of the precipitates thus obtained into fractions, and the isolation of products from these fractions by the action of phenylisocyanate and benzoyl and naphthalenesulphonyl chlorides. So far no very definite results have been obtained by these procedures. (Stookey, *Beitr. physiol. path. Chem.*, 1906, **7**, 590, Raper, *Ibid.*, 1906–7, **9**, 168; Rogozinski, *Ibid.*, 1908, **11**, 229 and 241.)

A number of digestion products in a pure state (some of which are crystalline), which contain amino-acids conjugated in the form of polypeptides have been obtained by E. Fischer and by Abderhalden and their co-workers. The preparation is in most cases difficult, and no general method has as yet been devised for the isolation of substances of this character. A few have also been isolated by other investigators. Some of the chief polypeptides isolated by the direct hydrolytic degradation of proteins are mentioned in the appended list with the names of the discoverers. Some of these products have also been obtained synthetically by E. Fischer and his pupils, and the elaboration of synthetic methods for preparation of the polypeptides has resulted in a considerable addition to our knowledge, not only on the chemical structure of the proteins, but on the processes involved in their digestion by enzymes. (Emil Fischer, *Untersuchungen über Aminosäuren, Polypeptide und Proteine*, Berlin, 1906.)

Polypeptides Obtained by Hydrolysis of Proteins.

{ Glycyl-*d*-alanineanhydride from silk fibrin, Fischer and Abderhalden (*Ber.*, 1906, **39**, 752).
{ Glycyl-*l*-tyrosineanhydride from silk fibrin, Fischer and Abderhalden (*Ber.*, 1906, **39**, 752).

Glycyl-proline anhydride from gelatine { Levene and Wallace (*Zeitsch. physiol. Chem.*, 1906, 47, 143).
{ Levene and Beatty (*Ber.*, 1906, **39**, 2060).

d-Alanyl-*l*-leucine
Glycyl-*l*-leucine anhydride [line } from elastin Fischer and Abderhalden (*Ber.*, 1906, **39**, 2315 and 1907, 40, 3544).
Anhydride yielding on hydrolysis alanine and pro-

l-leucyl-*d*-glutamic acid from gliadin, Fischer and Abderhalden (*Ber.*, 1907, 40, 3544).
Tetrapeptide (2 glycines, *d*-alanine and *l*-tyrosine from silk, Fischer and Abderhalden (*Ber.*, 1907, 40, 3544).
Dipeptide (phenylalanine and proline) from gliadin, Osborne and Clapp (*Amer. J. Physiol.*, 1907, 18, 219).
Glycyl-*l*-tyrosine } from silk. Abderhalden (*Zeitsch. physiol. Chem.*, 1909, 63, 401, and 1910, 65, 417).
d-Alanyl-glycine }
d-Alanyl-glycyl-*l*-tyrosine from silk, Abderhalden (*Zeitsch. physiol. Chem.*, 1911, 72, 1).

It is of interest to note that the tetrapeptide obtained from silk as well as several synthetic polypeptides, are precipitable from solution by ammonium sulphate, a property which is characteristic of the class of digestion products known as the albumoses (proteoses), which are discussed in some detail below.

The Nomenclature of the Digestion Products and the Methods of Characterisation.—As, in practice, the isolation of individual digestion products and their quantitative estimation is quite unfeasible, other methods must be employed for the chemical examination and for the determination of the degree of hydrolysis and character of the hydrolysis products contained in any given digest.

When a protein is submitted to digestion, certain progressive changes occur in the solution. Many of the ordinary protein reactions remain unchanged, as might be expected from the fact that these are due to individual amino-acids from which the protein is built up (see page 18). This remark refers especially to the various colour reactions. One of these, however, is especially characteristic of certain peptide groupings and is not yielded by the simple amino-acids, viz., the biuret reaction. The colour obtained in the biuret test is somewhat different with the digestion products to that given with the proteins from which these products are obtained, having a pinker tinge. It is possible by means of enzymes to digest a protein so far that the products no longer yield a biuret reaction, although certain peptides still exist in the mixture (Fischer and Abderhalden, *Zeitsch. physiol. Chem.*, 1903, **39**, 81). (The statement has also been confirmed by several subsequent observers.) The biuret test is the only colour reaction which is employed for the characterisation of a digest; a biuret-free product has formed the subject of many researches on animal metabolism, and may be regarded as a characteristic product of enzymatic digestion. It can be produced by a very prolonged action of pancreatin (trypsin) or by treatment of a protein first with pancre-

atin, and afterward with erepsin. It contains only relatively simple products.

Another series of changes occurs progressively during digestion, which are far more generally characteristic of the process than any of the changes in colour reactions, viz., changes in the precipitability of the digestion mixture with salt solutions.

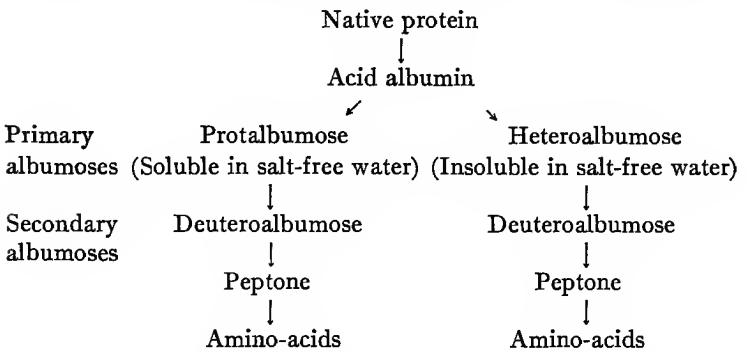
The subject of the precipitability of proteins has already been dealt with in a former article, and attention has there been drawn to the fact, that certain salts precipitate various proteins, the limits of concentration between which the precipitation occurs being characteristic both of the protein and the salt. As digestion proceeds the limits alter, the simpler degradation products being precipitable only by higher concentrations of salts or even remaining unprecipitated on complete saturation of the solution.

The first systematic application of the salt precipitation method to the study of the progression of digestion is due to Kühne and his pupils, especially Neumeister and Chittenden.

When a protein is submitted to peptic digestion (in the presence of acids) an insoluble but digestible substance, such as fibrin, first becomes soluble. If, after a short period of digestion, the mixture be neutralised, a protein is precipitated which dissolves in excess of acid or alkali. This is the so-called *acid albumin*, which after longer periods of digestion gradually disappears; no precipitate is formed at this stage on neutralisation. Along with the gradual disappearance of acid albumin, other products are formed, some of which are precipitable by saturation with sodium chloride, and others not precipitable by this salt, but precipitable by saturation with ammonium sulphate. The former are more complex, diminish in quantity and finally disappear as digestion proceeds; the latter substances at first increase and later diminish in quantity. As the digestion proceeds the amount of substance which is not precipitable by saturation with ammonium sulphate gradually becomes larger and larger. The substances which are precipitated by saturation with sodium chloride were designated by Kühne "*primary albumoses*"; the products which can be precipitated from the filtrate from these substances by saturation with ammonium sulphate were designated the "*secondary albumoses*" (deutero albumoses); the products which were not precipitable by salts, but which yield the biuret reaction were called peptones.

The protalbumoses were further separated by Kühne and his co-workers into two fractions, one of which was soluble in salt-free

water and the other insoluble. The former was designated "*protalbumose*," whereas the latter was called "*heteroalbumose*." According to the conception of Kühne, the native protein was first hydrolysed into a complex, and comparatively slightly degraded, product, which then broke down into a more degraded and less complex substance, the primary albumose, which itself degraded in stages into deutero-albumose, peptone and amino-acids. This conception as to protein degradation can be schematically represented in the following way:



The earlier work of Kühne and his school was subsequently submitted to revision, chiefly in the laboratory of Hofmeister, and the later investigations have shown that the older views as to the course of digestion are no longer tenable. Pick and Zunz, the two chief workers in the Strassburg laboratory, employed a somewhat different method for the examination of the digestion products. Instead of precipitating first with sodium chloride, and then with a salt like ammonium sulphate with a greater precipitating power they employed all the way through the same salt, and obtained fractions which were precipitated within different limits of concentration. Pick used ammonium sulphate, whereas Zunz employed zinc sulphate in acid solution. (Pick, *Zeitsch. physiol. Chem.*, 1897, **24**, 246; 1899, **28**, 219; *Beitr. physiol. path. Chem.*, 1902, **2**, 481. Zunz, *Zeitsch. physiol. Chem.*, 1899, **28**, 132; *Beitr. physiol. path. Chem.*, 1902, **2**, 435). In these experiments, the precipitability was investigated by preliminary trials (see article on proteins, page 65). In a series of test-tubes, 2 c.c. of the digestion mixture was diluted to 10 c.c. with varying amounts of saturated salt solution and water, and the filtrates from the precipitates tested by the addition of 1 or 2 drops of saturated salt solution. As an example of the fractions obtained by treatment of digestion products with acid zinc sulphate solution, the following from Zunz's paper may be cited:

PRECIPITATION LIMITS OF FRACTIONS OBTAINED FROM 2% PEPTIC DIGESTION MIXTURE OF VARIOUS PROTEIN FRACTIONS OF SERUM ALBUMIN.

Fraction	Euglobulin		Pseudoglobulin		Serum globulin	
	Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
I.....	24	46	24	46	26	46
II.....	54	68	54	68	58	72
III.....	76	84	76	84	74	84
IV.....	88	Saturation	88	Saturation	88	Saturation

The above numbers indicate the percentage saturation at which the precipitations of the various fractions begins and ends. Thus if 2 c.c. of the digest be examined, precipitation in the case of euglobulin would start when this quantity is diluted with 2.4 c.c. of saturated zinc sulphate solution and 5.6 c.c. water. The filtrate from this mixture would yield a further precipitate when treated with a drop of saturated zinc sulphate solution. The filtrate, however, from a mixture containing 2 c.c. digestion mixture, 4.6 saturated salt solution and 3.4 c.c. water would remain clear on the addition of a drop of the saturated salt solution. This would also be the case with mixtures containing slightly higher concentrations of salt solution (*e.g.*, 2 c.c. digestion mixture, 5 c.c. salt solutions and 3 c.c. water) until a greater concentration (in the case of euglobulin 2 c.c. digestion mixture, 5.4 c.c. salt solution, 2.6 c.c. water) was reached. At this point the filtrate would yield a precipitate on the addition of a drop of salt solution, indicating thus incipient precipitation of a second fraction. By proceeding in this way, Pick with ammonium sulphate, and Zunz with zinc sulphate, found that they could obtain four fractions by systematic precipitation of digestion mixtures with salt solutions. The limits of precipitation varied with the different proteins (digested in 2% solutions). Thus, for example, the first fraction in all cases was completely precipitated by half saturation with zinc sulphate; the second fraction of crystallised serum albumin and caseinogen digest was precipitated at 2/3 saturation; that of crystallized egg-albumin, pseudoglobulin and euglobulin at 6/7 saturation, and that of serum globulin at 73% saturation. At 83–85% saturation the third fraction was completely separated, and the fourth only on complete saturation.

The course of the formation of these various fractions was investigated by removal of portions of a digestion mixture after different intervals of incubation and estimating quantitatively the percentage of nitrogen precipitated in the various fractions. Of the substances

not precipitated by salts, the percentages precipitated by phosphotungstic acid were also estimated. As a result of these investigations, it was found that the earlier views of Kühne as to the mechanism of digestion could no longer be held, viz., that there is a gradual degradation of proteins through primary albumoses, secondary albumoses, peptones to the amino-acids, for it was found that the secondary products, and even monoamino-acids (not precipitated by either salts or phosphotungstic acid) were formed in quite early stages of the digestion. In view of more recent knowledge of the action of ferments, this result is not surprising, as it is now known that enzymes have a predilection for groups containing certain amino acids, and that, for example, tyrosine is set free very rapidly when certain proteins are submitted to tryptic digestion. A protein is therefore not attacked symmetrically by an enzyme, with scission in the first instance into two halves of approximately equal molecular weight. It might just as well be attacked at the end of the polypeptide chain with the scission of only a single amino-acid. The course of a digestion is illustrated by the accompanying table copied from Zunz's paper (*Beitr. physiol. path. Chem.*, 1902, 2).

A Peptic Digestion of Serum Albumin.

Portions of the digest were removed at the stated intervals and submitted to fractionation with zinc sulphate. The numbers express the percentages of the total nitrogen contained in each fraction.

Time of digestion	I. Acid albumin	II. Proteoses[1]				Total proteoses	III. Peptones and amino-acid		III Total
		(a)	(b)	(c)	(d)		(a)	(b)	
30 minutes....	4.58	44.03	0.00	39.15	0.00	83.18	2.64	9.60	12.24
1 hour.....	5.85	42.65	1.75	34.00	2.10	80.50	1.56	12.09	13.65
2 hours.....	2.78	39.89	3.02	35.20	3.14	81.25	2.89	13.08	15.97
4 hours.....	.....	37.10	4.93	29.46	3.28	74.77	1.83	23.40	25.23
8 hours.....	.....	30.12	8.64	25.82	4.86	69.44	2.65	27.91	30.56
22 hours.....	.....	14.94	7.12	16.51	5.80	44.37	5.12	50.51	55.63
2×24 hours...	.....	7.86	5.26	23.49	6.98	43.59	7.89	48.52	56.41
3×24 hours...	.....	3.71	3.05	18.95	8.28	33.99	19.06	46.95	66.01
6×24 hours...	.....	1.58	1.17	6.84	7.95	17.54	39.08	43.38	82.46
10×24 hours..	.....	0.64	0.00	2.96	6.64	10.24	.....	.....	89.76
15×24 hours..	.....	0.00	0.00	0.89	6.28	7.17	57.67	35.16	92.83
21×24 hours..	.....	0.00	0.00	0.00	6.17	6.17	55.64	38.29	93.83
30×24 hours..	.....	0.00	0.00	0.00	5.89	5.89	63.09	31.02	94.11

[1] For meaning of fractions (a), (b), (c) see page 476.

As quite simple substances such as amino-acids appear to be produced quite at the commencement of the digestion process, it seems somewhat illogical to subdivide the products into primary and secondary albumoses, as in this case the assumption is tacitly made that a protein gradually degrades through all these stages before it is broken down finally into the simple amino-acids. As furthermore each fraction obtained by salt precipitation is a complex mixture of several chemical individuals, it seems advisable to employ the precipitation process in a purely empirical manner when applying it to the examination of a digestion mixture and to relinquish any special designation for the fractions obtained, and to indicate their origin by simply stating the degree of salt saturation at which they were precipitated. All digestion products which are precipitable by salts are designated *proteoses;* those not so precipitable are called *peptones.* In addition the term *polypeptide* is sometimes employed. No hard and fast distinction between peptones and polypeptides can be drawn. It is perhaps convenient to confine the latter term to products which have been obtained synthetically, or which, having been obtained by a digestion process, have a known constitution. The peptones will then include these substances of polypeptide structure, which are not precipitable from salt solutions, and of which the actual chemical constitution is unknown.

In carrying out an examination of a protein digestion mixture, the following fractions may be conveniently separated.

I. Acid or alkali albumins.
II. Proteoses.—Fraction (*a*) precipitated in half saturation.
Fraction (*b*) precipitated between 50 and 70% saturation.
Fraction (*c*) precipitated between 70 and 85% saturation.
Fraction (*d*) precipitated between 85% and complete saturation by ammonium or zinc sulphate.
III. Peptones and amino-acids which may be subdivided into
Fraction (*a*) precipitated by phosphotungstic acid.
Fraction (*b*) not precipitated by phosphotungstic acid.

Fraction III (*a*) will contain the most of the peptones and the diamino-acids (see page 28). It must be remembered that the above separation is an empirical one, and the amounts obtained in each fraction will vary slightly under different conditions, of which the most important is the concentration of the solutions precipitated. Furthermore, as stated above, the limits between which the various fractions are obtained will vary with the different proteins and it may be occasionally advisable to vary them slightly in individual cases. Nevertheless, the above scheme is a convenient one for the majority of cases; suggestions as to a standard method for carrying out the separation are given in the section dealing with experimental details. The amount obtained in

each fraction is most conveniently expressed in terms of the amount of the nitrogen in each precipitate calculated as the percentage of the total nitrogen contained in the digestion mixture. The fractions obtained by salt precipitation may also be further subdivided by fractional extraction with alcohol or other solvents. By this method, Pick has separated Witte's peptone (a commercial peptic digest of fibrin) into the following fractions.

Fraction	Precipitation limits with ammonium sulphate	Solubility in alcohol
Aa	24–42%	Insoluble in 32% alcohol.
Ab	24–42%	Soluble in 80% alcohol.
Ba	54–62%	Insoluble in 60–70% alcohol.
Bb	54–62%	Soluble in 80% alcohol.
Ca	70–95%	Insoluble in 35% alcohol.
Cb	76–95%	Insoluble in 60–70% alcohol. (and other small fractions precipitated between 54 and 62% saturation).
D	100% + acid.	Soluble in 70–80% alcohol.

These fractions differ from one another very appreciably in their chemical reactions. Thus fraction Ba contains a large amount of sulphur, and Pick has designated it therefore, *thioalbumose*. Fraction Cb gives the various carbohydrate reactions strongly and it has therefore been called *glycalbumose*. Furthermore, the fractions differ from one another considerably in their yield of the different hydrolysis products. It cannot, however, be pretended that any of these fractions are chemical entitities and it seems somewhat inadvisable to give them definite names. If an alcohol fractionation be employed in addition to a salt precipitation, it should be used quite empirically, and as a method for comparing similar mixtures with one another.

In addition to the fractionation by salts, two other quite distinct chemical methods have recently been employed for examination of digestion products, and estimating the degree of hydrolytic dissociation, both of which are nearly allied to one another. If a polypeptide undergoes hydrolytic scission, the number of reactive amino- and carboxylic-acid groups increases. This is evident from the accompanying equation, which represents the hydrolysis of the simplest polypeptide, glycyl glycine

$$NH_2.CH_2.CO-NH.CH_2.COOH+H_2O=2NH_2.CH_2.COOH.$$

Whereas the polypeptide itself contains only one reactive amino and carboxylic group in the molecule, the hydrolysis products obtained from the same contain twice as many such groups as the

intact polypeptide. In other words, only half the nitrogen in the unchanged polypeptide is present in an amino group whereas all the nitrogen in the hydrolysis mixture is in this form. In the case of a tripeptide formed from monoamino-acids one-third of the nitrogen is present in an amino group, and in the products of complete scission the whole. If the scission is not complete, if, for example, only one amino group has been split off, then the amount of nitrogen contained in amino groups will lie between one-third and the whole of the total nitrogen. In the scission products a tetrapeptide, the amino-nitrogen will lie between one-fourth and the whole of the total, according to the degree of hydrolysis to which the peptone has been submitted. In general in an *n*-peptide formed from monoamino-acids the amino-nitrogen in the hydrolysis products will lie between $1/n$th and the total nitrogen. Like the reactive amino groups, the reactive carboxylic groups will progressively increase as a polypeptide undergoes hydrolytic scission.

The percentage of nitrogen present in reactive amino groups present in a digest mixture can be estimated by treating a known quantity of such a mixture with nitrous acid and measuring the amount of nitrogen evolved.

Still simpler is the method employed for following the course of digestion by estimating the changes in the number of reacting carboxylic-acid groups with progressing digestion. An amino-acid, owing to its amphoteric character cannot be directly titrated by a standard alkali solution in the presence of an indicator. If, however, formaldehyde in excess be added before the titration, the amino groups are converted into methyleneimino groups, and the substances lose their amphoteric character and can be directly titrated with standard alkaline solutions in the presence of phenolphthaleïn (compare p. 488).

These reactions can best be illustrated again by giving the reactions with the simplest polypeptide and its hydrolysis products.

Polypeptide $NH_2CH_2CO-NH.CH_2.COOH + HCHO = H_2O + CH_2(N:CH)CO-NH.CH_2.COOH$.

Hydrolysis products, $2NH_2CH_2.COOH + 2HCHO = 2CH_2(N:CH).COOH$.

From these equations it is obvious that the hydrolysis products after treatment with formaldehyde will require approximately twice as much alkali for neutralisation as the polypeptide from which they were obtained. To Sörensen (*Biochem. Zeitsch.*, 1908, **7**, 45), is due this formaldehyde titration method for following the course of digestion of a protein (see also page 488). The result of the titration is usually expressed

as the percentage amount of the total nitrogen in a mixture which enters into reaction with formaldehyde. Sufficient acid or alkali is first added to the mixture to render it neutral to a given indicator (litmus). Formaldehyde is then added, and owing to the reaction with amino groups, the mixture becomes strongly acid. The additional amount of acid necessary to neutralise this increased acidity gives the measure of the amino groups which have entered into reaction. The change in the number of reactive amino groups gives also the change in the number of reactive carboxylic groups as the scission of each peptide bond sets free one amino- and one carboxylic-acid group.

It has been already stated that the estimation of the reactive amino groups can also be applied to intact proteins (page 89). The amount of nitrogen (expressed as the percentage of the total) is, however, in these cases small. Furthermore, in applying the formaldehyde titration method to these cases there is an initial difficulty, in that the protein solution will as a general rule have an amphoteric character, and cannot therefore be accurately neutralised to an indicator (except in certain exceptional cases like that of caseinogen (see page 89). As the molecular weights of these substances are great, and as the number of reactive amino groups is small, the probabilities of error are great when the formaldehyde titration method is employed for the purposes of estimating the amino groups and obtaining a new chemical factor for characterising individual proteins. It is best used for estimating the *changes* which take place during the course of digestion, samples of the digest being withdrawn after different intervals of titration and then submitted to the formalin titration. Initial errors due to the difficulties of accurately neutralising before addition of formaldehyde are thereby avoided. The method can also be employed for estimating the number of peptide linkages still existing in a given digest, by determining the change which is produced in the formaldehyde titration number, by completing the hydrolysis of the mixture, by a known quantity of mineral acid.

The results in the above section may be summarised in the statement that it is not possible to isolate from any given digestion mixture the chemical entities which it contains. There are, however, certain analytical processes which can be applied to the examination of a digest, by means of which some idea can be formed as to the character of the material under examination. The chief of these consists in the fractional precipitation of the solutions with increasing grades of concentration of certain neutral salts, and the estimation of the percentage of the nitrogen carried down in each fraction. Although this process

is of empirical nature, it gives good comparative results, and throws much light on the degree of digestion to which a given sample has been submitted. In addition to these, the amount of nitrogen precipitable by phosphotungstic acid, or tannic acid can also be estimated.

Furthermore, the percentage of nitrogen present in amino groups can be estimated by determining the amount of nitrogen set free on treatment with nitrous acid, or approximately also by estimating the increased degree of acidity in the solution produced by the addition of formaldehyde. The percentage of nitrogen present in the form of peptide linkages can also be estimated by hydrolysing with mineral acids, and estimating the increase thereby produced in the "formalin-titratable" nitrogen (*i.e.*, the acidity produced by masking the basic action of the amino groups present treatment with formaldehyde).

The Preparation of Digests.—Digests of proteins are usually prepared by treating solutions of the substances, or when the latter are insoluble, suspensions of the same in water, with a solution or aqueous suspension of the ferment, at the temperature of 37.5°. For this purpose, either a thermostat or a biological incubator can be employed. Care must be taken as to the reaction of the medium. The conditions of optimum action have formed the subject of numerous researches in recent years (see especially Sörensen, *Enzymstudien, Biochem. Zeitsch.*, 1909, **21**, 131). For practical purposes, pepsin acts best in a solution containing 0.3–0.4% hydrochloric acid. During the course of the digestion the acidity changes, owing to the formation of amino-acids, etc. The proteoclastic enzyme from the pancreas[1] acts on the other hand best in alkaline solution (0.2–0.4% sodium carbonate) whereas erepsin acts in neutral solution. As already mentioned in the introduction, the enzymes differ from one another in respect to the peptide groupings which they attack, and they produce different amounts of degradation with the same protein. The pancreatic enzyme possesses the capacity of breaking down certain peptide linkages which still remain intact after a protein has been digested with pepsin, and erepsin possesses the property of hydrolysing peptones. By treating certain proteins consecutively with pepsin, trypsin, and erepsin (in each case under the correct conditions as regards acidity, etc.) a product which no longer yields the biuret test can be obtained, although hydrolysis is not complete, as it can be shown that the number of reactive amino groups increases if such a digest be heated with acids

[1] The pure pancreatic juice possesses no proteolytic activity, as the enzyme is present as a zymogen which can be activated, among other methods by the addition of the so-called enterokinase, which is contained in an aqueous extract of the mucous membrane of the small intestine.

(Henriques, and Gjaldbäk, *Zeitsch. physiol. Chem.*, 1910, **67**, 8, where references to earlier papers on this subject are given).

Although the ferments act catalytically, and a small amount of ferment should break down very large amounts of protein, they often, nevertheless, become inactive after the digestion has proceeded for some time. This is due to the fact that in presence of acids ar alkalis at 37° they are often themselves destroyed, and it is therefore necessary, when it is required to produce the maximum change in a protein by any given enzyme, to add fresh quantities of the latter from time to time, usually at intervals of a few days.

In all cases when a digest is prepared, it is necessary to guard against bacterial contamination. For this purpose, toluene or chloroform, or both these liquids are added to the mixture, which should be kept in closed flasks in the incubator or thermostat to prevent evaporation. The proteins should furthermore never be digested in too concentrated solutions or suspensions (5% is generally more than sufficiently concentrated) as the products of digestion inhibit the action of the ferments. The amount of ferment to be added will depend upon the quality of the preparation used. (For quantitative estimation of ferments see page 492.)

To obtain the digestion products in solid form, the digestion mixture should be evaporated *in vacuo* at a temperature not exceeding 40°. The concentrated residue is then thrown into several times the volume of a mixture of equal parts of alcohol and ether; it is then rubbed up with the same mixture to free it from water, and finally washed with ether and dried *in vacuo*. When the digestion has taken place in an acid medium, an acid should be employed which can readily be removed quantitatively; peptic digests can be conveniently carried out in solutions made acid with sulphuric acid, which can be quantitatively removed by barium hydroxide when it is required to isolate the digestion products in a solid form. Similarly pancreatic digestion can be carried out in a solution made alkaline with ammonia instead of sodium carbonate.

Methods for Examination of Digestion Products and for Tracing the Course of a Digestion.

For a complete examination of a digestion product (or of a sample of a digestion mixture removed after a stated interval of incubation) the following series of factors should be determined.

I. The amounts of nitrogen precipitated (*a*) by varying concentrations of certain salts (*b*) by certain alkaloidal precipitants.

II. The percentage of nitrogen in the form of reactive amino groups (*a*) by the nitrous acid method, (*b*) by the formaldehyde titration method. By these methods also, the percentage of nitrogen still existing in the form of peptide linkages can be estimated.

III. In following the course of digestion, certain changes in the physical characters of the digestion mixtures can be studied.

In actual practice, the two first named series of factors are most generally employed. The methods relating to the estimation of the nitrogen in the form of amino-derivatives and in peptide combination, are of recent introduction. These factors are, however, so important, and the methods are in many cases so simple, that there is no doubt that the processes employed should form part of the routine technique in the examination of the digestion processes. The measurements of the physical changes taking place in a mixture during the course of a digestion have thrown much light on the mechanism of digestion. The chief changes measured are those of electrical conductivity and viscosity. Those physical measurements, although of so much value in following out *changes*, taking place during digestion, are not much employed for the routine examination of digestion products, and need not be discussed in detail in this place. (See Bayliss, *The nature of enzyme action*, 2nd edition, London, 1911.) Digestive changes have also been studied by following the change in the optical rotation of the digestion mixture. (See numerous papers by Abderhalden and his co-workers in the recent numbers of the *Zeitsch. f. physiol. Chem.*)

I (*a*) The Estimation of the Nitrogen Precipitated with Varying Concentrations of Salts. (Fractional Salt Precipitation).—The salt most convenient for use for separating the various fractions of the proteoses is zinc sulphate; this salt can be used more advantageously than the corresponding ammonium salt, in that the filtrates from the precipitates can be directly used for the estimation of nitrogen. The reagent is, according to Baumann and Bömer who introduced it for the purpose of proteose separations (*Zeitsch. Untersuch. Nahr. u. Genussm.*, 1898, **1**, 106) employed in acid solution. Both to the solution of the digestion mixture and to the saturated zinc sulphate solution should be added sulphuric acid solution prepared by diluting 1 volume of the concentrated acid with 4 volumes of water. 2 c.c. of this mixture are added to each 100 c.c. of saturated zinc sulphate solution, and a solution of the digestion product.

The method of fractional precipitation by salts has been critically examined by Haslam (*J. Physiol.*, 1905, **32**, 267 and 1907, **36**, 164), who has pointed out that two sources of error are *a priori* conceivable

in effecting complete separation, viz.: (*a*) any fraction produced by a particular degree of saturation is not absolutely insoluble in a solution of the strength in which it is precipitated and (*b*) a precipitate may carry down with it a certain quantity of substance which belongs to a subsequent fraction. Haslam gives methods for separating fractions of constant composition. These are, however, inapplicable to a quantitative examination of digestion products. More recently Wiener (*Zeitsch. physiol. Chem.*, 1911, **74**, 29) in investigating the separation of globulin from albumin in blood-serum by fractional precipitation with ammonium sulphate, has shown that the separation is more complete in dilute than in concentrated solutions.

The variations of the results obtained by fractional salt precipitation under different conditions of experiment, emphasize a point to which attention has been already drawn, viz., that the quantitative separation of a digest into fractions by means of salt precipitation, must be regarded as an empirical process and that it is inadvisable to regard the various fractions to which such names as protalbumose, deuteroalbumose A, deuteroalbumose B, etc., have been assigned, as definite chemical products. Fractional precipitation, however, if carried out under definite standard conditions, affords valuable information as to the character of the material to be examined.

The following has been found to be a convenient method for the quantitative examination of a digest by means of salt precipitation.

If the substance is a solid, it is first dissolved in water, so as to make a solution of about 10% which is filtered clear from any undissolved matter, which is washed on a filter. This undissolved residue may be unchanged protein, and its quantity may be estimated, and the percentage of nitrogen which it contains. The clear solution is then carefully neutralised to an efficient glazed litmus paper. At this point, acid or alkali albumin may be precipitated. Sometimes nucleo-proteins (or nucleo-albumins) may be precipitated at this stage, and in this case it is advisable to estimate not only the nitrogen, but the organic phosphorus in the precipitate. The precipitate should, in any case, be washed and dried (the wash-water being added to the main bulk of the solution) and the nitrogen therein estimated, either in the whole or in a part according to the amount precipitated. If coagulable proteins be present in the filtrate, this should be acidified with butyric acid and heated and the coagulated protein filtered off, washed, dried and weighed, and the nitrogen contained in it estimated. If the quantity be small, the precipitate and filter paper (which should be nitrogen-free) can be directly transferred while moist to the Kjel-

dahl flask, and the nitrogen directly estimated without previous drying and weighing. As a rule, the quantity is only small. The filtrate, after the coagulable protein has separated, is then carefully neutralised again and the nitrogen percentage estimated therein by Kjeldahl's method by making it up to a definite bulk and incinerating a small aliquot part. If the solution is too dilute, it should be evaporated. A convenient solution at this stage is one in which 10 c.c. of the solution require, after Kjeldahl treatment, about 100 c.c. of $N/10$ solution to neutralise the ammonia (equivalent to about an 8.8% solution of protein). If the solution is much more dilute, it should be concentrated to about this strength. To the solution thus prepared, 2 c.c. of diluted sulphuric acid (1 volume concentrated acid, 4 volumes water) should be added to each 100 c.c. In this mixture the nitrogen should be again accurately estimated.

Into a series of 4 stoppered flasks of 100 c.c. capacity are then introduced four portions of the above solution, accurately measured or weighed, each portion being exactly equivalent to 100 c.c. of $N/10$ sulphuric acid after treatment by Kjeldahl method. The quantity should be about 10 c.c. This solution is then diluted with varying amounts of acidified water or acidified saturated zinc sulphate solution (always 2 c.c. of diluted acid to 100 c.c. of water or salt solution). To the first flask is added 50 c.c. of zinc sulphate solution, to the second 70 c.c., to the third 85 c.c. To the fourth is added enough solid zinc sulphate to saturate the solution, which is afterward diluted to the 100 c.c. mark with acidified saturated zinc sulphate solution. The other flasks are then made up to the 100 c.c. mark with acidified water. They are then stoppered, thoroughly shaken and allowed to stand. After a day, the precipitate is filtered off, and the nitrogen is directly estimated by Kjeldahl's method in 50 c.c. of the filtrates. In this way the amounts precipitated by 50, 70, 85 and 100% of saturation by zinc sulphate can be found. As 50 c.c. of the unprecipitated solution are equivalent to 50 c.c. $N/10$ sulphuric acid, the calculations are very simple. The volume of the precipitate may be neglected. It is often inconvenient to incinerate the filtrates with sulphuric acid directly, owing to the zinc sulphate they contain. Violent bumping often occurs, and the flasks have a tendency to break. This inconvenience can be obviated by a method suggested by Salkowski (*Biochem. Zeitsch.*, 1911, **32**, 355). Hydrogen sulphide is passed into the solution, and sodium carbonate solution is added from time to time to neutralise the acidity. The greater part of the zinc can thus be precipitated as sulphide, from which the organic matter can be readily

removed by washing. This is not the case if the zinc be precipitated as carbonate. If necessary, the filtrate from the zinc sulphide can be concentrated before incineration. By the above method, the following factors can be determined. (*a*) Insoluble matter (unchanged or coagulated protein), (*b*) acid or alkali albumin, (*c*) coagulable protein, (*d*) proteoses in four fractions. In the filtrate, amino-acids and peptones exist. Some conception as to the amount of peptones in this filtrate can be formed by estimating the percentage of nitrogen existing in peptide combination. This is done by estimating the increase in the nitrogen in reactive amino form after hydrolysis by either the nitrous acid or formaldehyde methods described in detail below.

In one case, which in practice is not infrequent, the above method does not yield satisfactory results, *i.e.*, where gelatin is present with digestion products. This substance cannot be separated as a coagulable protein, and it is precipitated with salts, together with the albumoses. Various methods have been suggested for separating gelatin from digestion products, but none have been found satisfactory. (See Greifenhagen, König und Scholl, *Biochem. Zeitsch.*, 1911, **35**, 217, when other references to the literature are given.)

It may be recalled here, that certain of the proteoses are precipitated with colloids (gum mastic, or ferric hydroxide) together with the proteins, so that by these methods, proteins cannot be separated from their digestion products.

(*b*) **The Estimation of Nitrogen Precipitated by Various Alkaloidal Reagents.** (i) *Precipitation by Phosphotungstic Acid.*—The technique in this case is exactly similar to that employed in estimating the "basic nitrogen" when determining the Hausmann numbers (see page 80).

(ii) *Precipitation by Tannic Acid Solution.*—The reagent can be made in the following way: 100 grm. tannic acid, 25 grm. sodium acetate, 75 grm. sodium chloride and 50 c.c. of acetic acid are dissolved in water, and the solution is made up to 1,000 c.c. (Hedin). Sufficient of the reagent is added to the solution of the digestion product, to completely separate the precipitable substances. Nitrogen can be estimated in an aliquot part of the filtrate.

The course of digestion can be conveniently followed by estimating, from time to time, the amount of nitrogen which is not precipitated by these reagents. This should increase with progressive degradation of the protein.

Tin salts have also been similarly employed. (See Scherning, *Zeitsch. analyt. Chem.*, 1893, **37**, 416.)

II. The Estimation of Nitrogen Existing in the Form of Reactive Amino Groups. (*a*) *The Nitrous Acid Method.*—When an amino group reacts with nitrous acid, nitrogen is evolved according to the equation $R.NH_2+HNO_2=R.OH+H_2O+N_2$, two atoms of the gas being evolved for each molecule of amino groups. The application of this reaction to digestion products has been already discussed in detail pages 82, and 86, and it was applied as long ago as 35 years by Sachsse and Kormann for quantitatively estimating amino groups. Various modifications of the original method and form of apparatus have been suggested (*e.g.*, that of H. Brown, *Trans. Guiness Research Laboratory*, 1903, **1**, Part I, 30), but quite recently D. D. van Slyke has suggested a process by means of which the estimation can be carried out within a few minutes (*Ber.*, 1910, **43**, 3170).

The main difficulty consists in obtaining the nitrogen set free from the amines free from atmospheric nitrogen and free from nitrous acid.

The apparatus employed is figured in the accompanying diagram.

The glass tubes fitting into the flask D are all capillary tubes of 6–7 mm. outer diameter, and with the exception of that leading to A, of 1 mm. lumen; this latter has a lumen of 2 mm. In order that the gas burette should remain clean, the water in E contains sulphuric acid to the amount of about 1%. The reaction is carried out in flask D, which is capable of holding about 35–37 c.c. The solution containing the amino-compounds, which should not contain more than about 20 mgrm. nitrogen in the amino-form, is introduced into the burette B, and about 5 c.c. of water are introduced into A. Into the flask D are introduced 28 c.c. of a solution of 3 parts of sodium nitrate in 10 parts of water, and then 7 c.c. of glacial acetic acid are added. Nitrous acid is immediately evolved. The stopper containing A B C is then inserted into the neck of D and secured by a wire and the tap *a* is opened. Water runs from A into D, and by this means, all the air is driven out through C. To drive out the air contained in the nitrous acid solution, the tap C is closed, the flask tap *a* is opened and the flask is shaken; an amount of nitric oxide is thereby evolved, and 10–15 c.c. of liquid are pressed back into A. C is then opened and the gas and the air which it has washed out of the apparatus is forced out through C. To ensure that the expulsion of air is complete, the last operations are repeated; *c* is then closed and the flask is shaken and a gas space of about 20 c.c. in D is thereby made; *a* is then closed, and *c* opened and connected with the gas burette E. The solution to be examined is then run out from B and mixed with the liquid in D. Nitrogen mixed with nitric oxide is immediately evolved. The reac-

tion is accelerated by shaking the flask about 4 or 5 times in a minute, and is complete after 4 or 5 minutes. The reaction is continued until an excess of 30–40 c.c. of nitric oxide are accumulated in the gas burette, *i.e.*, till the gas volume in E is at least 30–40 c.c. in excess of the expected amount of amino-nitrogen. By allowing the liquid from A to pass into D, all the gas from D and the capillary C is driven into E. From E the gas is driven into a Hempel pipette containing 2.5% sodium hydroxide saturated with potassium permanganate. After the absorption of all the nitric oxide, the residual gas is then driven back again into E and measured. It is advisable, until the operator

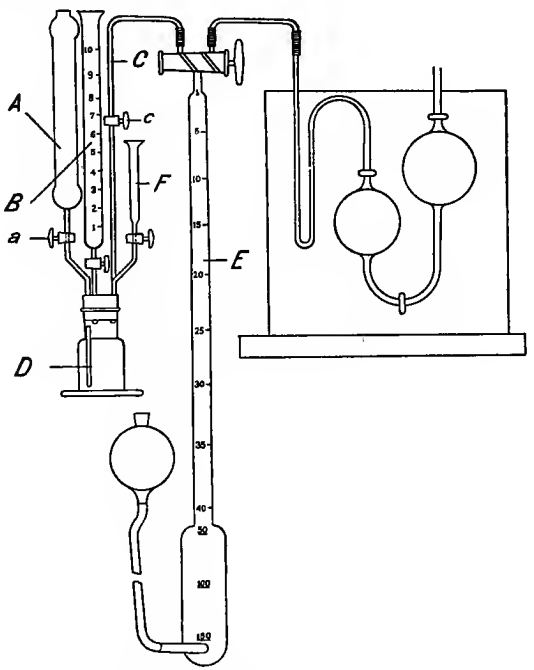


FIG. 28.—Van Slyke's apparatus for the estimation of amino-nitrogen.

is experienced with the use of the apparatus, to repeat the treatment of the gas with permanganate. If the analysis be carried out with fluids like protein solutions, which foam on shaking, a little amyl alcohol is introduced into the apparatus through F. By this means the frothing on shaking D can be inhibited. 1.7–1.9 c.c. of nitrogen are equivalent to 1 mgrm. of amino-nitrogen, according to the conditions of temperature and pressure. (For greater detail see Vol. 7, p. 264.)

In interpreting the results obtained by this process, it must be remem-

bered that some of the ultimate hydrolysis products do not contain all their nitrogen in the form of amino-nitrogen. Proline and hydroxyproline, for example, give off no nitrogen on treatment with nitrous acid. Arginine, histidine and tryptophan give off under the conditions of the experiment only 1/4, 1/3, and 1/2 of their total nitrogen. For this reason, the process can be employed for estimating approximately the amounts of these substances contained in the various fractions of the products of complete hydrolysis (phosphotungstic acid precipitate, ester fraction containing proline, etc.).

If the amino-nitrogen in a digestion mixture be estimated by this method, and an equivalent portion be hydrolysed by the method of Henriques and Gjaldbäk (see below, page 489) and the amino-nitrogen is estimated in the hydrolysed product, the difference between the two estimations gives the amount of nitrogen present in the peptide linkage.

(*b*) *The Formaldehyde Titration Method.*[1]—The theory of this method has been already given in detail above (pages 9 and 478). In practice it is carried out in the following manner (compare page 9). To 10 c.c. of a commercial formaldehyde solution is added 1 c.c. of a 1/2% phenolphthaleïn[2] solution dissolved in 50% alcohol. This mixture is then made faintly alkaline with either barium hydroxide or sodium hydroxide solution, the alkali being added till the mixture has a faint pink colour. This is then added to 20 c.c. of the digestive mixture, which is then titrated with $N/5$ barium hydroxide solution until a distinct red colour is produced. It is usual to titrate to the red colour and not to the initial pink, as Sörensen has shown that the theoretical amount of alkali has not been added when amino-acids are titrated until the solution is bright red, if phenolphthaleïn, under the given conditions of experiment, is used as the indicator. Where carbonates or phosphates are absent, $N/10$ sodium hydroxide can be used instead of barium hydroxide. As already mentioned, the nitrogen of the free amino-groups is estimated by determining the increase of acidity due to the reaction with formaldehyde, each c.c. of $N/10$ alkali solution being equivalent to 1.4 mgrm. of nitrogen existing in this form. When the method is applied directly to a single digestion mixture, there is a difficulty in obtaining an accurate result, arising from the circumstance that it is necessary to ascertain how much acid or alkali is necessary to neutralise the solution before the addition of the formaldehyde. This amount cannot be readily ascertained by titration in the presence of an indicator, owing to the amphoteric character of the mixture of amino-acids, peptides, proteoes, etc. It is

[1] Sörensen, *loc. cit.*, page 478.
[2] Thymolphthalein solution has also been employed for this purpose.

best applied therefore in estimating *changes*, when the error due to the initial neutralisation is obviated. Thus, for example, samples removed from a digest, after different intervals of incubation, can be directly titrated after treatment with formaldehyde, without initial neutralisation. The differences between the titration numbers of any two given samples removed at different intervals give an indication of the amount of digestion which has taken place within that interval.

The method is, however, best applied generally, when examining digestion products, by estimating the percentage of nitrogen still existing in the mixture in the form of peptide bonds. This can be done by titrating one portion by the formaldehyde process, and taking a similar portion, hydrolysing it in such a way as to completely break down all the peptide bonds, and titrating the hydrolysed product (after adding sufficient alkali to neutralise the hydrochloric acid present) by the formalin method. The difference of the titration numbers in the two samples is a measure of the nitrogen present in peptide form, each c.c. $N/10$ acid in this difference corresponding to 1.4 mgrm. nitrogen thus combined.

In carrying out this hydrolysis, care must be taken that the hydrolytic scission is complete, some preparations being hydrolysed by simply evaporating once or twice on a water bath, others requiring more drastic treatment. The technique of the process has been, however, elaborated by Henriques and Gjaldbäk (*Zeitsch. physiol. Chem.*, 1910, **67**, 8). They find that the hydrolysis is best accomplished by hydrochloric acid, when it is present, in the strength of three times normal in the digestion mixture at a temperature (in an autoclave) of 150°. Under these conditions, the hydrolysis is completed in 1.5 hours. The mixture thus obtained is often so highly coloured that an accurate titration is impossible. Sörensen and Jessen-Hansen have shown, however, that the pigment can be removed by precipitating silver chloride in the acidified solution (*Biochem. Zeitsch.*, 1908, **7**, 407). The amount of peptide nitrogen may be estimated, therefore, by combining the methods of Sörensen and Jessen-Hansen with those of Henriques and Gjaldbäk in the following way:

The solution of the digestion mixture in convenient concentration (*e.g.*, 10 c.c. containing 40 mgrm. N) is carefully neutralised to litmus and then acidified with $1/10$ the volume of N-hydrochloric acid. Two equal portions (of say, 50 c.c.) are taken of this solution which should be warmed in a current of carbon-dioxide-free air, to drive off any of this gas from solution. One portion is set aside and to the other portion is added so much hydrochloric acid that the concentra-

tion is about three times normal, and the mixture is then heated in an autoclave to 150° for about 1.5 hours. At the end of this period, it is transferred to an evaporating basin, and some of the hydrochloric acid is evaporated off on the water-bath. The residue is then diluted with water, until it reaches the original bulk (50 c.c.). The same series of estimations are then carried out in both the non-hydrolysed and the hydrolysed solutions. These are (*a*) the total nitrogen, (*b*) the amount of chlorine, (*c*) the acidity after formalin treatment in the solution decolourised by precipitation of silver chloride. The total nitrogen and chlorine is estimated in 10 c.c. of the solution, the chlorine being determined volumetrically by Volhard's method. The difference in the chlorine in the hydrolysed and non-hydrolysed samples indicates the extra acidity due to the addition of hydrochloric acid. This must be subtracted from the formalin titration number in the hydrolysed portion. The formalin titration is carried out in exactly the same method in both the hydrolysed and non-hydrolysed portions. To 25 c.c. of the solution (already acid) are added 4 c.c. of approximately $N/2$ barium chloride solution (244 grm. $BaCl_2.2H_2O$ in a litre) and 20 c.c. $N/3$ silver nitrate solution, and water is then added to make up the total bulk of the mixture to 50 c.c. 4 additional drops of water are added to correct for the bulk of the silver chloride. A known volume of the filtrate, which if not clear at first is thrown back on the filter (conveniently one of 11 cm. diameter) is then titrated with alkali after mixing it with half the bulk of neutralised formaldehyde in the method already described. The difference in the titration numbers between the non-hydrolysed and the hydrolysed portions (after allowing for the added acid as determined by the chlorine estimation) gives the amount of nitrogen in peptide combination.

The liquids, even after decolourisation with silver chloride, are often somewhat coloured. Errors in titration due to this factor may be eliminated by matching a control of water coloured by the addition of solutions of dyes. For this purpose 0.2 grm. Tropæolin O, Tropæolin OO, or Bismarck Brown or 0.02 grm. of Methyl-Violet in 1,000 c.c. of water may be employed. To the pigmented water is then added phenolphthaleïn, and sufficient sodium hydroxide solution to produce the requisite tint. This control serves as a standard tint in carrying out the titrations.

Silver chloride carries down a small amount of nitrogenous substance, and this is apt to cause an error. This can be determined by carefully washing the silver chloride precipitate with $N/5$ barium chloride solution, and estimating the nitrogen therein.

There is also one other factor that must sometimes be taken into account in estimating the peptide nitrogen. In carrying out the hydrolysis a certain amount of nitrogen may be set free in the form of ammonia ("amide" nitrogen, see page 84). This also reacts with formaldehyde to give hexamethylene tetramine, and will therefore affect the titration. Its amount should be estimated by making just alkaline a portion of the mixture to be used for the formalin titration, by the addition of a solution of barium hydroxide in methyl alcohol (to avoid frothing) and distilling off the ammonia *in vacuo* (at a temperature of the water bath not exceeding 40°) into an excess of standard acid. The nitrogen thus found should be subtracted from the peptide nitrogen. It is usually, however, quite small, but a certain quantity can be formed from amino-acids, if the temperature of hydrolysis is too high. (See Henriques and Gjaldbäk, *loc. cit.*).

In making an analysis of a digest, it must be borne in mind, that nitrogenous substances other than these derived from protein are often present. This is notably the case with digestion products of meat, the original material of which contains in addition to proteins numerous nitrogenous extractives. In such a mixture, by the method of analysis given above, the proteoses and peptones can be estimated. It is not as a rule necessary to separate the amino-acids derived from the digestion from the nitrogenous extractives. No simple method is as yet known for effecting this purpose.

The Proteoclastic Enzymes.

(See also article Enzymes, page 10.)

No enzyme has as yet been prepared in a pure state, although many attempts to effect this purpose have been made. The most successful investigations have been made by Peckelharing on pepsin (*Zeitsch. physiol. Chem.*, 1896, **23**, 233 and 1902, **35**, 8, see also Schrumpf, *Beitr. physiol. pathol. Chem.*, 1905, **6**, 396). It does not appear to be a protein. As the enzymes cannot be obtained pure, it is not possible to ascribe to them definite chemical properties. They are examined for technical purposes by estimating their digestive power.

The chief commercial preparations found on the market are pepsin, pancreatin (containing in addition to trypsin, the proteoclastic ferment, enzymes capable of acting on fats and carbohydrates) and papain or papayotin, derived from the unripe fruit of *carica papaya*.

Pepsin generally occurs in commerce as a yellowish-white or, amorphous powder, or sometimes in the form of scales. It is usually

prepared from the mucous membrane of pig's stomach, by extracting with acidified water containing glycerol, from which solution it can be precipitated by salts (sodium chloride or ammonium sulphate) and it can be purified by dialysis (to separate the salts) and precipitation from the dialysed solution by alcohol. It can also be prepared by extracting the mucous membrane with acidified water, evaporating the extract to a syrup *in vacuo* at a temperature not exceeding 45° and then scaling.

Pancreatin is extracted from the pancreas of pig by water, or very dilute acid, and the enzymes are precipitated from the aqueous solution by means of alcohol. The precipitate must be dried at a temperature not exceeding 40°. If very carefully prepared, such a preparation will contain no trypsin, but only trypsinogen, from which the active enzyme can be liberated by the addition of minute quantities of the ferment enterokinase, which is obtained by extracting the mucous membrane of the small intestine with water. As a rule, the pancreatin becomes contaminated with enterokinase during the course of preparation.

Papain is prepared by precipitating the plant juice with alcohol.

Various preparations of these enzymes are described in the British Pharmaceutical Codex (Ed. 1907). (Glycerin of pepsin, Glycerin of pancreatin, *Mistura pepsini composita*, etc.[1])

Methods for Estimating the Proteoclastic Power of Enzymes.[2]

Pepsin.

As pure ferments are not obtainable, only the *relative* digestive power of various preparations can be ascertained.

By the British pharmacopœia it is officially required that pepsin should be capable of digesting at least 2,500 times its weight of coagulated egg albumin. In recent years, various other methods have been devised, which are in many respects more satisfactory than the official method.

(*a*) **Pharmacopœial Method.**—The egg albumin employed for the test is made by boiling fresh eggs for 15 minutes, cooling, separating the whites from the yolks and membrane, and after drying the former with a cloth, sieving it through a wire gauze containing 12 meshes to the centimetre. If 12.5 grm. of this albumin be suspended in 125 c.c. of acidified water prepared by mixing 1 grm. of hydrochloric acid of

[1] For method, of employment of the various preparations, reference should be made to the Codex.

[2] See also article "Enzymes," page 10.

sp. gr. 1.160 with 156 c.c. water (0.2% HCl) and 5 mgrm. be added to the mixture and the whole be incubated for 6 hours, with frequent shaking, at 40.5°, the protein should dissolve, with the exception of a few flakes, to a clear solution. Relative strength of different preparations can be approximately ascertained by estimating how much more, or how much less than 5 mgrm. can produce the same result.

(*b*) **Mett's Method.**—In this method, egg-white is also used as a substrate for investigating the action of enzymes, but the method of manipulation is different. The fresh egg-white is drawn up into small tubes of about 2 mm. bore (care being taken that no air bubbles are drawn up at the same time) which are then placed in nearly boiling water for 2 to 3 minutes; the egg-albumin is thus coagulated. The tube is then divided into equal portions of about 10 mm., care being taken not to break up the coagulum when the lengths of tube are divided off from one another. Two or three lengths are then introduced into small flasks containing 0.4% hydrochloric acid solution and a known quantity of the enzyme solution dissolved in a volume of water equal to that of the acid solution. After 8–10 hours, the tubes are removed, and the length of the albumin dissolved from the ends of the column are measured by a millimetre scale. When comparing two pepsin preparations, experiments are carried out in two similar mixtures, and the square roots of the lengths digested are approximately proportional to the digestive activity of the enzymes.

This method has been very largely used, but in recent years, somewhat more convenient methods have been introduced, in one of which coagulated egg-white is used as a substrate (*Hata's method*), and in the other edestin. (*Fuld's method.*)

Hata's Method (*Biochem. Zeitsch.*, 1910, **23**, 179).—The substrate, in this method, is prepared in the following way. Egg-white is rubbed up in a basin until it is of uniform consistence. Five times the volume of water is then added in small portions at a time, the mixture being well stirred after each addition. The solution of egg-white is then filtered through gauze, and heated in a water-bath at 60° for about 20 minutes. A homogeneous turbid mixture is thus obtained, which can be kept under toluene. This mixture is diluted with 9 times its volume of water before use. 5 c.c. of the diluted mixture are then introduced into a series of tubes to each of which is added 1 c.c. of 0.4% hydrochloric acid; to each tube is then added varying portions (0.2, 0.4, 0.6, etc., c.c.[1]) of the enzyme solutions containing known quantities of the

[1] If less than 0.2 c.c. produces the full enzymatic effect, the solution should be diluted. If 1 c.c. is insufficient, the solution should be made stronger.

enzyme preparation. The tubes are then incubated in a water-bath at 40° for 15 minutes. The tube is then noted in which the smallest amount of enzyme has produced clarification within this time. If, for example, 0.2 c.c. of an enzyme *A* and 0.4 of enzyme *B*, have been just sufficient to produce this effect, then enzyme *A* is twice as active as enzyme *B*. That is, the active strength of an enzyme solution is inversely proportional to the amount necessary to produce clarification of the enzyme solution within 15 minutes.

Fuld's Method. (Fuld and Levison, *Biochem. Zeitsch.*, 1907, **6**, 473, and Blum and Fuld, *ibid.*, 1907, **14**, 62).—Edestin, the crystalline protein from hemp-seed, is in this process used as the substrate. This substance, on treatment with hydrochloric acid, is converted into edestan, which is thrown out of solution on addition of sodium chloride. If pepsin be present, the edestin is digested; if the ferment is present in sufficient quantity, then, after a given interval, the addition of sodium chloride no longer produces a precipitate. To carry out the reaction, portions of 5 c.c. of a 0.5% solution of edestin in 0.4% hydrochloric acid are introduced into a series of test-tubes to which varying amounts of enzyme solutions (0.1, 0.2, 0.3, etc., up to 1 c.c.[1]) are added. After keeping for 1 hour at ordinary temperatures, 1 c.c. of saturated sodium chloride are added to each tube. The tube is noted, which remains clear under these conditions with the smallest amount of enzyme present. The activity of two enzyme solutions will be inversely proportional to the smallest volume of the solution which will digest the edestin solution so far, that under the specified conditions of experiment, no precipitate is produced with sodium chloride within 1 hour.

Grützner's Method.—In this method, fibrin is used for the substrate. In the original process of Grützner, this was stained with carmine, which as digestion proceeded, was set free and coloured the supernatant liquid. This pigment can only be used in acid media, as it is dissolved out by alkalies, and for this reason, Roaf has recently substituted congo-red, which can be used both in acid and alkaline media. The principle of the method consists in determining colourimetrically the amounts of dye-stuff set free, when the same quantities of coloured fibrin are acted upon by the same volumes of the enzyme preparations under given conditions in a given time.

Trypsin.

The test for the tryptic activity of pancreatin suggested in the British Pharmacopœia Codex is the following: 28 cgrm. of the prepara-

[1] See preceding footnote.

tion and 1.5 grm. sodium hydrogen carbonate[1] are added to tepid water contained in a flask, and this solution is added to 400 c.c. milk, previously warmed to 38°; the mixture is kept at this temperature for for 30 minutes; the milk should in this time be so far digested, that a sample on mixing with nitric acid yields no coagulum of protein.

Trypsin preparations can also be compared by Mett's method as given above for pepsin. Gelatin coloured by an aniline dye (Methylene Blue) may be conveniently substituted for egg-white, the gelatin solution being drawn into the tubes while warm, and allowed to set. The digestion in this case is carried out in an alkaline medium (0.4% sodium carbonate).

The digestive activity of preparations can also be compared by measuring the rate of digestion of a 4% solution of caseinogen in 0.4% sodium carbonate by Sörensen's formaldehyde titration method, samples of the digest being removed from the digestion mixture and titrated from time to time (see page 488). The strengths of the preparation can be compared, by finding the relative amounts necessary to produce a given change within a certain time. This is perhaps the most convenient method for comparing tryptic activities.

Papain.

According to the British Pharmacopœia Codex, a good sample should digest 200–250 times its weight of blood fibrin in 4–5 hours at the temperature of 45–50°. It can act both slightly acid and neutral media.

The Plasteins or Coaguloses.

When ferments are allowed to act on digestion products in concentrated solutions, precipitates known as plasteins or coaguloses are formed of which the nature is not yet known, in spite of the fact that they have formed the subject of a large number of researches. (Laurow, *Zeitsch. physiol. Chem.*, 1907, **51**, 1; Sawjalow, *ibid.*, 1907, **54**, 119; Kurajeff, *Beitr. physiol. path. Chem.*, 1902, **1**, 121, 1902, **2**, 411; 1904, **4**, 1476, and other papers.) It has been suggested that the reaction taking place is a synthetical one, the enzyme accelerating a building-up process in concentrated solutions of degradation products. This view, which suggests analogies between certain enzymes acting on carbohydrates and proteoclastic enzymes, has been recently advanced by Henriques and Gjaldbäk (*Zeitsch. physiol. Chem.*, 1911, **71**, 485).

[1] Some trypsin preparations on the market contain carbonate admixed. See Codex.

HÆMOGLOBIN AND ITS DERIVATIVES.

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Blood as it circulates in the vessels of man and other vertebrates is a viscid liquid of a homogeneous red colour to the naked eye, of a bright scarlet hue in arterial blood, dusky red or claret-coloured in venous blood. Blood prevented from coagulating separates into a dark sediment formed of red corpuscles, each of which consists chiefly of a red colouring matter, *hæmoglobin;* the pale straw-coloured supernatant liquid is *liquor sanguinis*, or *blood-plasma.* Shortly after leaving the body, usually within 2 to 6 minutes, unless precautions are taken to prevent this, blood commences to coagulate; a pellicle of fibrin forms on the surface, this extends along the side of the containing vessel, and finally, in about ten minutes, the liquid is converted into a solid jelly. The process of coagulation is due to the formation of *fibrin* from fibrinogen, a protein of the plasma. The fibrin as it forms entangles the blood corpuscles. The jelly-like mass now shrinks, and a straw-coloured fluid, *blood serum*, exudes from the clot. Finally, at the end of 24 hours, the shrunken clot, often a third of its original bulk, remains adherent to the wall of the vessel, sunk in a quantity of serum. In sterile vessels this shrinking often occurs to only a small extent (Lister), therefore the yield of anti-toxic sera from blood collected in sterile flasks may be small. If shed blood is stirred with a bundle of wires or feathers for some minutes, as fast as the fibrin forms it collects on the wires. In this way all the fibrin, as it is formed, may be removed from blood during the process of coagulation. A red liquid is left; this is defibrinated blood—a suspension of corpuscles in serum.

Circulating blood = plasma + corpuscles (red corpuscles and leucocytes).

Clotted blood = serum + clot (fibrin and corpuscles).

Stirred blood = defibrinated blood (serum and corpuscles) + fibrin.

Reaction of Blood.—According to the indicator employed, the reaction of blood is either acid or alkaline (B. Moore, *Proc. Roy. Soc.*, 1905, 76B, 138). To litmus, methyl-orange or dimethyl-aminoazobenzene, blood-plasma is alkaline. To phenolphthaleïn it is acid. The grade of alkalinity, chiefly due to hydrogen disodium phosphate, determined by titration with tartaric acid (Engel, Loewy, Zuntz) or sulphuric acid (A. Wright, *Lancet*, 1897, Sept. 18) is equivalent to a solution of 0.4% sodium hydroxide (Loewy) after the corpuscles have been broken up.

The sp. gr. of defibrinated blood taken by weighing is 1.058. For drops of human blood by Hammerschlag's method, in 165 adult males the average was 1.0544. In 149 of these the variation was 1.050–1.065; in 57% of the whole from 1.054–1.060; in 4 from 1.046–1.049; in 9 from 1.068–1.070, and in 3 cases only 1.040–1.042 (G. N. Stewart, *Manual of Physiology*, 5th ed., 1906, page 19).

Hammerschlag's Method.—A drop of blood ejected from a capillary tube under the surface of a mixture of chloroform (sp. gr. 1.498) and benzene (sp. gr. 0.874) becomes a sphere. By cautious addition and mixing, either chloroform or benzene is added until the blood-drop is suspended in the mixture. The sp. gr. of this liquid, taken with the hydrometer or sp. gr. beads, gives the sp. gr. of the blood. Instead of benzene Inchley (*Proc. Physiol. Soc.*, Aug., 1904) suggests the use of Pratt's petrol A.

Enumeration of Red Corpuscles.—(1) *With Gowers' hæmacytometer* (*Lancet*, Dec. 1, 1877) (Fig. 29).[1]

A is a small pipette, which, when filled to the mark on its stem, holds exactly 995 mm^3.; B, a capillary tube marked to contain exactly 5 mm^3.; and C, a brass stage-plate, carrying a glass slip, on which is a cell 0.2 mm. deep. The bottom of this is divided into squares 0.1 mm. in diameter. Upon the top of the cell rests a cover-glass, which is kept in its place by the pressure of two springs proceeding from the ends of the stage-plate. D is a small glass jar, E, a glass stirrer, F, a sharp spear-pointed lancet.

In using the hæmacytometer, 995 mm^3. of an aqueous solution of sodium sulphate of 1,025 sp. gr. is sucked up by the pipette, A, and blown into the mixing jar, D. The finger, carefully cleaned with acetone, is then pricked with the lancet, the first drop of blood wiped

[1] Gowers' Hæmacytometer in made and sold by Messrs. Hawksley & Sons, 357 Oxford Street, London, W., to whom the authors are indebted for the illustration in the text.

away and 5 mm.^{3} of the second drop of blood sucked into the capillary pipette, B, and blown into the diluting liquid. The two liquids are well mixed by rotating the glass stirrer, E, between the finger and thumb, and a small quantity of the diluted blood placed in the centre of the glass-cell, a cover-glass gently placed on the top, and secured with the brass springs, which should be *lifted* and not slid into position. The plate is then placed on the stage of a microscope. In a few minutes the corpuscles will sink to the bottom of the cell, and, on focussing, can be seen to settle on the squares. The corpuscles in ten of the squares toward the centre of the cell are then counted, and their number, multiplied by 10,000, gives the number in a cubic millimetre of the

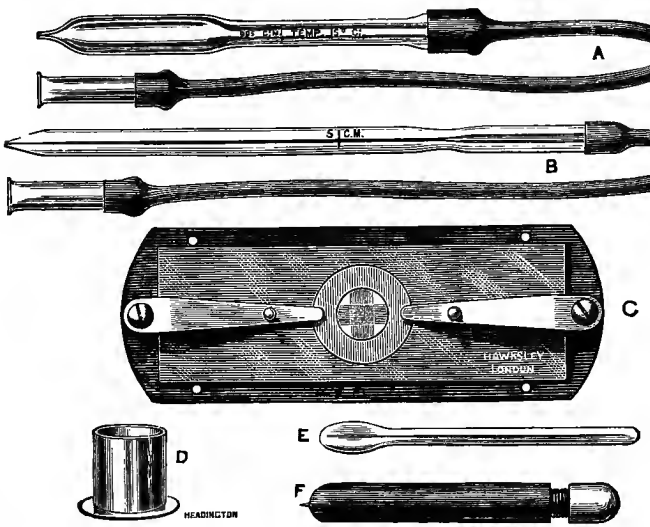


FIG. 29

blood examined. In each square, for normal blood, there is an average of about 50 corpuscles.

(2) *With the Thoma-Zeiss Hæmacytometer.* (Fig 30.)

The two parts of this apparatus are (*a*) a *counter*. Various forms may be used, such as the Thoma-Zeiss counter, Zappert counter, or Bürker counter; that of Thoma-Zeiss is a glass slide with a square affixed, so as to form a cell. Cemented to the floor of the cell and of less diameter is a circular disc B with ruled squares, a portion of which as seen under the microscope is shown at C; the area of each square is 1/400 sq. mm. When accurately covered with a flat square cover-glass, this ruled disc lies exactly 0.1 mm. from the under surface of the cover,

and the volume of the column of liquid standing upon a square is therefore $0.100 \times 1/400 = 1/4000$ mm.^{3} (*b*) *A Mixing Pipette.*—The capillary of this is expanded into a bulb E between the marks 1 and 101, containing a glass bead. Allowance is made for the size of bead, and the divisions on the capillary bear an exact ratio to the volume of the bulb. Thus at 05 or 1 the volume in the capillary is to the bulb when filled up to mark 101 as 1 : 200 or 1 : 100. A second pipette which allows of dilutions of 1 : 10 is used for enumeration of leucocytes, and the two are often supplied in one case.

To make a blood-count, suck up the blood using the second drop which exudes from a puncture on the skin which has been cleaned with acetone (root of finger-nail or ear) to the mark 05 or 1; wipe the ends of pipette clean with filter paper, and then with

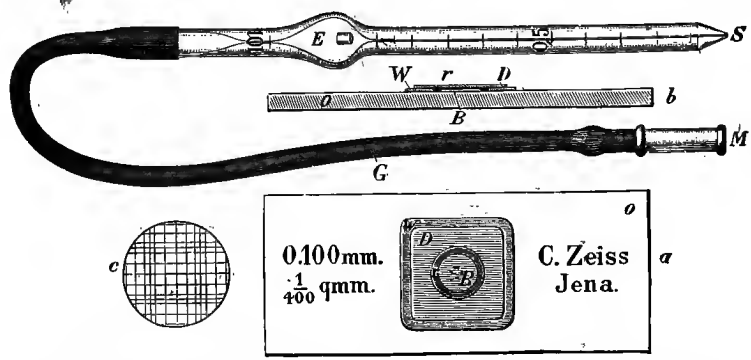


FIG. 30.—Mixing pipette and counter for red blood-corpuscles. After *Thoma*.

the pipette immersed in the diluting fluid[1] suck this up slowly while the pipette is continuously rotated between the thumb and finger so as to mix well. Stop when the liquid exactly reaches the 101 mark. Without removing the rubber tube, G, close with the fingers both ends of pipette, M and S, and shake vigorously for 1 minute. Next, by blowing, eject 3 or 4 drops, wipe end of pipette, and now blow out a minute drop on to the centre of the ruled disc of the counter. Cover carefully with the plane cover-glass. In doing this the liquid should not escape into the circular well round the disc, and Newton's rings should be seen as an interference phenomenon when the cover is accurately in contact with the square cell. Count the corpuscles in a number of squares, also including those which lie on the base line and left side of each square. Corpuscles which lie

[1] Diluting fluids for red corpuscles: Filtered Hayem's fluid; $HgCl_2$ 5 grm., Na_2SO_4 5 grm., NaCl 1 grm., H_2O 200 c.c. For white corpuscles: 3 % glacial acetic acid in water with 1 % of 1 % aqueous solution of Gentian-Violet or Methyl-Violet.

on the top and right-hand side do not belong to the square. In counting, count five squares left to right, next lower set right to left, then five below, left to right, and so on. As you proceed, note on paper the totals for each 5 or 10 squares. At least 40 squares should be counted. The number of corpuscles per cubic millimetre of blood is given by

$$\frac{4000 \times \text{degree of dilution} \times \text{total corpuscles counted}}{\text{No. of squares counted.}}$$

In sucking blood into the capillary, it is not necessary that the mark 05 or 1 should be exactly reached. Thus at 03, 05.4, 06, the ratio of blood volume to the bulb is $\frac{03}{1000}, \frac{05.4}{1000}, \frac{06}{1000}, \ldots$ and the degree of dilution is $\frac{1000}{03}, \frac{1000}{05.4}, \frac{1000}{06} \ldots$

Abbe has shown that when 16 squares are counted the error is 5%, when 100 about 2%, when 400 about 1%. However, a variation of 100,000 red corpuscles per cubic millimetre has no physiological interest.

Average number of red corpuscles per cubic millimetre of blood. *Man*, 5,000,000; *dog*, 6,500,000; *cat*, 9,000,000; *pig*, 6,000,000 to 8,000,000; *horse*, 7,700,000; *ox*, 6,000,000 to 8,000,000; *sheep*, 10,000,000; *goat*, 13,000,000 to 18,000,000.

Average Size of Red Corpuscles.—In the blood of all the mammals, except the *Camelidæ* which are oval, these are circular biconcave discs. The corpuscles of the blood of birds, reptiles, amphibia, and fish are oval (except in the lamprey), nucleated, and larger than mammals.

The average size of the blood-corpuscles from each species is approximately constant. Thus the corpuscles of human blood have an average diameter of about 1/3200 in. but in fresh human blood, corpuscles may always be found which vary very considerably from the mean value. The corpuscles of the human fœtus are usually larger, averaging 1/3000 in., and occasionally reaching nearly twice the diameter of adult human blood. At birth a few nucleated red corpuscles may always be found.

The following table shows the diameter of the red corpuscles of various mammalia, expressed both in fractions of an inch and in decimals of a millimetre. The blood was in each case allowed to dry in a thin layer on the slide. (Wormley, *Michrochemistry of Poisons*, Philadelphia, 1888; Gulliver, *Proc. Zool. Soc., Lond.*, 1875, 474.)

Source of blood	T. G. Wormley		Gulliver	
	Inch	Millimetre	Inch	Millimetre
Man	1/3250	.0078	1/3200	.0080
Man, fœtus	1/3000	.0085	.....	.....
Cat	1/4372	.0058	1/4404	.0058
Dog	1/3561	.0071	1/3532	.0072
Fox	.....	.....	1/4177	.0061
Pig	1/4268	.0059	1/4230	.0060
Elephant	1/2738	.0093	1/2745	.0092
Horse	1/4243	.0059	1/4600	.0054
Ass	1/3620	.0070	1/4000	.0064
Ox	1/4219	.0060	1/4267	.0059
Sheep	1/4912	.0052	1/5300	.0048
Goat	1/6189	.0041	1/6366	.0039
Deer	.....	.....	1/7060	.0036
Hare	.....	.....	1/3560	.0071
Rabbit	1/3653	.0069	1/3607	.0070
Mouse	1/3743	.0067	1/3814	.0066
Rat	1/3652	.0069	1/3754	.0067

The corpuscles of the blood of some of the quadrumana are very similar in size to human corpuscles. Gulliver has recorded the following measurements, in fractions of an inch.

Lemur anguanensis,	1/3976; 1/4003; 1/4440.
Ateles ater,	1/3368; 1/3342; 1/3412.
Chimpanzee,	1/3512; 1/3602.

The following are measurements by Gulliver of the corpuscles of the blood of certain oviparous vertebrate animals. The figures are in fractions of an inch.

Source of blood	Longer diameter	Shorter diameter
1. Fowl	1/2102	1/3466
2. Turkey	1/2045	1/3598
3. Pigeon	1/1973	1/3643
4. Duck	1/1937	1/3424
5. Goose	1/1836	1/3839
6. Turtle (green)	1/1231	1/1882
7. Frog	1/1108	1/1821
8. Toad	1/1043	1/2000
9. Trout	1/1524	1/2460
10. Pike	1/2000	1/3555
11. Eel	1/1745	1/2842

Composition of the Red Corpuscles.—For the dog Alderhalden (*Zeitsch. Physiol. Chem.*, 1898, **25**, 88) gives for 1,000 parts by weight:

Water	644.25
Solids	355.75
Hæmoglobin	327.52
Proteins	9.918
Cholesterol	2.155
Lecitbin	2.568
Fatty acids	0.088
Phosphoric acid as nuclein	0.110
Na_2O	2.821
K_2O	0.289
Fe_2O_3	1.573
MgO	0.071
Cl	1.352
P_2O_4	1.635
P_2O_5 (inorganic)	1.298

According to Brandenburg, 1,000 parts by weight of red corpuscles contain the following amounts of K_2O: *Cat*, 0.058, *dog*, 0.257; *man*, 4.294; *horse*, 4.957; *rabbit*, 5.229.

From the above table it will be seen that hæmoglobin, as it is known to the chemist, when separated from the corpuscles cannot be held in solution in the red corpuscles, for 32% of hæmoglobin cannot be in solution with 62% of water, since at 37° C. only 18% hæmoglobin can be held by water.

Laking of Blood.—When blood is treated with distilled water, both hæmoglobin and the electrolytes may leave the corpuscles. The liquid becomes transparent and is said to be *laked*, or the corpuscles to be hæmolysed. Within the body hæmolysis may occur. The hæmoglobin then passes into the plasma and may be excreted in the urine. The term hæmaturia indicates that blood corpuscles exist in urine; hæmoglobinuria that hæmoglobin is present in solution.[1] Intermittent hæmoglobinuria is not uncommon in individuals suffering from malaria, and blood may originate from hæmorrhage of the kidney, bladder or urethra. The mode of action of hæmolytic agents varies, but the phenomenon is produced by:

1. Distilled water.
2. Grinding up corpuscles with sand (Rywosch).
3. Substances such as urea, ammonium chloride, glycerol, alcohol, ether, bile salts.
4. Glucosides, such as saponin, cyclamin, githagin, and solanin (Kobert).
5. The toxins of bacteria such as tetanolysin of *bacillus tetani*, megatheriolysin of *bacillus megatherium*, or the toxic lysins of *staphylococcus* or *bacillus pyocyaneus*.
6. Not all, but many snake-venoms cause hæmolysis both inside and outside the body, *e.g.*, those of the cobra, rattle-snake, water-moccasin, copperhead, Daboia and Enhydrina.
7. The blood serum of one animal is often hæmolytic for the blood of a different species, *e.g.*, that of the dog for the rabbit. The serum of the eel (Mosso 1888) and of the frog (Krompecher, Buckmaster) is hæmolytic for most kinds of mammalian blood.
8. Abrin, ricin and robin are phyto-albumins which produce hæmolysis.
9. Hæmolytic sera may be experimentally obtained in a variety of ways (Belfanti and Carbone 1888, Bordet 1888, Ehrlich and Morgenroth

[1] Variable amounts of methæmoglobin are nearly always present in cases of hæmoglobinuria. Normal urine often contains traces of hæmatoporphyrin (A. E. Garrod).

1899). Thus, by repeated injections of guinea-pig's blood into the rabbit, the serum of the rabbit becomes hæmolytic for the red corpuscles of the guinea-pig. Similarly for goat's blood into rabbits, the rabbit serum is found hæmolytic for goat's corpuscles. This type of experiment can be varied for many animals, the blood being injected into the rabbit, and specific hæmolytic sera be obtained by this injection of foreign blood corpuscles (v. precipitation test for blood, page 577).

The substances in sera which cause hæmolysis are known as hæmolysins.

A number of drugs introduced into the body of man or animals cause not only hæmolysis but a conversion of oxyhæmoglobin into methæmoglobin, and in this latter form the pigment appears in the urine, so that this may appear almost black. Such drugs are: (1) Chlorates of potassium and sodium; (2) nitrites and nitrobenzene; (3) phenylhydroxylamine; (4) aniline derivatives in phenacetin, acetanilide; (5) quinine, according to R. Koch, Plehn and others, induces those attacks of black-water fever which are seen in tropical countries where a pernicious type of malaria is endemic. 5 grains of quinine will often be sufficient to cause the appearance of methæmoglobin in the urine of patients with malaria.

Blood in even a thin layer is opaque, dark when examined by transmitted light and clear by reflected light. From this fact alone it is obvious that blood in this optical property resembles those colours which are not true solutions but fine suspensions of colouring matter in a colourless medium, and hence it could be inferred that the blood-colouring matter is not in true solution. The colouring matter of the blood of vertebrate animals is a complex iron-containing protein, which can be obtained in a crystalline form. This is the chief constituent of the red corpuscles, and, owing to its presence, they act as the carriers of oxygen in the organism. It possesses the remarkable quality of linking to itself a molecule of oxygen to form an easily dissociable compound, known as *oxyhæmoglobin* ($Hb + O_2$); but oxyhæmoglobin and hæmoglobin (reduced hæmoglobin) exist side by side in various proportions in circulating blood, the former being more abundant in arterial, the latter in venous blood. Hæmoglobin is a protein, easily decomposed by moderate heat, weak acids or alkalies, into *globin* (a histone) and a relatively simple crystallisable prosthetic group containing the iron, spoken of as *hæmochromogen*, which easily unites with oxygen and is then known as *hæmatin*. The hydrochloride of this is crystalline *hæmin*. Removal of the iron from either of these three substances by strong acids yields coloured iron-free derivatives; of these *hæmatoporphyrin* and *hæmatoidin* are examples.

TABLE I.
ELEMENTARY ANALYSIS OF OXYHÆMOGLOBIN.

Source	C.	H.	O.	N.	S.	Fe.	P.	Observer	Reference
Dog	53.64	7.11	21.02	16.19	0.66	0.43	0.91 (P_2O_5)	C. Schmidt	Hoppe-Seyler's *Med. Chem. Unter.* 1867, **2**, 169
Dog	53.85	7.32	21.84	16.17	0.36	0.43	.....	F. Hoppe-Seyler	*Ibid.*, 1867, **2**, 189.
Goose	54.26	7.10	20.69	16.21	0.54	0.43	0.77 (P_2O_5)	F. Hoppe-Seyler	*Ibid.*, 1867, **2**, 194.
Guinea-pig	54.12	7.36	20.68	16.78	0.58	0.48	.....	F. Hoppe-Seyler	*Ibid.*, 1868, **3**, 370.
Squirrel	54.09	7.39	21.44	16.09	0.40	0.59	.....	F. Hoppe-Seyler	*Ibid.*, 1868, **3**, 370.
Horse	54.87	6.97	19.73	17.31	0.65	0.47	.....	Kossel	*Zeit. f. Physiol. Chem.*, 1878, **2**, 150.
Dog	54.00	7.25	21.45	16.25	0.63	0.42	.....	J. Otto	*Ibid.*, 1882, **7**, 61.
Pig	54.17	7.38	21.36	16.23	0.66	0.43	.....	J. Otto	*Ibid.*, 1882, **7**, 61.
Horse	54.76	7.03	19.81	17.28	0.67	0.45	.....	J. Otto	*Pflüger's Arch.*, 1883, **31**, 243.
Horse	54.40	7.20	19.67	17.61	0.65	0.47	.....	M. Bücheler	*Dissert.*, Tübingen, 1883.
Horse	51.15	6.76	23.43	17.94	0.39	0.34	.....	O. Zinoffsky	*Zeits. f. Physiol. Chem.*, 1885, **10**, 32.
Ox	54.66	7.25	19.54	17.70	0.45	0.40	.....	G. Hüfner	*Ludwig's Festgabe*, 1890, **80**.
Pig	54.71	7.38	19.60	17.43	0.48	0.40	.....	G. Hüfner	*Ludwig's Festgabe*, 1890, **80**.
Dog	53.91	6.62	22.62	15.98	0.54	0.33	.....	A. Jaquet	*Zeit. f. Physiol. Chem.*, 1888, **12**, 288.
Dog	54.37	7.22	21.93	16.38	0.57	0.34	.....	A. Jaquet	*Zeit. f. Physiol. Chem.*, 1889, **14**, 291.
Fowl	52.47	7.19	22.50	16.45	0.86	0.34	0.20	A. Jaquet	*Zeit. f. Physiol. Chem.*, 1889, **14**, 295.
Horse	54.56	7.15	.....	17.33	0.43	.....	.....	F. N. Schulz	*Zeit. f. Physiol. Chem.*, 1898, **24**, 469.
Horse	54.75	6.98	20.12	17.35	0.42	0.38	.....	E. Alderhalden	*Zeit. f. Physiol. Chem.*, 1903, **37**, 494.

Occurrence.—Hæmoglobin and allied colouring matters are found in animals[1] either (1) in special morphological elements of the blood or perivisceral fluids, (2) in a state of solution in the liquids of the body, (3) in muscular tissue, (4) in nervous tissue, (5) in the urine and fæces (pathological).

The hæmochromogen moiety of hæmoglobin appears to be identical in all vertebrates, though the blood-colouring matter itself exhibits differences in physical characters and chemical composition. The published elementary analyses of crystallised purified oxyhæmoglobin, dried between 100–110°, given by various observers, do not completely agree, as will be seen in Table I.

The globin of hæmoglobin has generally been regarded as a histone, but the presence of only 20% of diamino-acids is against this view; further, the principal diamino-acid is histidine (β-iminazole-α-amino-propionic acid), whereas in other histones it is arginine (α-amino-δ-guanidine-valeric acid).

TABLE II.

PRODUCTS OF THE HYDROLYSIS OF THE HISTONE MOIETY OF HÆMOGLOBIN FROM HORSES' AND DOGS' BLOOD, ACCORDING TO ABDERHALDEN.[2]

Products of hydrolysis	Globin of horses' blood	Globin of dogs' blood
Glycine	—	—
Alanine	4.19	3.0
Valine	—	1.0
Leucine	29.04	17.5
Phenylalanine	4.24	5.0
Tyrosine	1.33	—
Serine	0.56	—
Cystine	0.31	—
Proline	2.34	4.5
Oxyproline	1.04	—
Aspartic acid	4.03	2.5
Glutamic acid	1.73	1.2
Tryptophane	+	—
Arginine	5.42	—
Lysine	4.28	—
Histidine	10.96	—
Total	69.87	34.7

On the assumption that the molecule of hæmoglobin contains only 1 atom of iron and that the percentage of iron is 0.336, the molecular weight of oxyhæmoglobin is 16,662, and calculating from the fact that 1 grm. of hæmoglobin from ox blood unites with 1.338 c.c. of CO at 0° and 760, and assuming that Hb and CO unite in molecular proportion, the molecular weight would be 16,736.

[1] Preyer, "*Die Blutkrystalle*," page 6. E. Ray Lankester, *Proc. Royal Soc. Lond.*, 1872, 21, 70–81. R. Hertwig, *Lehrb. v. Zool.*, 6 Auf., Jena 1903. O. v. Fürth, *Vergleich. Chem. Phys. v. nied. Tiere*, Jena 1903. P. Nolf, Art. *Hæmoglobine*, Richet's *Dict. de Physiol.*, 1908, page 321. Gamgee, *Textbook of Physiology*, ed. by Schäfer, 1898, 1, page 156.

[2] *Zeit. f. Physiol. Chem.*, 1903, 37, 454; 1907, 51, 397.

The molecular weight has been determined from the osmotic pressure. Assuming that 1% solution of oxyhæmoglobin is undissociated and, as shown by the ultra-microscope, to be a true solution, an osmotic pressure of 10.77 mm. of mercury corresponds to a molecular weight of 16,669. After three recrystallisations without alcohol, Hüfner[1] for oxyhæmoglobin of the horse found a molecular weight between 14,630 and 15,840, the mean of 4 experiments gave 15,115; for oxyhæmoglobin from the ox 15,500 and 18,370, and the mean of 11 experiments 16,321. For 1% oxyhæmoglobin solution (Hüfner) the osmotic pressure in millimetres of mercury is,

Horse	Ox	Ox
11.90	10.18	11.03
11.14	10.61	10.45
11.64	10.59	9·31
12.10	10.67	10.55
	10.78	10.29
		11.00

The values found by Waymouth Reid[2] for oxyhæmoglobin of the dog are only 3.5 to 3.8 mm. of mercury after one crystallisation, 4.1 to 4.3 mm. after a second crystallisation.

Preparation of Oxyhæmoglobin: Method of Preparation of Crystals in Small Quantities for Microscopic Examination.

1. *Funke's method*, the simplest and oldest, for the blood of horse or rat, consists in mixing a drop of blood with a drop of distilled water on a slide by means of a needle. When the edges commence to dry, apply a cover-glass. Crystals form at once.

2. *Rollett's Method.*—Defibrinated blood is repeatedly frozen and thawed in a platinum crucible placed in a mixture of ice and salt or, better, snow and calcium chloride. After the last gradual thawing the laked blood is poured out into a glass vessel, forming a layer 15 mm. deep, and set aside in a cool place. In a short time crystals form, most easily from guinea-pigs' blood, and for other animals in the following order:—squirrel, cat, dog, man, rabbit, pig. Frog's blood yields no crystals by this method.

3. *Gscheidlen's Method.*—Defibrinated blood, which has been exposed to air for 24 hours, is sealed in narrow glass tubes (vaccine tubes) and then placed in the incubator at 37° for some days. On opening the tubes, the contents are spread on a slide and allowed slowly to evaporate. From the dog prismatic crystals, 3.5 cm. in length, may be obtained.

[1] Hüfner and E. Gansser. *Arch. f. Anat. u. Physiol.*, 1907, 209.
[2] W. Reid, *J. Physiol.*, 1905, **33**, 12.

4. A drop of blood is allowed to dry somewhat at the edge, surrounded with a ring of Canada Balsam dissolved in xylene, benzene, or chloroform, and then covered. Within half an hour crystals form.

5. Leeches which have sucked blood, some 3 weeks later are found frequently to have masses of hæmoglobin crystals in the crop.

Reichert states that when the blood of the dog, horse, rat, guinea-pig, or a cold-blooded animal like necturus, is laked with water, ether, or, best of all, ethyl-acetate, and before or after laking ammonium oxalate to the amount of 1 to 5% added, hæmoglobin crystallises quickly and easily.[1]

S. M. Copeman[2] found that if the blood from the finger of patients suffering from pernicious anæmia was allowed to fall on a glass slide and then, when the edge of the drop had dried somewhat, a cover-glass was gently placed on it, crystals of hæmoglobin gradually formed in the film in 10 to 24 hours without any further preparation. After treatment with arsenic, the blood of such patients yielded no crystals. Bond found in cases of septic anæmia and pernicious anæmia, that hæmoglobin crystals formed spontaneously when blood was slowly dried. He also obtained crystals from normal human blood by mixing this with putrid human serum.

The most reliable method is that given by Copeman for the blood of the following animals: horse, bullock, sheep, pig, dog, cat, squirrel, rabbit, guinea-pig, rat, mouse and fowl. To defibrinated blood add 1/16 part of its volume of ether, and shake for some minutes until it becomes perfectly transparent or laky. Then set aside in a cool place for a period varying from a few hours to a few days (Gamgee, *Physiol. Chem.*, 1, page 87). The shaking is done in a stoppered bottle, and the air allowed to escape gradually as the ether volatilises. By this means the air is replaced by ether vapour, while at the same time the small portion of blood which is forced out around the stopper of the bottle dries and so prevents ingress of air. The bottle is best kept at even temperature, not in a cool place. How long the blood should be left (in the case of most animals 2 days at least) is known by removing a drop, and then when the edges are slightly dry, gently lowering a cover-glass on to the surface. Crystals commence to form an hour or so later. Human blood mixed with putrid sheep's serum invariably yielded hæmoglobin crystals. These are in the state of reduced hæmoglobin.

[1] *Amer. J. Physiol.*, 1903, 9, 97. See also *The crystallography of Hæmoglobin* by E. Tyson Reichert, and A. P. Brown, *Publication* 116 *Carnegie Inst.*, Washington, 1900 (contains 100 plates illustrating crystals from different kinds of animals).

[2] *J. Physiol.*, 1890, 11, 401.

Method of Preparation of Hæmoglobin Crystals in Quantity.

Perfectly fresh defibrinated horse or ox blood is strained through muslin and centrifugalised (2,000–2,400 revolutions per minute), the serum is poured off and the deposit of blood corpuscles mixed with a volume of sodium chloride 0.85% equal to the volume of serum, and then again centrifugalised. The sediment is then dissolved in the least possible quantity of distilled water at 37°. The solution is then cooled to 0°, and mixed with half its volume of pure cold ether, transferred to a stoppered separating funnel and thoroughly shaken several times during the day. It is then allowed to stand for 24 hours or longer, and the content separates into three layers, the lower one a clear aqueous solution of hæmoglobin, a middle gelatinous layer with corpuscle residues, and an upper one of ether. The aqueous solution is run off into a beaker, and what is allowed to flow out must be free from the slightest turbidity. The solution is filtered into a large flask and freed from ether by drawing a current of air through the flask. The air current must be purified by passing through concentrated solutions of potassium permanganate and concentrated sulphuric acid. The solution at 0° is now cautiously mixed, gently shaking the flask, with pure alcohol in proportions depending on the solubility of the hæmoglobin. For the horse the alcohol added equals one-fourth of the volume of the solution, for the ox one-third. All these operations must be conducted in a cold room and the solution kept at 0° as nearly as possible. The whole is placed in a good freezing mixture (−20°) which is constantly renewed morning and evening. The hæmoglobin gradually crystallises out, for the horse in about 12 hours, for the ox in about 24 hours or longer. The crystals are separated from the mother-liquor by a centrifuge kept at 0°. They are then recrystallised without alcohol. The crystals are first washed with pure ice-cold boiled-out distilled water and then dissolved in the least possible quantity of of pure boiled-out distilled water at 30°. They are then cooled to −3° and seeded with a small portion of washed crystals of the original crystallisation, and left to crystallise. The crystals are again separated by the centrifuge and the operation repeated. Gamgee[1] recommends thorough washing by decantation with 20% alcohol after each successive crystallisation and washing the ultimate product many successive times with large quantities of the purest distilled water at 0°. If the crystals are desired dry, the moist mass is spread out on a Schleicher and Schull thick filter-paper plate and dried *in vacuo* for 6 hours over sulphuric acid. The mass is powdered in an agate mortar and

[1] *Proc. Royal Soc.*, 1902, 70, 81.

finally dried in a current of pure hydrogen in a flask kept at 105° in an air-bath or in the vapour of boiling toluene.

In the case of human blood it is not possible to crystallise the hæmoglobin by alcohol in the above manner, but crystallisation may be brought about by salting out with ammonium sulphate. The hæmoglobin obtained in this way is washed on the filter with ice-cold water and again dissolved.[1]

A convenient method for the preservation of small quantities of oxyhæmoglobin is given by Uhlik.[2] Some drops of moist crystals are allowed to fall into a few cubic centimetres of 25% alcohol in a test-tube and gently mixed by movement. The crystals settle, the alcohol is poured off and replaced with 50% and again mixed. The procedure is repeated with replacement with 75% alcohol, and finally with absolute alcohol. The sediment is placed on a slide, and when quite dry covered with a drop of Canada Balsam.

The crystals of hæmoglobin of man and different animals belong, with one exception—the squirrel—to the rhombic system. In this animal they are hexagonal tables, but after many recrystallisations they change into rhombic needles and tetrahedra. Hæmoglobin crystals exhibit the phenomena of pleomorphism.

The water of crystallisation is usually determined in the following way. Pure hæmoglobin is dried *in vacuo* at 0° and the water of crystallisation driven off by heating to 115°. The accurate determination is difficult, but there can be no doubt that the crystals of hæmoglobin from different animals possess different amounts of water.

Oxyhæmoglobin	Water of crystallisation, %	Authorities
Dog	3.4	Hoppe-Seyler.
Horse	3.94	Hüfner.
Pig	5.9	Otto.
Guinea-pig	6	Hoppe-Seyler.
Squirrel	9	Hoppe-Seyler.

Solubility.—The determination of the solubility of hæmoglobin is difficult, owing to the readiness with which the crystals undergo change, and also to the difficulty of avoiding traces of the reagents employed in the preparation of crystals. In the present state of knowledge, hæmoglobin from different animals differs markedly in solubility. The hæmoglobin of man is most soluble in water, then follow in order that of the pig, ox, birds, horse, dog, squirrel, guinea-pig and rat. The crystals are soluble in highly diluted solutions of

[1] *Zeits. Physiol. Chem.*, 1909, 62, 193.
[2] *Pflüg Arch.*, 1904, 104, 64.

ammonia, alkali hydroxides and their carbonates. Such solutions resist decomposition much longer than pure aqueous solutions. Stronger solutions decompose hæmoglobin. The crystals are soluble in highly diluted alcohol, but by contact with this, they become less soluble in water. The crystals are insoluble in absolute alcohol.

Diffusibility.—Oxyhæmoglobin is a typical colloid in the sense of its absolute indiffusibility through animal membrane and parchment paper, but differs from most colloids in the facility with which it crystallises. When pure solutions of oxyhæmoglobin are subjected to electrolysis there occurs a separation of oxyhæmoglobin in a colloidal but perfectly soluble form. Gamgee, employing an electrolytic cell in which the anode is separated from the cathode by an animal membrane (sheep's intestine or pig's bladder), noticed that the first action of the current is to cause a separation of colloidal hæmoglobin in the anode cell. This colloidal hæmoglobin falls as a beautiful red cloud, leaving a perfectly colourless supernatant liquid. On stirring, it instantly dissolves. The further action of the current is to cause a rapid and entire transfer of the colloidal hæmoglobin from the anode to the cathode cell. With an electrolytic cell, of which each compartment had a width of 5 mm. and contained 2.5 c.c. of a 1% solution of oxyhæmoglobin, complete precipitation and transfer occurs within 60 minutes. On reversal of the current the hæmoglobin returns again in the direction of the positive current into the original cell from which it started. Gamgee considers that it is highly probable that within the corpuscle hæmoglobin is present in a colloidal form.[1]

The conductivity of oxyhæmoglobin solutions is low. It increases rapidly with increase of temperature and undergoes remarkable and permanent changes when the solution is kept, even for short periods, at any temperature above 0°. It is therefore impossible to obtain very reliable data as to the absolute specific resistance of oxyhæmoglobin solutions. Gamgee,[2] as the result of laborious investigations, gives the following numbers, expressed in reciprocal ohms, for the specific conductivity of solutions of oxyhæmoglobin.

Temp.	Solution of O_2Hb contains 3.07 % or 1 grm. mol. in 542,900 grm. Conductivity	Contains 2.235 % O_2Hb or 1 grm. mol. in 745,800 grm. Conductivity
0°	$10^{-5} \times 2.626$	$10^{-5} \times 2.23$
18°	$10^{-5} \times 4.432$	$10^{-5} \times 3.25$
25°	$10^{-5} \times 5.19$	$10^{-5} \times 4.27$
39°		$10^{-5} \times 7.47$

[1] *Proc. Royal Soc.*, 1902, 70, 83.
[2] *Proc. Royal Soc.*, 1902, 70, 82.

Optical Properties of Oxyhæmoglobin.—As first described by Rollett and v. Lang, the crystals of oxyhæmoglobin exhibit a marked pleochroism. This is best seen with hæmoglobin and carboxy-hæmoglobin, less obviously with oxyhæmoglobin and methæmoglobin. This phenomenon is apparent when the crystals are rotated on a slide on the stage of the polarising microscope, only the lower nicol being used, and without an analyser in the eye-piece. Crystals of oxyhæmoglobin of the dog present a more or less permanent tint, according to their position with reference to the plane of polarisation. Some are scarlet-red, others orange or yellow. On rotation of the slide, each crystal passes insensibly from one to other of these colours. Hæmochromogen crystals also show pleochroism.

Examined with crossed nicols the crystals show double refraction. Those of the squirrel are an exception. Within the corpuscle hæmoglobin exhibits no double refraction. Pleochroism characterises the crystals more definitely than double refraction.

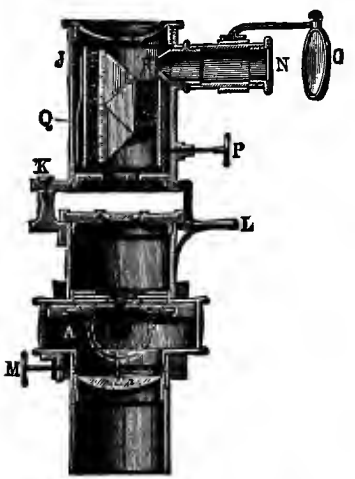
Specific Rotation.—Gamgee and Croft Hill determined this for oxyhæmoglobin and carboxyhæmoglobin. They filtered the light of an arc-lamp through a Landolt's filter, one compartment of which contained a solution of 0.05 grm. of hexamethylpararosaniline, dissolved in 1 litre of dilute alcohol, the second compartment being filled with 10% aqueous solution of potassium chromate. Only a narrow band of the spectrum passed through $\lambda 718\mu\mu - \lambda 639\mu\mu$. For oxyhæmoglobin the mean value was $[\alpha]_c = +10°$. For Hb-CO $[\alpha]_c = +10.8°$. Globin, like other proteins, is lævo-rotatory $[\alpha]_c = -54.2°$.

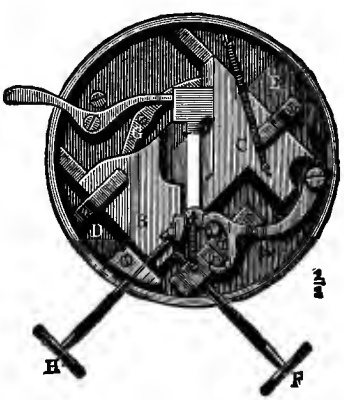
Spectroscopic Properties of Oxyhæmoglobin.—The spectroscope commonly employed in physical and chemical laboratories may be used for studying the visible spectra of hæmoglobin and its derivatives. The absorption spectra of these are best investigated with an instrument provided with a single good flint glass prism rather than with a train of prisms since, with the greater dispersion of the latter, the absorption bands are less clearly defined.

Dual vision spectroscopes of the Browning or Hilger patterns or direct vision spectroscopes, adapted to the eye-piece of the compound spectroscope, may be employed. This class of instrument may be utilised for the investigation of minute quantities of colouring matters. In Abbe's micro-spectroscope (Figs. 31 and 32) made by Zeiss the direct-vision prism can be moved away from or brought over the eye-piece at will, and is secured in position by means of a catch (L). By means of a screw and spring the micrometre-scale can be readily adjusted to the standard position with regard to the spectrum, namely, that in

which the Fraunhofer-line D corresponds with the division 58.9. This arrangement allows the wave-lengths of all other lines and bands to be read off at once without the necessity of referring to a table. The length and width of the slit can be adjusted by the screws F, H.

For fine work, the spectroscope employed should be one furnished with means for determining accurately the position of any line or the boundaries of any absorption band observed in the spectrum, it being usual to express the position in terms of the wave-length of the light corresponding to it. For this purpose the spectrum of sunlight is observed, and the position of the chief Fraunhofer lines in reference to the divisions of the graduated circle of the instrument is determined.





The Zeiss micro-spectroscope.

FIG. 31.—Section of instrument. FIG. 32.—Mechanism of slit.

From such observations a curve may be plotted, enabling the observer to convert the readings of the arbitrary divisions of his scale into wave-lengths. For accurate work, the eye-piece of the instrument should be provided with cross-threads, and for use in the investigations of absorption spectra, provided with a variable slit, such as is used in the spectrophotometer for isolating any desired region of the spectrum.

As a source of light, sunlight reflected from a white surface may be employed, or an incandescent burner. The latter is the best of all lamps for the examination of absorption spectra.

In examining the absorption spectra of liquids, these are placed in small cells with parallel glass or quartz sides which are a known width apart. Such vessels are known as hæmatinometers. The simplest is

that devised by Hoppe-Seyler, and consists of a rectangular glass cell in which the internal surfaces of the larger glass plates are 1 cm. apart. In the hæmatoscope of Hermann (Fig. 33) the distance of the plates can be varied. The instrument consists of a glass plate A forming the anterior wall of tube B supported on a stand. The metallic tube C, sliding in front of tube B, is closed anteriorly by a glass plate parallel to A. A funnel D communicates with a space between the plates; by sliding the piston C in and out of B the distance between the two plates can be varied and the depth of the stratum of liquid read off on a millimetre scale engraved on C. With the hæmatoscope we can obtain, within limits, the same results with a solution of constant concentration as with a large number of solutions in which the concentration varies in known proportion, since the absorption of light passing through a coloured liquid depends upon the number of absorbing molecules in its path.

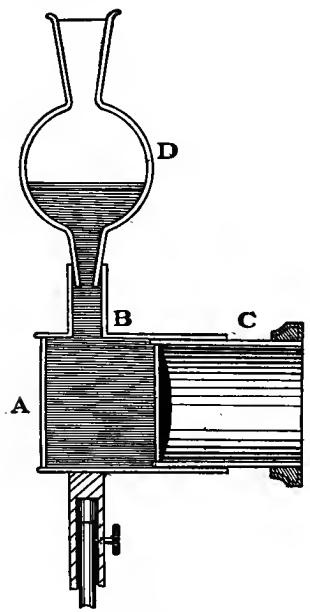


FIG. 33.

Specially characteristic of an absorption band is its point of maximum absorption, which does not change with different concentrations of the colouring matter, whereas the width of the bands changes very considerably. The point of maximal absorption of any band or bands in the spectra to be described later will be always indicated by the wave-length value of this region of maximal absorption.

In the case of hæmoglobin and its derivatives, some bands are visible with the naked eye and some can only be detected by using fluorescent eye-pieces or screens, or by special photographic methods: since these lie in or near the ultra-violet region of the spectrum. (For details of these methods see J. L. Soret, *Arch. phys. et nat. Geneva*, 1878, p. 322–357. A. Gamgee, *Proc. Roy. Soc.*, 1896, **59**, 256; Lewin, Miethe and Stenger, *Pflüger's Archiv.*, 1907, **118**, 80; *Beiträge zur Physiologie der Blutspectra;* Rost, Franz and Heise, *Arbeiten aus d. Kaiserlich. Gesundheitsamt*, 1909, **22**, Heft 12. Schumm, *Klinische Spectroskopie*, Jena, 1909; also article in Abderhalden's *Handbuch des Biochem*

Arbeitsmethoden, Band 6, p. 389. Mees, An Atlas of Absorption Spectra London, 1909. W. J. Dilling, *Die photographie der Blutspektra, Zeitsch. f. biolog. Tech. u. Math.*, 1912, Bd. II, No. 8, 353.) According to Gamgee, the bands in the ultra-violet are even more distinctive for blood colouring matter than the bands of the visible spectrum. At a meeting of the International Physiological Congress at Berne in 1895 he described the following simple arrangement, which requires merely an electric arc lamp and an ordinary laboratory spectroscope of the Bunsen type, by which the bands in the ultra-violet may be rendered visible. "The telescope of the spectroscope is removed and a beam from the positive pole of the arc is allowed to fall on the slit of the collimator. The spectrum is focussed on a fluorescent screen, made by coating a white surface, such as cardboard, with barium platino-cyanide, and then the slit is opened very widely. If the spectrum be a continuous one (which is the case if it be that of the positive pole of the electric arc), the blood solution is then interposed in the path of a beam falling on the slit."

Recent advances on the sensitising of photographic plates have enabled spectrograms to be made without the use of fluorescent screens. The following account is taken from Lewin, Miethe and Stenger (*Pflüg. Archiv.*, 1907, **118**, 82). For the blue and violet parts of the spectrum alone an ordinary silver bromide gelatin plate suffices. In order to be able to photograph on one plate fine and coarse absorption bands in any part of the spectrum, the plate must be as far as possible equally sensitive to the whole spectrum, and no absorption bands, due to any material employed in making the plate, must be apparent. Such a continuous spectrum, extending from $\lambda = 375\mu\mu$ to $\lambda = 690\mu\mu$, is obtained either by the use of Wratten and Wainwright's panchromatic spectral plates or an isokol-bath plate.

Among coloured substances which can be used to sensitise a plate, *isokol*, prepared by Bayer & Co., Elberfeld, is the best. The highly sensitive dry-plates prepared by Perutz are placed in the dark for 2 minutes with constant movement of the dish in an isokol-bath:

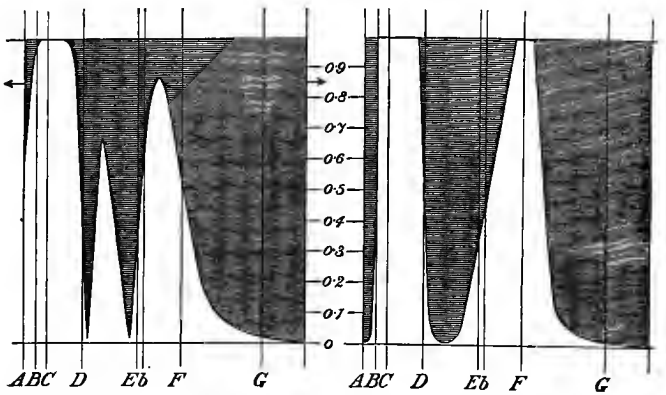
Isokol (1 : 1,000 alcohol)	5 c.c.
Alcohol	5 c.c.
Ammonia conc.	2 c.c.
Distilled water	200 c.c.

Franz, Rost and Heise use the "Agfa Plates" of the *Actiengesellschaft für Anilinfabrikation in Berlin*, and place these for 4 minutes in

Isokol (1 : 1,000 of 90% alcohol)	5 c.c.
Alcohol, 90%	70 c.c.
Ammonia sp. gr. 0.960	2 c.c.
Distilled water	130 c.c.

The plates are washed in the dark in running water for 3 minutes,

then placed in a perfectly dark drying chamber, through which dust-free air at 20° is aspirated. They should be dry in 20 to 30 minutes, as they are damaged by longer drying. The plates are put into the dark slide, and the development must commence in the dark, since the sensitiveness for red is so great. But after a minute the developing can be controlled with careful use of dark red light, since the plate, as it slowly develops, loses its marked sensitiveness to red light. For developing, 4 to 5% rodinol is used; it is then fixed in an acid bath. Instead of a prism, a diffraction grating is used, about 15,000 lines to an inch. As a source of light, a "Nernst" lamp or incandescent burner serves very well. The time of exposure depends on the kind of plate, the



Oxyhæmoglobin. Hæmoglobin (reduced).

FIG. 34.—Graphic representation of the visible spectrum after A. Rollett. The figures represent percentages of the colouring matter.

source of light, and the concentration of the solution investigated, and must obviously be determined by trial.

Spectrum of Oxyhæmoglobin.—The two characteristic bands, in the visible spectrum, are seen in greatest perfection in a stratum 1 cm. thick of a solution containing 1 per 1,000 of oxyhæmoglobin or 0.12–0.14% of blood. They are still perceptible in solutions of 1 in 100,000.

Fig. 34 illustrates the variations in the spectrum of the blood colouring matter corresponding to all concentrations, a stratum of 1 cm. being examined. In this diagram, the position of the Fraunhofer lines is shown; the numbers indicate percentage of oxyhæmoglobin, HbO_2, and reduced hæmoglobin. The shaded part indicates the absorption of light. By drawing lines parallel to the abscissæ we at once obtain the character of the absorption spectrum, corresponding to the concentra-

tion indicated by the vertical column of figures. The spectroscopic properties of the oxyhæmoglobin of all animals are the same, two absorption bands between D and E. With dilution the right-hand band disappears and only the left remains. According to Lewin, Miethe and Stenger, the mean of 112 measurements of different bloods gives maxi-

$$\lambda = 577\mu\mu \text{ and } \lambda = 537\mu\mu$$

mal points for the oxyhæmoglobin of horse blood; the mean of 42 measurements was

$$\lambda = 579\mu\mu \text{ and } \lambda = 542\mu\mu$$

for the ultra-violet line (Soret's band); 50 determinations of rabbit's blood gave

$$\lambda = 415\mu\mu$$

and a similar value for the blood of man, pig and earthworm.

In the case of horse blood the mean of 42 measurements gave $\lambda = 413\mu\mu$. And for the mean of 134 measurements of pure oxyhæmoglobin, $\lambda = 415\mu\mu$. Lewin has examined blood 29 years old, which in solution was flocculent, brownish-black and showed no visible spectrum, and found the ultra-violet absorption, $\lambda = 408\mu\mu$. Another dried preparation of ox blood which was so insoluble in water that the extract in hot water showed no absorption even in a thick layer and gave no hemin crystals by Teichmann's method, still showed the ultra-violet band $\lambda = 415\mu\mu$. Also the yellow filtrate of blood or oxyhæmoglobin solutions which had been boiled repeatedly over a flame, still showed the violet absorption $\lambda = 415\mu\mu$.

In Fig. 35 we give the spectrum of oxyhæmoglobin (Rost, Franz and Heise). A series of solutions of rabbit's blood of varying concentration taken with a panchromatic plate by Wratten and Wainwright.

Spectrophotometric Properties of Oxyhæmoglobin.—The impression obtained by the unaided eye as to the intensity or breadth of an absorption band is often very fallacious. It is, therefore, necessary to determine quantitatively the amount of light actually absorbed by any medium, the absorption spectrum of which is being investigated, instead of trusting to the unaided sense of sight.

The qualitative estimation of hæmoglobin and its derivatives spectrophotometrically depends upon the determination of the ratio of the extinction coefficients in two definite parts of the spectrum of the colouring matter, on the assumption that this colouring matter undergoes no change during the experiment. This ratio is more or less characteristic

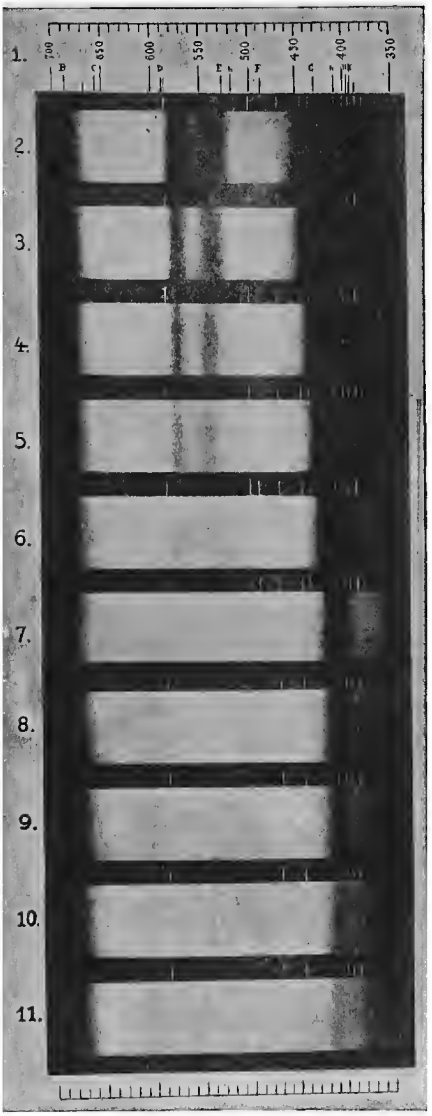


FIG. 35.—Spectrum of oxyhæmoglobin from Rost, Franz and Heise.

1. Scale of wave lengths.
2. Dilution, 1 : 70.
3. Dilution, 1 : 100.
4. Dilution, 1 : 150.
5. Dilution, 1 : 200.
6. Dilution, 1 : 300.
7. Dilution, 1 : 500.
8. Dilution, 1 : 800.
9. Dilution, 1 : 1,000.
10. Dilution, 1 : 1,500.
11. Dilution, 1 : 2,000.

for any given colouring matter, as Hüfner and his pupils have shown. If ε' is the extinction coefficient in one part of the spectrum and ε that in the other part of the same spectrum, the quotient $\frac{\varepsilon'}{\varepsilon}$ for a definite derivate is constant, even for different concentrations and is different for different derivatives. If the molecular weight of hæmoglobin and its derivatives were exactly known, the molecular extinction, that is to say, the extinction which 1 molecule or a definite fraction of 1 molecule dissolved in 1 litre of water exerts for a definite thickness of stratum in a definite region of the spectrum, would serve for its characterisation. Bunsen and Roscoe defined the extinction-coefficient as the reciprocal of that thickness of solution by which light of intensity J is reduced by 1/10 in passing through the absorbing body. It is not necessary to know the absolute value of the intensity of the original light, since an absorbing body which weakens light of 1 by 1/10 weakens light of intensity 2 by 2/10, of 3 by 3/10 . . . . . The coefficient of light diminution remains the same. Let ε be the extinction coefficient and m the thickness of solution by which light is reduced by 1/10, then

$$\varepsilon = \frac{1}{m}.$$

For the determination of the extinction coefficient we can also make use of the fact that the coefficient is, under the above assumption, a function of the thickness of the stratum of liquid, but with a constant thickness of stratum we can indirectly ascertain the extinction coefficient by measurement of the amount of light unabsorbed in the following manner

Monochromatic light of intensity J passes through a stratum of coloured liquid of unit thickness, and is thereby weakened by $\frac{J}{n}$, in passing a second layer of the same thickness then according to Beer's law it suffers a further weakening of $\frac{1}{n}$, so that the remaining light is now of intensity $\frac{J}{n^2}$. By passing through m such layers the residual light is now only $\frac{J}{n^m}$. In other words, as the thickness of the stratum of liquid increases in arithmetical progression, the intensity of light decreases in geometrical progression.

Let the original intensity of light $J=1$, and intensity of the light after passing through m strata, J', then

$$J' = \frac{1}{n^m} \text{ or } n^m = \frac{1}{J'} \quad (1)$$

Taking logarithms and transposing, we get

$$\log n = \frac{-\log J'}{m} \quad (2)$$

From the definition of the extinction coefficient, it follows that, $m = \frac{1}{\varepsilon}$ since $J' = 1/10$, and substituting these values in equation (2); we obtain

$$\log n = \varepsilon$$

and, therefore, also

$$\varepsilon = -\log \frac{J'}{m}$$

If we choose the thickness of m as 1 cm,

$$\varepsilon = -\log J',$$

that is to say, the extinction coefficient in this case is equal to the negative logarithm of the intensity of the residual light.

If one determines with the spectrophotometer the intensity of the residual light after absorption by a stratum of hæmoglobin or one of its derivatives, a simple calculation is only necessary for ascertaining the extinction coefficient. But for such measurement it presupposes that light as monochromatic as possible is used.

The Spectrophotometer of Hüfner.—In this instrument many of the inconveniences and sources of error in the original form employed by Vierordt are eliminated. (For description of the form supplied by Hilger, see Vol. 1, page 38.) In this instrument light, polarised by a Nicol, falls on one half of the slit of a spectroscope, while unpolarised light falls on the other half. The slit should not be open more than 0.5 m.m. The unequal brightness of the two beams is compensated by a smoked glass wedge. After the brightness of the two beams has been rendered unequal by the interposition of the absorbing liquid, the second Nicol in the telescope is rotated until the two fields appear equally bright. The angle of rotation φ is measured, and the extinction coefficient found as follows. If J° is the intensity of the polarised ray before rotation and J' the intensity after rotation, then

$$J^\circ - J' = \sin^2 \varphi$$

and if $J^{\circ}=1$, then $J'=-\cos^2\varphi$, and therefore the extinction coefficient $\epsilon=-\log J'=-\log\cos^2\varphi=-2\log\cos\varphi$.

The hæmoglobin solution is placed in a glass cell with parallel walls 11 mm. apart; in the lower half of this cell is a glass block known as "der Schulz'sche Glaskörper" exactly 10 mm. broad and half the height of the interior of the cell. It rests upon the floor of the cell, so that the anterior and posterior surfaces of the blood shall be parallel to the glass plates of the trough. When light traverses the lower half it passes through a stratum 1 mm. in thickness; when the upper half, 11 mm. in thickness.

TABLE III

Colouring matter	Wave-length of spectral region in $\mu\mu$ For ϵ' 531.5–542.5 (a^1) or 534.0–542.0 (b^1) For ϵ 534.0–565.0 (a) or 556.5–564.5 (b)	Quotient	Author
HbO_2[1]	a^1 or b^1 a or b	$\frac{\epsilon'}{\epsilon}=1.578$	Hüfner, *Arch. f. Physiol.*, 1894, 130.
Met-Hb alkaline	a^1 and a	$\frac{\epsilon'}{\epsilon}=1.19$	Zeynek, *Arch. f. Physiol.*, 1899, p. 460.
Met-Hb neutral	b^1 and b	$\frac{\epsilon'}{\epsilon}=1.58$	Bürker. *Tigerstedt's Handb. f. Physiol. Methode*, 1910.
Hb-CO	a^1 and a	$\frac{\epsilon'}{\epsilon}=1.10$	Hüfner, *Arch. f. Physiol.*, 142.
Hb-NO	D63E-D84E and D32E-D53E	$\frac{\epsilon'}{\epsilon}=1.05$	Hüfner and Kulz, *Zeits. f. Physiol. Chem.*, 1883, 7, 366.
Hb-CN	a^1 and a	$\frac{\epsilon'}{\epsilon}=1.21$	Zeynek, *Zeit. f. Physiol. Chem.*, 1901, 33, 426.
Hb (reduced)	a^1 and a	$\frac{\epsilon'}{\epsilon}=0.76$	Hüfner, *Arch. f. Physiol.*, 1894, 130.
Hæmatin alkaline	b^1 and b	$\frac{\epsilon'}{\epsilon}=1.09$	Bürker, *ibid.*
Hæmatin acid	b^1 and b	$\frac{\epsilon'}{\epsilon}=1.25$	*Ibid.*
Hæmochromogen	b^1 and 534–562	$\frac{\epsilon'}{\epsilon}=0.50$	*Ibid.*
Hæmatoporphyrin alkaline.	b^1 and b.	$\frac{\epsilon'}{\epsilon}=1.11$	*Ibid.*
Hæmatoporphyrin acid	b^1 and b	$\frac{\epsilon'}{\epsilon}=0.76$	*Ibid.*

The spectrophotometer constants of oxyhæmoglobin are given in Table III, taken from K. Bürker, along with the constants of the other derivatives. The determination of these is an essential method for the accurate qualitative recognition of hæmoglobin and its derivatives. Butterfield (*Zeits. f. physiol. Chem.*, 1909, **62**, 178–204) has shown that the constancy of the quotient $\frac{\epsilon'}{\epsilon}$ holds not only for fresh normal blood

[1] Hb = Hæmoglobin.

of men and animals but for human blood in pathological states, such as polycythæmia, chlorosis, pernicious anæmia and scurvy.

The Heat of Combination of Hæmoglobin and Oxygen.—According to Barcroft and A. V. Hill (*J. Physiol.*, 1910, **39**, 427) the mean value for 1 grm. of hæmoglobin is 1.85 calories. According to Berthelot, the value is 14.77 calories. Torup's value is similar.

Chemical Properties.—Hæmoglobin dissolved in water or, better, in 0.1% sodium hydroxide solution, shaken with air unites with a definite quantity of oxygen. At 0° C. and 760 mm. 1 grm. of hæmoglobin unites with 0.001915 grm. = 1.34 c.c. of oxygen. Oxyhæmoglobin is possibly a peroxide, Hb$\begin{smallmatrix} O \\ | \\ O \end{smallmatrix}$, and is a dissociable compound, so that diminution of the oxygen tension in the atmosphere to which oxyhæmoglobin is exposed results in a certain degree of dissociation. The oxygen tension can reach zero by passage of indifferent gases, such as hydrogen or nitrogen, by exposure to a vacuum, or by reducing agents. Various curves, indicating the grade of dissociation of oxyhæmoglobin, or what is the same, the extent of saturation of hæmoglobin with varying pressures of oxygen, have been published by Hüfner,[1] Krogh,[2] Bohr,[3] and Barcroft and Roberts.[4] The dissociation curves of the blood of different animals show some difference, attributable to difference in the saline constituents of the red corpuscles. A clear discussion of the evidence for and against the chemical nature of specific oxygen capacity in hæmoglobin has been recently published by Peters (*J. Physiol.*, 1912, **44**, 131).

Oxyhæmoglobin has the character of a weak acid and apparently forms with correspondingly weak alkaline solutions a salt; with stronger alkali it is decomposed.

Blood colouring matter, especially in the form of oxyhæmoglobin, acts as a *catalyst*, decomposing hydrogen peroxide into water and oxygen; and the purer the hæmoglobin the less active is it in this respect. In connection with this property, a number of chemical tests for blood have been devised. These are of some considerable diagnostic value, and are given by hæmoglobin and its compounds and all its iron-containing hydrolytic derivatives. Substances such as hæmatoporphyrin, hæmatoidin or bilirubin, which are iron-free derivatives, do not give the tests now to be described.

1. **The guaiacum reaction** of Schönbein first described in 1858. In

[1] *Arch. f. Physiol.*, 1901, Suppl. Bd., 187.
[2] Krogh, *Skand. Archiv.* 1903, 16, 400.
[3] *Skand. Archiv. f. Physiol.*, 16, 409.
[4] *J. Physiol.*, 1910, 39, 118.

later communications (1863) Schönbein directed attention to the delicacy of the guaiacum-peroxide test for blood, and to its value in forensic investigations. A sapphire-blue colour (guaiacum blue) develops when blood, hydrogen peroxide, and alcoholic solution of guaiacum are mixed in a test-tube. The guaiacum test is also known as Van Deen's, Almen's, or Vitali's test. Instead of hydrogen peroxide, ozonised ether or ozonised oil of turpentine have been employed; and instead of alcoholic solution of guaiacum resin, an alcoholic solution of guaiaconic acid, C_{20} H_{24} O_5, is to be preferred. (Van Deen, *Arch. f. d. holländ. Beitr. z. Natur. u. Heilkunde*, Utrecht, 1861–1864, **3**, 228; Taylor, Guy's Hosp. Reports, 1868, **13**, 431; 1870, **15**, 273; 1874, **19**, 517; Buckmaster, *J. Physiol.*, 1897, 35; Carlson, *Zeits. physiol. Chem.*, 1906, **48**, 69, and 1908, **55**, 260; Whitney, *Brit. Med. and Surg. J.*, 1909, **160**, 202. Lesser, *Zeits. f. Biol*, 1907, **49**, 571; Liebermann, *Pflüger's Arch.*, 1904, 227. See also J. H. Kastle, Chemical Tests for Blood, *Hygienic Lab. Reports of the Public Health and Marine Hospital Service*, 1909, Bull. 51.

The general theory of the reaction is a matter of uncertainty. The presence of iron in the blood pigment is essential, and the test must be performed with some care and with controls. Blood exposed to a temperature of 200° still gives the test. If the material to be tested (blood) is left in contact with even weak hydrogen peroxide solution and then, 5 minutes later, guaiaconic acid is added, the reaction fails; the products of the chemical change, therefore, do not blue guaiacum.

The guaiacum-peroxide test for blood is not due to any ferment. It resembles, but is in its nature quite dissimilar to any of the peroxydase reactions hitherto described. For convenience, the reaction with blood may be termed a pseudo-peroxydase reaction.

The test is carried out as follows:

1% solution of guaiaconic acid (Merck) in equal parts of alcohol and water is freshly prepared. A few cubic centimetres are placed in a test-tube, then half this volume of 3% hydrogen peroxide. On adding the blood solution, the guaiacum blue colour develops, and slowly disappears in about 30 minutes. O. Schumm regards ozonised turpentine oil as a better reagent than peroxide. For his method of preparing an active ozonised oil, see *Zeitsch. f. physiol. Chem.*, 1906, **50**, 374. Bürker, Carlson, and Buckmaster consider that weak peroxide solutions are preferable.

Delicacy of the guaiacum test for detection of blood and water.

1–5,000 of water, Adler, *Zeits. f. physiol. Chem.*, 1904, **41**, 59.

1–40,000 of water, Limon, *Vrtljhrsch. f. ger. u. off. Med.*, 1863, **24**, 193.

1–40,000 to 100,000, Schumm, *Arch. Pharm.*, 1907, **247**, 1.
1–20,000 to 40,000 (urine) Schumm, *Arch. Pharm.*, 1907, **247**, 1.

In the writers' experience the guaiacum test is of especial value in the detection of blood. It is given by blood-solutions which have been boiled, and this should be done before carrying out the test so as to avoid any possibility of the colour being produced by ferments. The test is more sensitive with great dilutions of laked blood compared with unlaked blood. The test can be obtained with dilutions of 1 in 100,000.

The Benzidine Test of O. Adler and R. Adler (*Zeit. physiol. Chem.*, 1904, **41**, 76). Benzidine or paradiamino-diphenyl, $NH_2C_6H_4C_6H_4NH_2$, preparations are not all of equal value for this test (O. Schumm and C. Westphal, *Zeitsch. physiol. Chem.*, 1905, **46**, 510). The solution should be fresh. A few milligrm. of Merck's benzidine are dissolved in 2 c.c. of glacial acetic acid. 10 drops are placed in a clean test-tube, and then 30 c.c. of 3% hydrogen peroxide added, when no colour should appear. Next add the blood or suspected fluid, when a blue-green colour develops. Einhorn (*Deutsche med. Woch.*, 1907, 1089) soaks white filter paper with the acid solution and dries, then dips a strip in the fluid to be tested, and drops peroxide solution on this, or the paper may be laid in the solution.

The reaction is a delicate one; blood can be detected in a dilution of 1 : 100,000 (Adler) or 1 : 200,000 (Schumm), 1 : 300,000 (Ascarelli), when using Einhorn modification in 1 : 80,000. With two of Kahlbaum's preparations the limit of the reaction was 1 part to 2,000,000, with some others of the same firm 1 : 50,000 (Schumm).

The aloïn test of Rossel (*Deutsche Archiv. f. klin. Med.*, 1903, **76**, 505). Solutions of aloïn (Merck's preparation from Curaçoa aloes) react exactly like solutions of guaiaconic acid to hæmoglobin, its derivatives and hæmatoporphyrin. The aloïn solution must always be freshly prepared; 0.25 grm. to 50 c.c. of equal parts of water and alcohol. The clear yellow liquid is the test solution. 10–20 drops of this in a test-tube are mixed with twice its volume of 3% hydrogen peroxide. There is no change in colour. Next add the suspected blood. A purple-red colour gradually develops. The colour is much more stable than that of guaiacum blue, but the aloïn reaction is less sensitive. (Schaer, *Arch. Pharm.*, 1900, **238**, 42).

The malachite green reaction of O. Adler and R. Adler (*Zeit. physiol. Chem.*, 1904, **41**, 59). The leuco-base of malachite green (tetramethyldiaminotriphenylmethane) is a white crystalline substance insoluble in water, but soluble in weak acids. Such solutions are easily

oxidised to a peacock blue colour (malachite green). Adequately diluted solutions give a distinctive absorption spectrum and the centre of the band nearly coincides with the sodium line *D*. From this property, as v. Czyhlarz and v. Fürth have shown, it is possible to measure with accuracy the rate at which leuco-base is oxidised by hæmin in the presence of hydrogen peroxide. (*Hofmeister's Beitr.*, 1907, **10**, 363; Buckmaster, *J. Physiol.*, 1908, 37.)

0.25 grm. of leuco-base in 163 c.c. of water and 12 c.c. of glacial acetic is prepared. 10 drops of this solution and three times the quantity of 3% peroxide solution are placed in a perfectly clean test-tube. Then the blood is added. This should be boiled before the test is performed.

The test solution slowly turns green when kept in a stoppered bottle. This can be to some extent obviated by saturating the liquid with carbon dioxide and keeping in an atmosphere of this gas. The reaction is exceedingly sensitive, at least equal to, or more so than the guaiacum test; 1 part of blood in 100,000 of water can easily be detected. The leuco-base, unlike guaiaconic acid, is not oxidised by 3% hydrogen peroxide. Buckmaster has shown, (1) That the extent to which leuco-base is converted into malachite green depends entirely on the quantity of blood-pigment present; (2) The reaction, occurring with boiled blood pigment, is not due to a ferment; (3) Removal of the iron from hæmoglobin, forming hæmatotoporphyrin or hæmatoidin, abolishes the reaction.

The **paraphenylenediamine hydrochloride**, $(C_6H_4(NH_2)_2, 2HCl)$, test of J. Boas (*Centralbl. f. inner. Med.*, 1906, **27**, 601). 0.5% aqueous solution of this substance is used. 1–2 drops of reagent placed in a test-tube (with the addition if acid of sufficient $N/2$ alcoholic potash to neutralise this) then 15–20 drops of 3% hydrogen peroxide and finally the solution to be tested. If hæmoglobin or any of its iron-containing derivatives are present an olive-green colour develops.

Schumm and Remstedt advise the addition of 1–5 drops of weak acetic acid to the mixture. The olive-green colour gradually passes into a brown red.

The test is not so delicate as the guaiacum or benzidine reaction.

Phenolphthalin Test for Blood.—Utz (*Chem. Ztg.*, 1907, **31**, 737) regards phenolphthalin as a more delicate test for blood than either guaiacum or aloin. Blood at a dilution of 1 : 1,000,000 could be detected (Delearde and A. Benoit, *Compt. Rend.*, 1908, **64**, 960–992). J. H. Kastle (*Ibid*, page 30) using a Duboscq's colorimeter states that he recognised blood at a dilution of 1 : 80,000,000.

Phenolphthalin is the leuco-compound of phenolphthaleïn. It is colourless in alkaline solutions, but on adding alkali to the substance after oxidation, or if it be oxidised in the presence of alkali, the purple-red colour of phenolphthaleïn in alkali is produced. (For the preparation of phenolphthalin see Kastle.) The reagent is prepared by dissolving 0.032 grm. phenolphthalin in 21 c.c. $N/10$ sodium hydroxide, and making up to 100 c.c. with pure redistilled water. To this 0.1 c.c. of $N/1$ hydrogen peroxide is added.[1] On the addition of diluted blood the colour develops at room temperature in 5 minutes. Using an alkaline solution of phenolphthalin without peroxide the colour develops in 18 hours at room temperature. 1 part of blood in 8,000,000 could be detected. Moreover, the quantity of phenolphthalin oxidised under the influence of blood is proportional to the quantity of blood present.

The test is interfered with, or retarded by, substances present in tissues, such as liver, spleen, pancreas or urine. In the latter fluid about 1 part of blood in 390,000 of urine could be detected.

The phenolphthalin test for blood is exceedingly delicate, but copper salts, chromic alum, mercuric chloride, lead chloride and potassium ferricyanide all show in some degree a similar oxidation of this reagent.

The chief objection urged against the guaiacum test for blood, most observers considering that its value lies in indicating the absence of blood when the reaction is negative, is that a large number of substances other than blood give a positive reaction, the following among others: all oxidising agents, the halogens, finely divided metals such as copper, iron, and compounds of cobalt, chromium, copper, gold, iron, manganese and lead. Also hypochlorites, iodides, bromides, nitrates, nitrites, and sodium chloride. Milk, pus, saliva, fæces and sweat, together with many substances of vegetable origin derived from asparagus, potatoes, flour, tomatoes, oats, peas and meal also may give the guaiacum reaction.

The general consensus of opinion among those who have worked at this reaction is that the guaiacum test, performed as above directed, is a valuable one. If no reaction, then the absence of blood can be inferred. If a positive reaction occurs, care should be taken to exclude any ferment action due to oxidases or peroxidases, by boiling for some minutes and then subjecting the material to confirmative tests.

Hæmoglobin (Hb, reduced hæmoglobin, or purple cruorin).—In 1864 Stokes, in a communication to the Royal Society, showed that when blood is treated with certain reducing agents the colour of the liquid and the spectrum undergo remarkable changes, and concluded

[1] The purest sodium hydroxide must be used, and redistilled water for all dilutions.

from his experiments "that the colouring matter of blood, like indigo, is capable of existing in the state of oxidation, distinguishable by a difference of colour and a fundamental difference in the action on the spectrum. It may be made to pass from the more to the less oxidised state by the action of suitable reducing agents, and recovers its oxygen by absorption from the air."

Methods of Reducing Oxyhæmoglobin to Hæmoglobin.—In all these experiments diluted blood may be substituted for solutions of the pure colouring matter. Instead of very pure water it is advisable to use 0.1% of sodium hydroxide. It is essential that the reducing agent should not act destructively on the hæmoglobin. The reduction of oxyhæmoglobin seldom proceeds quantitatively (Hüfner). Generally, secondary reaction takes place to some degree, whereby part of the loosely combined oxygen is liberated and oxidises not only other substances but the molecule of colouring matter itself.

1. *Stokes' Reagent.*—(Alkaline solutions of ferrous salts.) To a solution of ferrous ammonium sulphate, tartaric or citric acid, or one of the alkaline salts of these acids is added, and then ammonia until the reaction is just alkaline. The light green solution obtained reduces oxyhæmoglobin rapidly, even in the cold. This solution presents the disadvantage that it itself becomes oxidised and therefore coloured, and absorbs the more refrangible rays of the spectrum and interferes with the exact study of the absorption bands. To get over this difficulty, Stokes subsequently substituted stannous chloride for a ferrous salt.

2. *Alkaline sulphides* also effect the reduction, but more slowly than Stokes' reagent. Ammonium sulphide is a good reagent, and is best prepared by saturating strong ammonia with hydrogen sulphide and then adding an equal volume of strong ammonia. The solution must be freshly prepared and colourless, since if yellow it absorbs the violet end of the spectrum. Ordinary solutions of ammonium sulphide should not be used, as they lead to the formation of sulph-methæmoglobin. Many workers prefer to use a freshly prepared solution of 40% potash saturated with hydrogen sulphide. The solution should be clear and colourless.

3. Ludwig and Schmidt used *ferrum reductum.*

4. *Sodium hyposulphite,* $Na_2S_2O_4$, has intense reducing power. It possesses the advantage of not greatly increasing the volume of the blood solution, and its solution is also clear and colourless.

5. *Hydrazine.*—The 50% solution of hydrazine hydrate of commerce easily reduces oxyhæmoglobin, and has the advantage that the only

products of its decomposition are nitrogen and water, $NH_2NH_2,H_2O + O_2 = N_2 + 3H_2O$. If we know the quantity of oxyhæmoglobin to be reduced, the calculated quantity of hydrazine hydrate can be added. It must not be used in excess, otherwise the hæmoglobin is decomposed into hæmochromogen and globin.

6. *Auto-reduction by bacteria.*—The solutions are sealed up in tubes and kept at 40°, when reduction rapidly takes place.

FIG. 36.—(From Franz, Rost and Heise.)

1. Scale of wave lengths.
2. Normal rabbit's blood, 1 : 170.
3. Normal rabbit's blood reduced by ammonium sulphide.
4. Normal rabbit's blood, 1 : 100.
5. Normal rabbit's blood reduced by ammonium sulphide.
6. Normal rabbit's blood, 1 : 150.
7. Normal rabbit's blood reduced with ammonium sulphide.

7. By removal of the oxygen of oxyhæmoglobin in a Torricellian vacuum.

8. By the passage of such gases as nitrogen or hydrogen through solutions of oxyhæmoglobin. The hydrogen must be purified by passing through concentrated potassium permanganate and then concentrated potassium or sodium hydroxide.

The crystals of hæmoglobin were prepared by Kühne by placing a concentrated solution of pure oxyhæmoglobin in very dilute ammonia in a gas chamber and passing pure dry hydrogen over it; as the solution evaporated the crystals separated as hæmoglobin. Gamgee placed a magma of pure hæmoglobin crystals together with some of the mother-liquor from which they had separated in a glass tube and then sealed it off just above the surface of the liquid. He then placed this in an incubator at 35° for some days, then set aside in an ice chamber, and after many weeks the tube was found to contain crystallised and perfectly reduced hæmoglobin. The crystals are doubly refracting, pleochromatic, and practically identical in form with those of oxyhæmoglobin.

In Fig. 36, we give comparative spectra of oxy- and reduced hæmoglobin.

In thick layers, solutions of hæmoglobin are dark claret-coloured; in dilute solutions greenish. This dichroism is characteristic of the blood of asphyxiated animals.

Spectrum.—When oxyhæmoglobin is reduced, the two absorption bands disappear, and are replaced by a single band between D and E, which is less deeply shaded and possesses less sharply defined edges. (The right hand diagram of Fig. 34 shows the various degrees of absorption of light by various strengths of solution of hæmoglobin.) Lewin, Miethe and Stenger give the point of maximum absorption as $\lambda = 558\mu\mu$ for crystallised hæmoglobin, prepared from oxyhæmoglobin and $\lambda = 559\mu\mu$ for reduced hæmoglobin from blood.

The photographic spectrum, taken with a perortho plate gives a band $\lambda = 429\mu\mu$, so that the band characteristic of oxyhæmoglobin is displaced toward the red end of the spectrum by $14\mu\mu$. For the spectrophotometric constants see Table III.

Carbon-monoxide Hæmoglobin (Carboxyhæmoglobin).—When a current of carbon monoxide is led through a solution of oxyhæmoglobin or blood, the solution acquires a cherry-red colour in contrast to the scarlet of oxyhæmoglobin. If the original solution was sufficiently concentrated, then on cooling to 0°, and adding a quarter of its volume of alcohol also at 0°, and setting aside to cool in a chamber below 0°, after some hours or days crystals of CO-Hb separate. They are identical in form with oxyhæmoglobin, but are less soluble.

Hüfner has shown that exactly the same volume of carbon monoxide combines with hæmoglobin as oxygen; 1 grm. hæmoglobin uniting with 1.338 c.c. of CO at 0° and 760.

Carboxyhæmoglobin is more stable than oxyhæmoglobin. The affinity of hæmoglobin for carbon monoxide is 154 times that which it possesses for oxygen (Haldane, *J. Physiol.*, 1895, **18**, 430). Solutions of carboxyhæmoglobin yield up carbon monoxide to the vacuum of the blood pump, but less readily than oxyhæmoglobin; and if present in small quantity carbon monoxide is difficult to recover by this method unless the blood is acidified. The continued bubbling of indifferent gases through solutions of CO-hæmoglobin results in finally driving out the carbon monoxide, a solution of hæmoglobin remaining. (Gamgee, *J. Anat. Physiol.*, 1867, 1, 339). The gas can also be extracted from carboxyhæmoglobin by treatment with a strong solution of potassium ferricyanide. The solutions are cherry red in tint, and in great dilution typically pink. If concentrations of equal strength, of oxy- and carboxyhæmoglobin are compared by means of the spectroscope, it will be found that the CO compound absorbs the blue rays of the spectrum to a less degree than the oxyhæmoglobin.

Spectrum.—Carboxyhæmoglobin shows two bands of almost equal intensity and breadth in the yellow-green part of the spectrum. A band can also be detected in the violet. As a mean of 60 measurements of the spectrograms, Lewin, Miethe and Stenger give as the points of maximal absorption $\lambda = 570\ \mu\mu$, $\lambda = 542\ \mu\mu$, $\lambda = 416\mu\mu$.

The position of these bands is not altered by the addition of reducing reagents. The spectrophotometric constants are given in the table III, (page 521).

In Fig. 37 we give the spectrum of carboxy-and oxyhæmoglobin, taken from Rost, Franz, and Heise, rabbit's blood being diluted and then converted into carboxyhæmoglobin with carbon-monoxide gas.

The addition of a concentrated solution of sodium hydroxide (sp. gr. 1.3) to a solution of blood saturated with carbon monoxide in the proportion of 10 to 1, causes the blood to assume a fine cinnabar red colour and deposit a cinnabar red precipitate. The same precipitate is produced with solutions of pure carboxyhæmoglobin. Normal blood or oxyhæmoglobin solutions treated in the same way gives a shiny black mass, which is greenish-brown when spread out on a porcelain slab.

According to Kunkel, tannic acid is useful for the detection of Hb-CO. With diluted normal blood this gives a greenish-brown, with Hb-CO a bright carmine-red precipitate.

Pure aqueous solutions of carboxyhæmoglobin when boiled give a

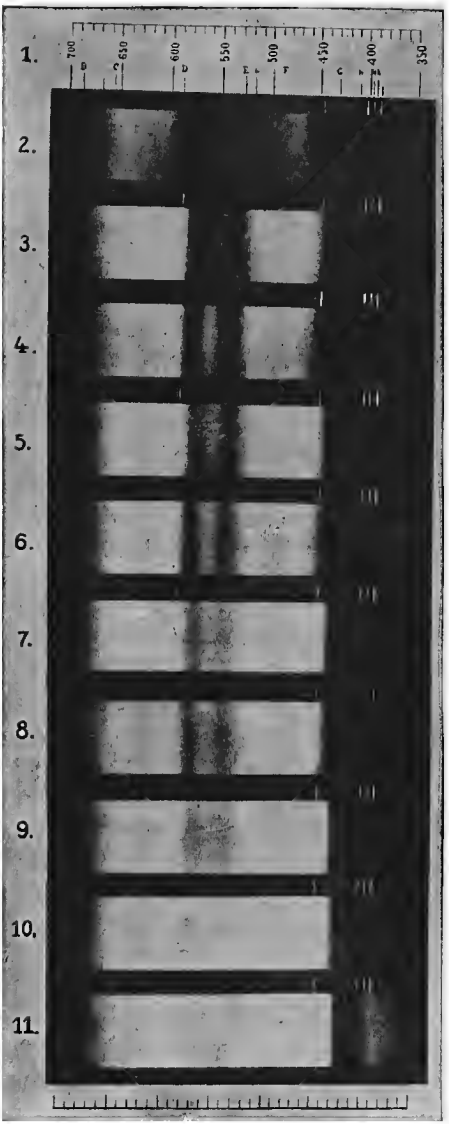


FIG. 37.

1. Scale of wave-lengths.
2. HbO_2. 1 : 70.
3. HbCO. 1 : 70.
4. HbO_2. 1 : 100.
5. HbCO. 1 : 100.
6. HbO_2. 1 : 150.
7. HbCO. 1 : 150.
8. HbO_2. 1 : 200.
9. HbCO. 1 : 200.
10. HbO_2. 1 : 500.
11. HbCO. 1 : 500.

bright red precipitate, composed, according to Hoppe-Seyler, of coagulated protein and CO-hæmochromogen. Solutions of carboxyhæmoglobin exposed to nitric oxide in the absence of oxygen, are at once decomposed. Nitric oxide hæmoglobin is formed and carbon monoxide liberated.

Apart from its behaviour to hæmoglobin, carbon monoxide is a perfectly indifferent gas for respiration. Since the relative affinities of carbon monoxide and oxygen for hæmoglobin are as 154 : 1, in an atmosphere of 1 volume CO and 154 volumes O_2 the hæmoglobin present in blood takes up equal quantities, and the hæmoglobin is half saturated with O_2, half with CO. If the normal alveolar air of the lungs contains 15% of oxygen, then 0.1% carbon monoxide in the respired air will half saturate the available hæmoglobin; with 0.3% of carbon monoxide the saturation of blood with this gas would be three-quarters.

In any sample of blood which is suspected to contain carboxyhæmoglobin this can be estimated by Haldane's carmine method.[1] As it is known that oxyhæmoglobin sufficiently diluted is yellow, while carboxyhæmoglobin is pink, this latter tint can be easily matched with carmine solution.

Narrow test-tubes about 0.5–0.75 cm. diameter are used. In each of three of these, A, B, C, 2 c.c. of water is placed. 20 c.c. of the blood to be examined is mixed with water in A. Normal blood similarly diluted is placed in B and also in C. Tube B is then saturated with carbon-monoxide gas, so that all the blood-colouring matter is in the state of carboxyhæmoglobin. To tube C (normal diluted blood) standard carmine is added, 0.1 c.c. at a time, until it equals the tint of A; then more, until it equals the tint of B. Then the amount of carboxyhæmoglobin in tube A can be calculated. Suppose 0.5 c.c. of standard carmine must be added to C to make its tint equal to A, and 2.2. c.c. of standard carmine to produce equality of tint with B, then since 2 c.c. of water is in each tube, $A=\frac{0.5}{2.5}\times\frac{4.2}{2.2}\times 100=38\%$ saturation or of 100 parts of hæmoglobin 38 parts are carboxyhæmoglobin and 62 parts oxyhæmoglobin.

Carboxyhæmoglobin can be dissociated by means of potassium ferricyanide in the same manner as oxyhæmoglobin (Haldane).

For obtaining the gases,—oxygen or carbon monoxide, about 20 c.c. of blood are mixed with 30 c.c. of 1/500 ammonia. The blood is thereby laked and 4 c.c. of a saturated solution of potassium ferri-

[1] Carmine solutions: 1 grm. pure carmine is mixed in a mortar with a few drops of ammonia and dissolved in 100 c.c. of glycerin. The standard solution is made fresh by adding 5 c.c. to 500 c.c. of water.

cyanide is mixed with this. All the associated oxygen or carbon monoxide is evolved and collected over water. The amount of gas so liberated is equal to that obtained by exposure of the blood at a temperature of 40° C. to a Torricellian vacuum. This experiment can be carried out with the ordinary Dupré's apparatus for determination of urea or with the original apparatus (Fig. 38) used by Haldane. For small quantities of blood the apparatus of Barcroft and Haldane can be employed (Barcroft and Roberts, *J. Physiol.*, 1909, **39**, 429).

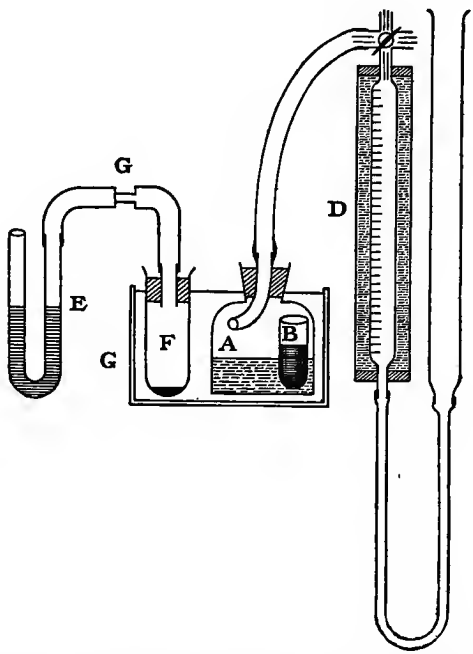


FIG. 38.—A is a corked bottle holding blood and ammonia; B, a test-tube holding ferricyanide of potassium prior to mixing, C is a water-bath; D, gas burette and levelling tube; E, a water-gauge attached to the temperature and pressure control tube F, which is adjusted by sliding the rubber tubing backward or forward on the glass tube G.

Buckmaster and Gardner (*Proc. Roy. Soc.*, 1909, **B. 81**, 575) found that minute quantities of carbon monoxide in the blood, too small to be recognised by other methods, can be detected as follows. The suspected blood is treated with potassium ferricyanide, as described above, and the gases which are evolved are allowed to escape into a closed space containing about 50 c.c. of air. The form of apparatus is a small bottle in which the blood and ferricyanide are mixed, connected with a

Hempel gas-burette fitted with a 3-way tap. The gas mixture is displaced into the burette and then bubbled very slowly through a solution of blood and distilled water in a narrow test-tube. The solution is so dilute that in looking down the tube it appears a reddish-yellow tint. The tint of this is then compared with a control sample of the same diluted blood solution. For comparisons simple dilutions are made, containing the same quantity of colouring matter as the control sample, and of the sample through which the gas has been bubbled. The latter tube shows the characteristic bluish-pink tint of carboxyhæmoglobin. The two tubes are examined side by side, or in a tintometer by looking through the column of liquid against a white surface. An even more delicate way of detecting any difference in the two samples is to fill 20 cm. polarimeter tubes, placed side by side in a dark box and viewed through a ground glass screen suitably illuminated with blue light. In this way minute quantities of carboxyhæmoglobin can be detected. For instance, 0.5 c.c. of blood from a cat was saturated with carbon monoxide and reintroduced into the blood stream of the animal. Half an hour later the animal was killed, and by the method just described carboxyhæmoglobin recognised with certainty in the blood. (The blood volume of a cat weighing 3 kg. is about 100 c.c., so that the hæmoglobin was not more than 1/200 saturated.)

Also carbon monoxide was easily recognised by this method in 5 c.c. of air containing 0.8% of the gas and quite readily in 10 c.c. of air containing 0.2% of carbon monoxide.

A. P. Fokker (*Chem. Centralb.*, **20**, 380; *J. Soc. Chem. Ind.*, 1884, p. 579) has described the following process, by which it is claimed that it is possible to detect carbonic oxide in a single drop of blood. From 1 to 2 c.c. of the blood to be examined is placed in a small deep beaker, clamped between three bent brass wires. The upper ends of these wires support a watch-glass containing a solution of palladious chloride, while the lower ends are soldered to a round brass plate. The arrangement is placed in a porcelain vessel filled with water, and covered with a narrow bell-glass. About two-thirds of the water is then sucked out of the bell-jar by means of an india-rubber tube. The water rises in the bell-jar, and the beaker, kept upright by the brass plate, swims on its surface. The water is then heated to boiling, when the blood coagulates, and the palladious chloride is reduced by the liberated carbonic oxide. If only traces of this gas are present, the reduction is not complete, and the arrangement must be allowed to stand for 24 hours. Free ammonia, if present, will produce a yellow amorphous compound with the palladium chloride, but carbonic oxide forms a

brilliant black metallic mirror. A similar reaction is produced by sulphuretted hydrogen, which is, however, rarely present in fresh blood.

Desgrez and Nicloux stated as the result of experiments carried out on dogs in Paris that carbon monoxide is not only a normal constituent of the blood-gases, but that the blood of these animals once anæsthetised by chloroform contained an augmented quantity of the gas. (*Arch. Physiol.*, 1898, **2**, 377) Nicloux also states that when blood is incubated *in vitro* with chloroform, carbon monoxide is produced. The method these observers used for the detection of carbon monoxide was to pass the evacuated blood-gases over solid potash to absorb the carbon dioxide and then over iodine pentoxide at 150°. The carbon monoxide decomposes iodine pentoxide according to the following equation:—

$$I_2O_5+5CO=I_2+5CO_2.$$

The iodine liberated is collected in a suitable solvent and estimated. The writers have been unable to confirm the existence of carbon monoxide either in blood of cats in London, or during chloroform narcosis, or by incubating chloroform and blood together, and their experiments show that the carbon monoxide found by Nicloux in the blood of chloroformed animals is due to the carbon monoxide produced when the chloroform vapour which is contained in the blood gases is passed over potash to absorb the carbon dioxide. Chloroform vapour is very readily decomposed either by solutions of potash or by solid potash yielding carbon monoxide;

$$CHCl_3+3KOH=3KCl+CO+H_2O.$$

The small quantities of carbon monoxide found in the blood of normal animals is probably due to the fact that iodine pentoxide itself undergoes a slight decomposition at temperatures below 100° and 150° with evolution of minute traces of iodine (*Proc. Roy. Soc.*, 1909, B. **81**, 515–528).

The fatal accidents due to the inhalation of coal gas, blast-furnace gases, lime-kiln gases, products of combustion of charcoal, or the combustion products that have passed through red-hot iron stoves, or from gas produced in coal-dust explosions in mines, are due to various degrees of saturation of the hæmoglobin of the blood with carbon monoxide.

Experiments by J. S. Haldane (*Jour. Physiol.*, 1895, **18**, 430) on himself show that the symptoms caused by carbonic oxide depend on

the extent to which the hæmoglobin has been saturated. During rest the symptoms do not become sensible till the corpuscles are about one-third saturated. With half saturation, the symptoms (headaches, respiratory distress, etc.), become urgent. About one-half of the carbonic oxide contained in the respired air is absorbed.

The time required for the production of sensible symptoms varies in different animals with the respiratory exchange per unit of body-weight, and is about twenty times as long in a man as in a mouse. Hence a mouse can be used as an indicator of the respirability of a vitiated atmosphere before men venture into it.

With a given small percentage of carbonic oxide in air, a certain percentage saturation of the blood is reached in about 150 minutes, and is not afterward exceeded, however long the breathing of air vitiated to the same extent is continued. Distinct symptoms of poisoning, appreciable during rest, are produced when the proportion of carbonic oxide reaches 0.05%, and with about 0.2% urgent symptoms are produced. The disappearance of the carbonic oxide from blood when fresh air is again breathed is always much slower than its absorption, and is chiefly due to the dissociation of carboxyhæmoglobin by the mass-influence of the oxygen in the pulmonary alveoli.

Nitric Oxide Hæmoglobin, Hb-NO.—This compound was first described by L. Hermann in 1865. Nitric oxide possesses an even greater affinity for hæmoglobin than carbon monoxide, and will displace this from carboxyhæmoglobin. Owing to the great affinity of nitric oxide for oxygen, this compound is best prepared by passing nitric oxide through a solution of carboxyhæmoglobin. In order to carry out the experiment, the solution is placed in a flask with some arrangement for permitting the whole of the air above the solution to be driven out and replaced by a neutral gas before allowing access to the nitric oxide. After the latter has exerted its action, the neutral gas must again be passed through to remove all traces of nitric oxide.

It may be prepared from oxyhæmoglobin in a similar way, but in this case sufficient alkali must be added to the solution to neutralise the nitric acid produced by the action of nitric oxide in the oxygen of the hæmoglobin.

Blood saturated with nitric oxide possesses almost as florid a colour as CO blood, but does not possess the slight bluish shade of the latter. It is not dichroic. The solutions are very stable, and keep even longer than those of carboxyhæmoglobin.

It can be crystallised; the crystals are identical in form and aspect with those of oxy- and carboxyhæmoglobin.

Spectrum.—The bands in the visible spectrum occupy the same position as the two bands of oxyhæmoglobin, and in the photographic spectrum the band in the extreme violet is absolutely coincident with that of carboxyhæmoglobin.

Nitric oxide hæmoglobin is recognised by the following tests: On boiling, a red coagulum is produced which remains unchanged in the air. While oxyhæmoglobin is converted into methæmoglobin by the action of potassium ferricyanide, followed by acetic acid, in the case of the nitric oxide compound, this change proceeds very slowly and the colour remains brownish-red, and if examined with a spectroscope the band of methæmoglobin is seen in the red, together with both the bands of nitric oxide hæmoglobin.

Nitrites are very poisonous when taken by the mouth, and the blood contains a mixture of methæmoglobin, nitric oxide- and oxyhæmoglobin (Haldane, *J. Physiol.*, 1898, **22**, 298). Haldane also found that the red colour of meat pickled by potassium nitrate is due to nitric oxide hæmoglobin (*J. Hygiene*, 1901, **1**, 115).

Cyanhæmoglobin.—According to Preyer, when an aqueous solution of oxyhæmoglobin is treated with potassium cyanide or prussic acid and allowed to stand at room temperature, or is warmed to about 40°, the solution assumes an orange-yellow shade. This can be instantly produced, according to Kobert, if a solution of methæmoglobin is treated with 0.5 % hydrogen cyanide. Von Zeynek (*Zeits. physiol. Chem.*, 1901, **33**, 426–480) found that crystallised methæmoglobin dissolves in 0.5 % solution of hydrogen cyanide, yielding a reddish liquid having a characteristic broad absorption band in the green. When diluted and mixed with alcohol at −10° microscopic crystals are obtained. As a rule, these are long prisms, terminated by pyramids which occasionally are rhomboidal in shape. The same compound is also obtained by adding potassium cyanide to alkaline methæmoglobin solution. The compound is stable, and may be heated to 40° in a stream of indifferent gas, or its solution may be boiled at 40° in vacuo without loss of prussic acid. The compound contains 0.158 % of CN, a result indicating the presence of one CN group in the molecule. Boeck's photo-methæmoglobin, obtained by exposing weak solutions of methæmoglobin made by the action of ferricyanide of potassium on oxyhæmoglobin to sunlight, has been shown by Haldane (*J. Physiol.*, 1900, **25**, 230) and von Zeynek to be cyanhæmoglobin.

In Fig. 39 is shown the spectra of hæmatin in alkaline solution, of cyanhæmoglobin, of cyanhæmatin and cyanhæmochromogen (Rost, Franz and Heise).

Reduced hemoglobin does not combine with hydrogen cyanide.

Spectrum.—This resembles hæmoglobin (reduced). Compare Figs. 39, 2, with Fig. 36. Ziemke and Muller give $\lambda = 579$–520μ for the extent of the band of cyanhæmoglobin and $\lambda = 596\mu\mu$, $\lambda = 543\mu\mu$ for hæmoglobin (*Arch. f. Physiol.*, 1901, Suppl. Bd., 177).

By the addition of Stokes' reagent or ammonium sulphide, the spectrum undergoes a change. Two bands, all but indistinguishable from those of hæmochromogen, are seen. The bands of cyanhæmo-

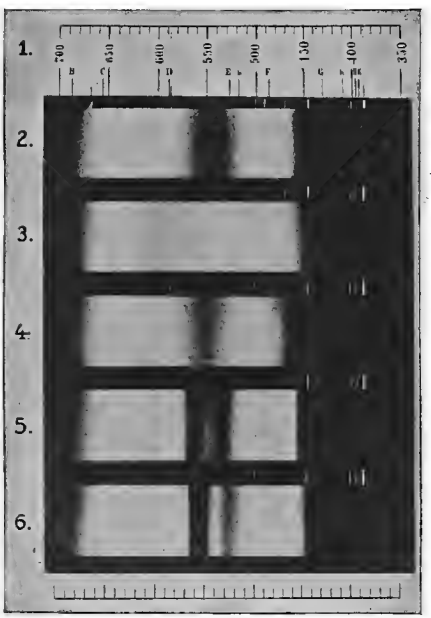


FIG. 39.

1. Scale of wave-lengths.
2. Dilute rabbits' blood, 1:80, treated with ferricyanide of potassium and hydrocyanic acid added.
3. Dilution, 1:80. Converted into hæmatin with sodium hydroxide.
4. Solution 3 plus hydrogen cyanide (cyanhæmatin).
5. Solution 4 plus ammonium sulphide (cyanhæmochromogen)
6. Solution 3 plus ammonium sulphide (hæmochromogen).

chromogen, according to Ziemke and Muller, are of equal density and extend more toward the red part of the spectrum.

In cyanhæmochromogen the bands extend from $\lambda = 577\mu\mu$ to $\lambda = 562\mu\mu$, and from $\lambda = 548\mu\mu$ to $\lambda = 592\mu\mu$.

Sulphhæmoglobin, or Sulphmethæmoglobin.—This compound

was believed by Hoppe-Seyler to be the cause of the greenish colour of the skin of corpses. This is well seen on the abdomen. It is produced by passing hydrogen sulphide through blood and in putrefaction by the formation of this gas by bacteria. The solution is green in thin layers, red in thick layers, and somewhat turbid. Such a solution shows a well-marked absorption band $\lambda=610\mu\mu-622\mu\mu$. Gamgee considers there is not the slightest ground for believing that the spectrum described is due to a definite body, but is caused by the decomposition products of oxyhæmoglobin. (*Text-book of Physiology*, Ed. by Schäfer, **1**, 251, 1898).

Clarke and Hurtley (*J. Physiol.*, 1907, **36**, 62) regard sulphhæmoglobin as a definite compound and not the result of any decomposition products of oxyhæmoglobin. They draw attention to a characteristic test for this substance. When acid-free carbon monoxide is passed into a solution of sulphhæmoglobin, all the bands are shifted toward the violet. The bands of oxyhæmoglobin become those of carboxyhæmoglobin, while the band in the red moves from $\lambda=610\mu\mu$ to $\lambda=625\mu\mu$ and $\lambda=605\mu\mu$ to $\lambda=620\mu\mu$. This is due to the production of a new compound, COSHb.

Hydrogen selenide behaves in a similar way to sulphuretted hydrogen; in this case the band apparent is $\lambda=613\mu\mu-\lambda628\mu\mu$ (selenhæmoglobin).

Methæmoglobin, Met.-Hb.—It was observed as long ago as 1864, by Hoppe-Seyler, that solutions of oxyhæmoglobin on exposure to the air assume a brown colour, become acid, and give a spectrum in which, in addition to the two bands of oxyhæmoglobin, a band is seen in the red in much the same position as the band of acid hæmatin. He called the indefinite substance in such solution methæmoglobin, and considered it to be a product of the partial reduction of oxyhæmoglobin and derived from it by the removal of a portion of the dissociable oxygen. It has since been discovered that a large number of substances—organic and inorganic, oxidising agents and reducing agents—act upon solutions of oxyhæmoglobin, converting them into methæmoglobin. Such substances are: potassium ferricyanide, hydroxylamine, nitrites, chlorates, iodine, formaldehyde, nitrobenzene, pyrogallol, pyrocatechol, acetanilide, etc., and also the β-rays of radium. Even violent agitation with mercury will bring about some degree of change. In order to study the spectroscopic character of methæmoglobin, a solution of diluted blood is treated with a few drops of a strong solution of potassium ferricyanide or shaken with a few crystals, when the change in colour and spectrum

is seen to occur almost instantly. In order to prepare the crystalline colouring matter, 2–3 c.c. of a saturated solution of potassium ferricyanide or of a nitrite is added to a litre of saturated aqueous solution of crystals of oxyhæmoglobin, and after the conversion has taken place, alcohol is added to the extent of 25%. The liquid is then left to stand in a chamber below 0° when crystals gradually separate. The crystals are more difficultly soluble in water, and form more readily than is the case for oxyhæmoglobin.

When oxyhæmoglobin is changed into methæmoglobin, the oxygen is not removed, but passes into such a condition that it can no longer be removed by boiling *in vacuo*, or by the action of carbon monoxide, though it can be replaced by nitric oxide to produce nitric oxide hæmoglobin.

The substances which convert oxyhæmoglobin into methæmoglobin do not act catalytically (Babel, *Arch. Sc. phys. nat.*, 1896; series 4, **22**, 216–239), because, if care be taken to avoid secondary action, it is not possible to recover the whole of such substances at the end of the reaction, and further, there exists for each substance a limiting quantity, below which it is incapable of effecting any change in the oxyhæmoglobin. In the change of oxy- to methæmoglobin by the action of potassium ferricyanide, Haldane (*J. Physiol.*, 1898, **22**, 298) has shown that a volume of oxygen equal to the volume of the oxygen combined in oxyhæmoglobin is evolved. The action may be represented by the equation:

$$2(K_3Fe'''(CN)_6) + \begin{matrix} H{-}OH \\ H{-}OH \end{matrix} = 2(K_3HFe''(CN)_6) + \begin{matrix} OH \\ OH \end{matrix}$$

$$Hb\begin{matrix} O \\ | \\ O \end{matrix} + \begin{matrix} OH \\ OH \end{matrix} = Hb\begin{matrix} OH \\ OH \end{matrix} + O_2$$

The formula $Hb\begin{matrix} OH \\ OH \end{matrix}$ for methæmoglobin is open to the objection that the acid characters of methæmoglobin are not sufficiently marked, and Haldane prefers the formula $Hb\begin{matrix} O \\ O \end{matrix}$.

Methæmoglobin will also combine with nitric oxide. Hüfner and Reinbold (*Archiv f. Anat. u. Phys.*, 1904, Supp., Bd., 391) have determined for this compound that 1 grm. of methæmoglobin associates 2.66 c.c. of oxygen at 0° and 760 mm., exactly double the quantity of

oxygen or carbonic oxide associated with 1 grm. of hæmoglobin or hæmochromogen.

Reducing agents, *e.g.*, Stokes' reagent, convert methæmoglobin into hæmoglobin. According to Gamgee, this change is preceded by the momentary formation of oxyhæmoglobin.

Solutions of hæmoglobin and oxyhæmoglobin are not precipitated by either neutral or basic lead acetate, but these reagents, if added cautiously so as to avoid excess, precipitate methæmoglobin, hæmatin and hæmatoporphyrin, and may be used for the separation and detection of traces of oxyhæmoglobin when mixed with and concealed by any of the above-mentioned bodies.

Spectrum.—The characters of the spectrum of methæmoglobin have been a matter of considerable controversy, since different methods have been employed in the production of this body. In blood that has stood some weeks exposed to the air, or when the bloody fluids

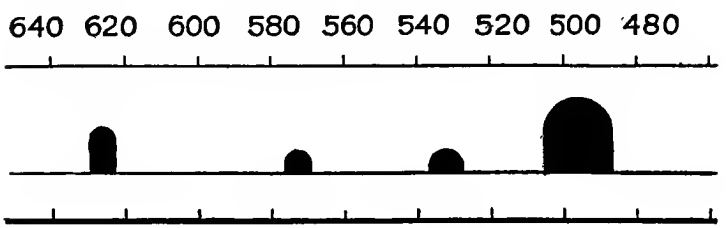


FIG. 40.—Neutral methæmoglobin.

of the cavities of the thorax or abdomen of corpses are examined, the spectrum generally shows a feeble absorption band in the red, two wider bands occupying a position similar to those of oxyhæmoglobin, and a fourth band in the green-blue region. A similar spectrum is seen when methæmoglobin is made artificially by the reagents mentioned above and is given by pure solutions of methæmoglobin crystals. It is certain that if the solutions of methæmoglobin are neutral the first and fourth bands belong to methæmoglobin; but it is a disputed matter, as Lewin, Formanek (*Zeit. Analyt. Chem.*, 1901) and others contend, whether the two middle bands represent the spectrum of unchanged oxyhæmoglobin, or are features of methæmoglobin itself. The weight of evidence appears to favour the view that only the two extreme bands, the first and the fourth, are characteristic of methæmoglobin, and that when animals are poisoned with nitrites or hydroxylamine, just as in carbon monoxide poisoning, a part only of the hemoglobin is converted into methæmoglobin. (See Appendix.)

Acid or neutral solutions of methæmoglobin are coffee coloured. Lewin, Miethe and Stenger, using 2.5% solutions in a stratum 1 cm. thick and with 0.5–3.0 min. exposures of the photographic plates, obtained five bands. The first four are represented graphically in the diagram (Fig. 40).

The positions are as follows:

Met-Hb.	$Hb\text{-}O_2$.
$\lambda = 626\mu\mu$	
$\lambda = 575\mu\mu$	$\lambda = 577\mu\mu$
$\lambda = 533\mu\mu$	$\lambda = 537\mu\mu$
$\lambda = 499\mu\mu$	
$\lambda = 410\mu\mu$ (violet band)	

The violet band was taken using 1% solution in a stratum 1 cm. thick. The second and third bands are to be regarded as the bands of oxy-

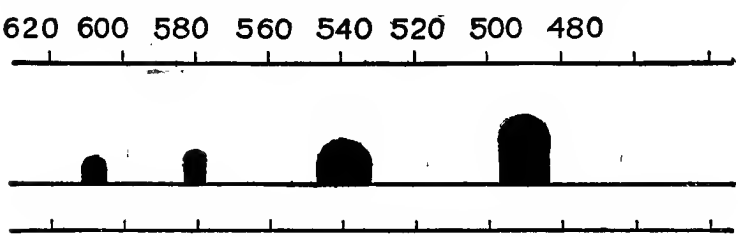


Fig. 41.—Alkaline methæmoglobin.

hæmoglobin, although they are slightly shifted toward the violet end of the spectrum.

An alkaline solution of methæmoglobin is chestnut brown, and the absorption bands characteristic of it are somewhat different in form and position. This is graphically represented in Fig. 41.

The positions are different from those of neutral methæmoglobin. The five bands are:

Alkaline met-Hb.	HbO_2.
$\lambda = 608\mu\mu$	
$\lambda = 578\mu\mu$	$\lambda = 579\mu\mu$
$\lambda = 540\mu\mu$	$\lambda = 540\mu\mu$
$\lambda = 493\mu\mu$	
$\lambda = 415\mu\mu$	

The second and third bands correspond with those of oxyhæmoglobin.

In Fig. 42, taken from Bürker, we give the comparative spectra of HbO_2 and neutral methæmoglobin, and in Fig. 43 that of HbO_2 and alkaline methæmoglobin.

The spectrum of methæmoglobin is not always easy to recognise, and the observation of Kobert, that the addition of hydrogen cyanide

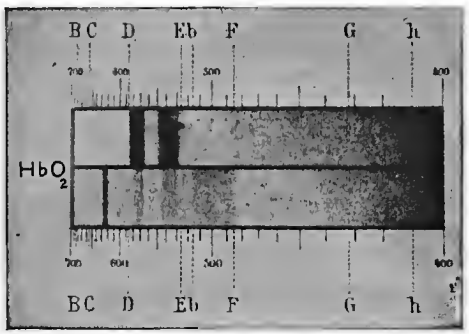


FIG. 42.

or cyanogen gas to methæmoglobin forms cyanmethæmoglobin, renders this reaction of considerable diagnostic importance. The brown-colour of the solutions or of the organs of corpses change to red.

The spectra of methæmoglobin and acid hæmatin are very similar,

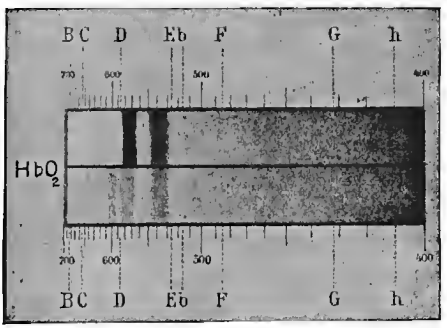


FIG. 43.

each showing a band in the red. The acid hæmatin spectrum remains unchanged on the addition of Stokes' reagent, that of methæmoglobin is replaced by the spectrum of reduced hæmoglobin.

Fluormethæmoglobin.—The addition of alkaline fluorides or fluor-silicates to solutions of methæmoglobin causes the band in the

red to shift toward the yellow ($\lambda=612\mu\mu$). This so-called "band of Menzies," even if the band in the methæmoglobin is not very marked, is, in great dilution, readily seen, and can be used for the identification of methæmoglobin. Fluormethæmoglobin can also be crystallised, but the crystals are unstable (*J. Physiol.*, 1895, **17**, 402–422).

The spectrophotometer constants are given on page 521. See Table III.

The Products of the Hydrolysis of Hæmoglobin.—Oxyhæmoglobin is a salt or perhaps ester-like substance, and, when treated in aqueous solution with dilute acids or alkalies, or when the solution is simply warmed to 64–69°, breaks up into globin—a histone of basic character—and hæmatin, a colouring matter of acid character. Small quantities of simple substances, such as formic acid, butyric acid, etc., are also formed.

Schulz[1] from pure oxyhæmoglobin obtained 86.5% of globin, 4.2% of hæmatin and 9.3% of indeterminate matter. Lawrow,[2] however, obtained 94.09% proteins, 4.47% hæmatin and only 1.44% of other constituents.

The most important derivatives related to hæmatin are hæmin, hæmochromogen and hæmatoporphyrin.

Hæmatin, $C_{34}H_{34}O_5N_4Fe$.

Hoppe-Seyler prepared hæmatin by the action of strong acetic acid on blood in the presence of ether. From the ethereal solution, after filtration, the hæmatin separates as an amorphous precipitate. It is, however, difficult to prepare the pure substance from blood, and hæmin is now used as the starting material.

The hæmin is dissolved in the cold in a 10% solution of sodium hydroxide, and the hæmatin formed is precipitated by exact acidification with hydrochloric acid and well washed with hot water.

It is an amorphous blue-black substance, insoluble in water, alcohol, chloroform or aqueous solutions of acids, but easily soluble in acetic acid, alcoholic solutions of acids, alkalies, and pyridine. It has not hitherto been obtained in the crystalline form. It can be heated to 180° without decomposition, but above this temperature carbonizes with the production of some hydrogen cyanide and pyrrole.

The calcium and barium salts are precipitated when ammoniacal solutions of hæmatin are treated with calcium or barium chloride. The acid solutions are brownish-red in colour, but the alkaline solu-

[1] *Zeitsche. für physiol. Chem.* 1898, **24**, 449.
[2] *Zeitsche. für physiol. Chem*, 1898,, **26**, 343.

tions are olive-green and are dichroic. The spectrum in acid solution is different from that in alkaline solution.

Figures 44 and 45 taken from Bürker show the spectrum of an acid alcoholic solution of hæmatin compared with oxyhæmoglobin (Fig. 44) and the comparative spectra of HbO_2 and alkaline hæmatin (Fig. 45).

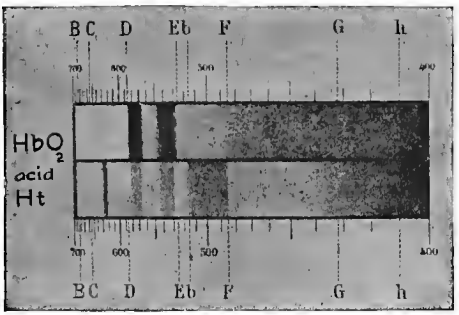


FIG. 44.

Acidified blood, or acidified oxyhæmoglobin solutions show in suitable concentration a characteristic absorption band in the red, which in concentrated solution widens out over the whole of the outer part of the red. Using a 0.18% solution of oxyhæmoglobin acidified with

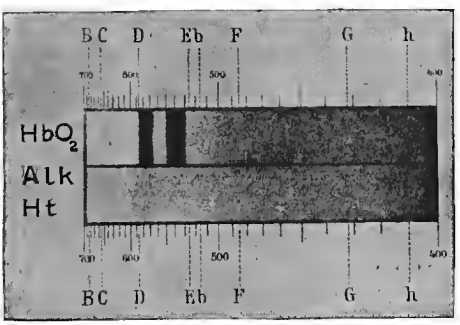


FIG. 45.

hydrochloric acid in a layer of 1 cm., a slit of 0.2 cm. and a variable exposure of 2 to 8 minutes, Lewin, Miethe and Stenger find a maximal absorption at $\lambda = 659\mu\mu$. In the case of alcoholic or acetone solutions of pure hæmatin, rendered acid with hydrochloric acid, a marked displacement of the maximal point was observed. In alcoholic acid

solution it was at $\lambda=632\mu\mu$ and in acetone solution at $\lambda=630\ \mu\mu$. Besides this, the spectrum contains three further bands, a faint band near D with a maximum point at $\lambda=578\mu\mu$, a wider one near E at $\lambda=540\mu\mu$, and a still wider one near F at $\lambda=502\mu\mu$.

In the ultra-violet portion of the spectrum, acid blood shows a band $\lambda=390\mu\mu$. Using an acetone solution of pure hæmatin, the point of maximal absorption is $\lambda=402\mu\mu$. For alkaline hæmatin, using 0.1 grm. hæmatin dissolved in 15 c.c. alkaline water, a slit of 0.1 mm., a Nernst lamp as a source of light and an isokol plate, the above-mentioned observers find the maximal absorption in the characteristic band at $\lambda=616\mu\mu$. Besides this there are two further bands, difficult to photograph and difficult to measure. Their positions are $\lambda=568\mu\mu$ and $\lambda=540\mu\mu$. If acetone solution is used a remarkable displacement of all the bands is noticed. The three corresponding positions of the maximal points are now $\lambda=580\mu\mu$, $\lambda=560\mu\mu$ and $\lambda=524\mu\mu$.

In the ultra-violet part of the spectrum there is a single band, with a maximal point in an aqueous alkaline solution $\lambda=428\mu\mu$, and in acetone solution $\lambda=380\mu\mu$.

Hæmin, $C_{34}H_{33}O_4N_4FeCl$.

This substance is of considerable theoretical and practical importance, and may be regarded as hæmatin in which an (OH) group has been replaced by chlorine. Hæmin may be prepared by any of the following methods:

(1) *Nenčki and Sieber's Method.*[1]—Blood corpuscles are washed, with physiological saline, and coagulated by alcohol. The moist material is then boiled with amyl alcohol with the addition of hydrochloric acid. The crystals, which separate on cooling, are washed successively with alcohol, chloroform, and water. Yield: 1 grm. from 1 litre of blood.

(2) *Method of Nencki and Zaleski.*[2]—An improvement of a method originally devised by Schalfejeff. 1 litre of glacial acetic acid, saturated at ordinary temperature with sodium chloride, is heated to 90–95°, and 200 c.c. defibrinated blood added with stirring. After 10 minutes the liquid is again raised to 90° and filtered. The crystals, which separate from the filtrate on cooling, are washed with water, then with 65% alcohol, and dried in a vacuum. The dried crystals are then recrystallised from 50 c.c. of the following mixture: 15 parts

[1] *Untersuchungen über den Blutfarbstoff, Archiv. f. Experim. Path. u. pharmak*, 1884, 18, 401.
[2] *Untersuchungen über den Blutfarbstoff, Zeit. physiol. Chem.*, 1900, 30, 384.

of 95% alcohol, 4 parts water, and 1 part of an aqueous solution of ammonia (sp. gr. 0.91). The crystals dissolve in this at room temperature in about 30 minutes. The solution is added to 4 to 6 times its volume of glacial acetic acid, saturated with salt, and kept at 110°. On cooling crystals separate, which are washed with 2% hydrochloric acid and dried *in vacuo*. The yield is 3–4 grm. per litre of blood. The product is called acethæmin.

(3) *Method of K. A. N. Mörner* (Küster, *Zeitschr. physiol. Chem.*, 1900, **29**, 187).—1 litre of blood is boiled with 3 litres of water and 10 c.c. of 1% sulphuric acid. The coagulum so produced is stirred with half a litre of 90% alcohol, then filtered and pressed. It is then ground with 1.75 litres of 90% alcohol, to which 35 c.c. of a mixture of equal parts of concentrated sulphuric acid and 90% alcohol are added, and allowed to digest for 1 hour at room temperature. The liquid is strained off and squeezed out of the solid matter, and raised to the boiling-point. To the boiling liquor hydrochloric acid and alcohol are now added in the proportion of 8 c.c. of 25% acid and 12 c.c. of 90% alcohol to every litre of blood, and the whole cooled in water. After standing two days, the crystals which have been deposited are filtered off, washed with alcohol, then with water and dried. The dried crystals are finally washed with petroleum ether. The yield is 3 grm. per litre of blood. Mörner named the product β-hæmin, as it differed somewhat from other products.

(4) *Method of von Zeynek.*[1]—In this method the protein constituent of hæmoglobin is got rid of by digestion with pepsin and hydrochloric acid. The product is then treated as in Nencki and Zaleski's process.

The hæmins prepared by these various methods were supposed to be different, but Küster[2] has shown that the differences were due to impurities, and they consist of one and the same hæmin. Hæmin is most readily purified by warming in chloroform containing quinine (1 grm. hæmin in 50 c.c. chloroform containing 1 grm. of quinine) until dissolved. The solution is then filtered, and on treatment with alcohol containing hydrochloric or acetic acid to neutralise the quinine, hæmin crystallises out. Pyridine may be advantageously used instead of quinine (1 grm. hæmin in 20 c.c. chloroform and 2 c.c. pyridine).

Hæmin is a blue-black crystalline powder with a metallic lustre. It is insoluble in water, alcohol or ether, slightly in dilute mineral acids, alcohol containing hydrochloric or acetic acid, and moderately easily soluble in alkalies and alkaline carbonates.

[1] *Zeitschr. physiol. Chem.*, 1900, 30, 126 and 1906, 49, 472.
[2] *Zeitschr. physiol. Chem.*, 1904, 40, 391–422.

The change of hæmin into hæmatin takes place according to the equation

$$C_{34}H_{33}N_4FeClO_4 + KOH = C_{34}H_{34}N_4FeO_3 + KCl.$$

The reverse change of hæmatin into hæmin can be brought about quantitatively by adding a solution of hæmatin in chloroform containing quinine to a hot glacial acetic acid solution of sodium chloride, and stirring well. Crystals of hæmin separate on cooling. After repeating the above operation, steel-blue glistening crystals of pure hæmin are obtained.[1]

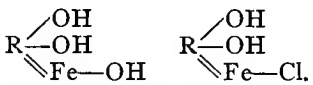
Hæmin contains two hydroxyl groups of a phenolic nature, and a number of ethers and esters have been prepared.[2] The dialkyl ethers, unlike hæmin, are insoluble in alkalies, and their nitrogen can be split off as ammonia, so that the alkyl groups are not in combination with the nitrogen. Hæmin, on treatment with aniline, loses the elements of hydrogen chloride and yields, after extraction with dilute acetic acid and ether, an amorphous product—*dehydrochlorhæmin* ($C_{34}H_{32}O_4N_4Fe$), which, if fresh, can be converted again into hæmin by the action of hydrochloric acid, but on keeping it undergoes some change which prevents this.

Dehydrochlorhæmin, when dissolved in alkali and reprecipitated by acid, is transformed into an isomeride dehydrohæmatin, since it is no longer capable of being converted into hæmin.

Hæmin forms salts with three molecules of alkali hydroxide and dehydrochlorhæmin with two molecules. These salts can be dialysed in 1% solution without any pigment coming through. Hæmin only takes up two molecules of sodium carbonate, and sodium hydrogen carbonate appears in the outer water on dialysis. On precipitating these alkali salts by means of solutions of other metallic salts, the precipitates show very varying quantities of metal. Both hæmin and hæmatin are insoluble in acid carbonate and di-acid phosphates, but with normal carbonate, acid carbonates are formed. The di-sodium salt of hæmatin gives up a molecule of sodium hydroxide on prolonged dialysis. Solutions of the alkali salts of hæmatin polymerise on keeping. The two phenolic hydroxyl groups in hæmin are also present in hæmatin, though so far no hæmatin ethers have been prepared. The third hydroxyl in hæmatin which is readily replaced by chlorine, is removed when iron is withdrawn from the hæmatin molecule, and is, therefore, presumably attached to the iron atom.

[1] O. Piloty, *Annalen*, 1910, 377, 314–369.
[2] Nencki and Zaleski, *Zeitsch. physiol. Chem.*, 1900, 30, 384–435; Küster, *Ber.*, 1910, 43, 2960–2962.

The relationship between the two substances is represented by the following schemes,



Hæmin and hæmatin do not appear to contain free carboxyl groups, but when the iron is removed from hæmin the product—hæmatoporphyrin is both distinctly acid and basic, as it dissolves readily in both dilute acid and alkali, to form well-defined salts. The iron is removed as a ferric salt only, so that the iron in the hæmin molecule is in the trivalent condition.

The ready formation of hæmin from hæmoglobin is taken advantage of in Teichmann's well-known test for blood stains. (See p. 574.) The spectrum of hæmin in alkaline or acid solution corresponds with that of hæmatin in like solvents.

Hæmochromogen, $C_{64}H_{64}N_8Fe_2O_7$.

This substance—the reduced alkaline hæmatin of Stokes—is formed when blood or a solution of methæmoglobin is boiled with a dilute solution of potassium hydroxide, or treated in the cold with 32% potassium hydroxide or concentrated sodium hydroxide. These operations must be carried out in an atmosphere free from oxygen. Beautiful ruby red crystals are obtained as a microscopic preparation when a drop of blood is mixed with pyridine on a slide and then ammonium sulphide added. The crystals are most easily obtained by mixing a drop of fresh blood with an equal amount of pure pyridine, then adding but not mixing a drop of 50% hydrazine sulphate, covering and quickly raising just to the boiling-point. The preparation must not be boiled. For other methods see Nippe (*Deutsche med. Wochensch.*, Nov., 1912, No. 47); also W. J. Dilling, *Atlas du Kristallformen und d, absorptionsbänder der Hæmochromogen*, Stuttgart, 1910. (Fig. 46.) Pure hæmatin is not reducible, but hæmochromogen is formed when it is reduced by Stokes' reagent (see this) or hydrazine hydrate in presence of a trace of albumin. It is also formed when hæmatin is reduced by means of potassium hydrogen sulphide. It is not possible to eliminate the oxygen of hæmatin in the blood-pump to produce hæmochromogen. If hæmatin is suspended in alcoholic ammonia and reduced with hydrazine in an atmosphere of hydrogen, and precipitated by ether (still in a hydrogen atmosphere) a pure ammonium compound of hæmo-

chromogen is obtained. v. Zeynek[1] considers that in the reduction of the hæmatin one atom of oxygen is eliminated from two molecules, and the two hæmatin residues combine together with two NH_4 groups, forming $C_{64}H_{72}O_7N_{10}Fe_2$. Hæmochromogen itself would then be $C_{64}H_{72}O_7N_8Fe_2$. According to Küster, the iron of the hæmochromogen is in the ferrous state.[2]

The spectrum of hæmochromogen is exceedingly characteristic. It consists of two bands, one a dark, sharply defined band between the position of the bands of oxyhæmoglobin, and a much feebler, somewhat wider band about E. Lewin, Miethe and Stenger, using hæmochromogen obtained from oxyhæmoglobin through hæmatin, give the following situations of the maximal absorption in the bands: $\lambda = 556\mu\mu$

FIG. 46.

and $\lambda = 530\mu\mu$, and, using hæmochromogen from pure hæmatin $\lambda = 558\mu\mu$ and $\lambda = 526\mu\mu$. The band in the ultra-violet is also characteristic, and persists in dilutions in which the other bands cannot be distinguished. Gamgee gives the position between G and h $= 420\mu\mu$. Lewin, Miethe and Stenger give $\lambda = 411\mu\mu$. The spectrum of hæmochromogen is of high diagnostic value, for if the solution under examination consists of a mixture of oxyhæmoglobin, methæmoglobin and hæmatin, and the spectrum is consequently of a mixed character, since all these substances pass under suitable reduction into hæmochromogen, which is the basic substance of this colour group, the well-

[1] *Zeit. physiol. Chem.*, 1898, 25, 492.
[2] *Zeit. physiol. Chem.*, 1910, 66, 165–249.

defined absorption spectrum of this substance can be produced. Further, as the dark band is very persistent, it often appears in the reduction of solutions too dilute to show the bands of hæmoglobin or hæmatin.

In Fig. 47 we give the spectrum of solution of rabbit's blood converted into hæmatin by an alkali and reduced to hæmochromogen by ammonium sulphide.

Hæmochromogen forms compounds with carbon monoxide, nitric oxide and the cyanogen group.

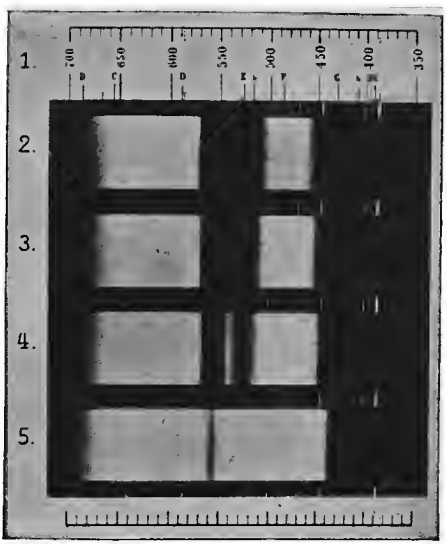


FIG. 47.—From Franz, Rost and Heise.

1. Scale of wave lengths.
2. Dilution, 1 : 60.
3. Dilution, 1 : 80.
4. Dilution, 1 : 100.
5. Dilution, 1 : 300.

Carboxyhæmochromogen.—If an ammoniacal solution of hæmin is reduced with hydrazine hydrate and saturated with carbon monoxide in an atmosphere of this gas, and the solution so obtained mixed with an equal volume of a concentrated sodium chloride solution also saturated with carbon monoxide, a compound of hæmochromogen and carbon monoxide is precipitated. The precipitate is washed with half saturated sodium chloride solution, then with alcohol, finally with ether, and then dried. All the operations must be conducted in an atmosphere of carbon monoxide free from air. The dry powder of CO-

hæmochromogen is stable in dry air, but in moist air changes to hæmatin (Pregl, *Zeit. f. physiol. Chem.*, 1905, **44**, 173).

Linossier, and also Gamgee, obtained nitric oxide hæmochromogen by the action of nitric oxide on hæmochromogen, but this substance has not been purified. Haldane also found it in meat salted with nitrates.

Cyanhæmochromogen is obtained by adding 0.5% aqueous prussic acid to alkaline hæmatin and reducing the cyanhæmatin with ammonium sulphide. It has never been purified.

Hæmatoporphyrin, $C_{34}H_{38}O_6N_4$ (P. Eppinger).[1]

When blood or hæmoglobin, or any of its iron-containing derivatives—moist or dry—is mixed with concentrated sulphuric acid and warmed on the water-bath, iron is split off from the hæmatin molecule with the production of a beautiful red-green solution of acid hæmatoporphyrin. If this solution is poured into water, hæmatoporphyrin is precipitated in the form of brown flocks. Laidlaw has shown that the iron is much more readily split off from reduced than from oxyhæmoglobin (*J. Physiol.*, 1904, **31**, 464).

Nencki and Zaleski[2] recommend the following method of preparation from hæmin:

5 grm. of hæmin (it is best to work with small quantities) are added gradually with vigorous shaking, to 75 c.c. of glacial acetic acid previously saturated at 10° with hydrogen bromide. The liquid is allowed to stand at room temperature for 3 or 4 days with occasional shaking. When all the hæmin has dissolved and the solution acquired the beautiful red tint of hæmatoporphyrin, the contents of the flask are poured into distilled water, and after standing several hours are filtered. The filtered solution is now treated with sodium hydroxide solution, until all the hydrobromic acid is neutralised, when the colouring matter, which is quite insoluble in dilute acetic acid, is precipitated. The precipitate is allowed to settle and thoroughly washed, at first by decantation, until the filtrate no longer reacts with silver nitrate. The washed but still moist hæmatoporphyrin is then digested on a water-bath with a pure dilute solution of sodium hydroxide for 15 minutes, and the coloured solution filtered from the precipitated oxide of iron. The colouring matter is then precipitated from the

[1] *Philos. Dissertations*, 1907, München.
[2] *Zeit. Physiol. Chem.*, 1900, 30, 423; and *Annalen*, 1910, 377, 314–364.

filtrate by means of acetic acid, again filtered and well washed. The precipitate is now made into a paste with a little water in a dish, and ground up with small portions of hydrochloric acid until the colouring matter goes into solution. The solution is then filtered from any resinous matter and more hydrochloric acid added. Should any more resin be precipitated, it is again filtered and then evaporated *in vacuo* over sulphuric acid until crystals of hæmatoporphyrin hydrochloride separate out. The crystals are separated from the mother-liquors on the pump and well washed with 10% hydrochloric acid. After again recrystallising, the hæmatoporphyrin hydrochloride is obtained in the pure state.

Pure hæmatin, on standing with a glacial acetic solution of hydrogen bromide at room temperature, also gives hæmatoporphyrin.

The *hydrochloride*, $C_{34}H_{38}O_6N_4$, 2HCl, of hæmatoporphyrin forms star-shaped aggregates of needles. It is readily soluble in dilute hydrochloric acid, but the solubility decreases with the strength of acid. On treating a solution with sodium acetate the hæmatoporphyrin is thrown out of solution in brown amorphous flocks which, after drying in the cold, have the composition $C_{34}H_{38}O_6N_4$. Hæmatoporphyrin is easily soluble in a solution of alkali hydroxides and alkali carbonates, dilute mineral acids, and alcohol, but with difficulty soluble in ether, amyl-alcohol and chloroform. It is insoluble in water and dilute acetic acid, and is precipitated in acid solution by means of sodium chloride, magnesium chloride, ammonium sulphate and lead acetate.

Solutions of hæmatoporphyrin in acidified alcohol have a beautiful purple colour, and assume a bluish-violet tint when the solution is made strongly acid. Alkaline solutions are a fine red, but with an excess of alkali become more violet in tint. Solutions of hæmatoporphyrin also, even if extraordinarily dilute, exhibit a magnificent red fluorescence.

Hæmatoporphyrin behaves as an amino-acid with two phenolic hydroxyl groups. Dimethyl- and diethyl-ethers and a monoacetyl derivative have been prepared.[1] The presence of ethylene linkages is proved by the readiness with which it is reduced by sodium amalgam, one molecule of the compound taking up 6 to 8 atoms of hydrogen. The solution of the leuco-base thus obtained reduces Fehling's solution, and ammoniacal silver nitrate, and on addition of acid yields a white precipitate of the base, which turns brown on exposure to air. The leuco-base cannot be prepared in the pure state, but on oxidising the

[1] Nencki and Zaleski, *Zeit. physiol. Chem.*, 1900, 30, 429.

solution by air and acidifying the product, a solution is obtained which appears to be dioxyhæmatoporphyrin.[1]

Spectrum.—The spectra of acid and alkaline hæmatoporphyrin differ from one another in the position of their absorption bands, and, further, both products show differences according as the solution examined is prepared from animal and human blood or from pure previously prepared hæmatoporphyrin. According to Lewin, a hæmatoporphyrin solution prepared from blood by means of concentrated sulphuric acid shows 3 absorption bands, one in the orange, a second feeble one, and a third in the green. Lewin, Miethe and Stenger give the positions of maximum absorption as $\lambda = 598\mu\mu$, $\lambda = 575\mu\mu$ and $\lambda = 533\mu\mu$. In the violet there is a band $\lambda = 404\mu\mu$.

If pure hæmatoporphyrin hydrochloride is used in aqueous hydrochloric acid solution, the 3 bands above mentioned are seen, but if a solution of the dry hydrochloride in alcohol is used there are 5 bands, but two vanish again on adding a mineral acid. A specimen obtained by Miethe, Lewin and Stenger from Frau Sieber showed in aqueous hydrochloric acid solution 6 bands, of which the first four were easily recognisable, but the other two in the green required special care for recognition. The positions of the maximal absorption of the 6 bands are $\lambda = 593\mu\mu$, $\lambda = 571\mu\mu$, $\lambda = 550\mu\mu$, $\lambda = 540\mu\mu$, $\lambda = 520\mu\mu$, and $\lambda = 510\mu\mu$. The two bands in the violet and ultra-violet are at $\lambda = 403$ $\mu\mu$ and $\lambda = 380\mu\mu$.

Solutions of alkali hæmatoporphyrin, prepared from dry blood or from the urine of a patient who had taken sulphonal, gave four distinct bands. The positions of the maximal points were $\lambda = 624\mu\mu$, $\lambda = 574$ $\mu\mu$, $\lambda = 544$ $\mu\mu$, and $\lambda = 509$ $\mu\mu$. The band in the violet, obtained by means of an isokol plate, is at $\lambda = 404$ $\mu\mu$.

Pure hæmatoporphyrin hydrochloride in alkaline solution shows five bands, extending from red to blue, as follows: $\lambda = 614\mu\mu$, $\lambda = 563$ $\mu\mu$, $\lambda = 535\mu\mu$, $\lambda = 501\mu\mu$, and $\lambda = 461\mu\mu$.

The violet band can be detected by using zircon light and a perortho plate $\lambda = 388\mu\mu$.

Fig. 48 taken from Rost, Franz, and Heise, shows the spectrum of hæmatoporphyrin hydrochloride in alcohol, ammonia, and sulphuric acid.

Hæmatoporphyrin, according to MacMunn[2] occurs in the integuments of certain molluscs and echinoderms. It is also found in normal human urine[3] (2–10 mgrm. per day) and in increased quantity in the

[1] Piloty and Stein, *Annalen*, 1910, 377, 314–369.
[2] *J. Physiol.*, 1886, 8, 240, and 1887, 8, 384.
[3] Schulz, *Ergebn. f. Physiol.*, 1903, 2, 159.

urine in certain diseases, and especially in cases of sulphonal and trional poisoning. In the latter case it is found in the bile, liver and urine[1].

The method recommended by Garrod[2] for its detection in urine is as follows: The earthy phosphates are precipitated from 500 c.c. urine by the addition of 100 c.c. of 10% alkali. The precipitate, which contains the hæmatoporphyrin, is washed and dissolved in an alcoholic solution of hydrochloric acid. The hæmatoporphyrin is again precipitated by the addition of ammonia, redissolved in acetic acid, filtered and extracted with chloroform. The chloroform is evaporated and

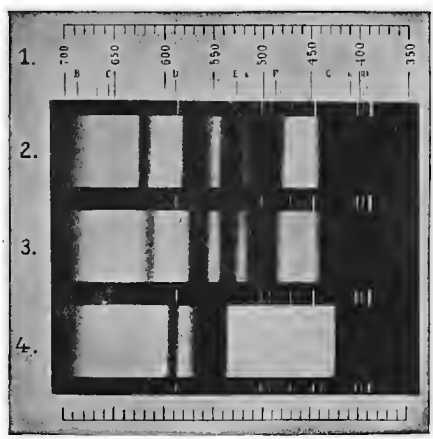


FIG. 48.

1. Scale of wave lengths.
2. 1.5 mgrm. hæmatoporphyrin in 10 c.c. 96% alcohol.
3. 1.5 mgrm. hæmatoporphyrin in 20 c.c. 5% NH_3.
4. 1.5 mgrm. hæmatoporphyrin in 10 c.c. 1 % H_2SO_4.

the residue washed with alcohol and dissolved in acidified alcohol. This solution is examined spectroscopically.

Mesoporphyrin, $C_{34}H_{38}N_4O_4$.

This substance was prepared by Nencki and Zaleski[3] by the reduction of hæmin. 55 grm. hæmin were dissolved in the water-bath in 75 c.c. of glacial acetic acid and 15 to 20 c.c. of concentrated hydrochloric acid (sp. gr. 1.96) and heated 1/4 hour. This was then diluted with 6

[1] Neubauer, *Arch. exp. path.*, 1900, 43, 541: Salkowski, *Zeit. physiol. Chem.*, 1891, 15, 286.
[2] *J. physiol.*, 1892, 13, 598; 1894, 17, 349.
[3] *Ber.*, 1901, 34, 997–1010.

to 10 c.c. of water, and at 70°, 5 to 8 grm. of phosphonium iodide were gradually added with constant stirring. After standing for 1/2 hour in the bath, 2–3 times its volume of water were added and the whole poured into 2–3 litres of water. To this solution sufficient of a solution of sodium hydroxide was added to render it feebly alkaline, and a precipitate of mesoporphyrin was thrown down. For purification this was dissolved in 30 c.c. of hydrochloric acid (sp. gr. 1.124), filtered, and then heated 2 hours on the water-bath. On cooling 100 c.c. of hydrochloric acid (sp. gr. 1.19) were added and on standing mesoporphyrin hydrochloride crystallised out. The yield was 40%. To obtain the substance quite pure, it must be recrystallised several times.

Mesoporphyrin is very similar in properties to hæmatoporphyrin, but according to Nencki and Zaleski the ethers differ somewhat in solubility and melting point from those of hæmatoporphyrin.

In acid solution the spectrum has four bands, $\lambda = 608\mu\mu$, $\lambda = 589\mu\mu$, $\lambda = 567\mu\mu$, and $\lambda = 546\mu\mu$, the last being the most marked.

In the violet the maximal point of the band is $\lambda = 399\mu\mu$. In alkaline solution, using an isokol plate, seven bands have been distinguished, $\lambda = 633\mu\mu$, $\lambda = 615\mu\mu$, $\lambda = 583\mu\mu$, $\lambda = 560\mu\mu$, $\lambda = 535\mu\mu$, $\lambda = 501\mu\mu$, and $\lambda = 463\mu\mu$. With a zircon light and a perorthic plate a band $\lambda = 402\mu\mu$ was obtained.

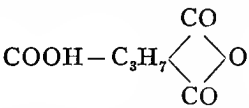
Hæmatoidin.—This iron-free derivative of hæmoglobin may occur as microscopic crystals in old blood extravasations within the body; for example, clots of blood within the brain or the contents of cysts in the liver. The crystals are orange-coloured, rhombic plates, soluble in chloroform. Hæmatoidin solutions show no spectrum, but the ultra-violet and blue rays are largely absorbed. It is generally considered that hæmatoidin and bilirubin are identical from their crystalline characters, and both give Gmelin's test—a play of colours when solutions are treated with nitric acid containing nitrous acid.

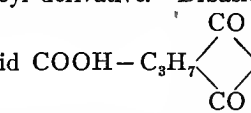
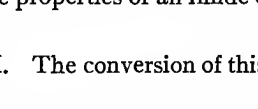
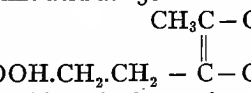
Phylloporphyrin, $C_{34}H_{38}O_2N_4$.

This substance was obtained by Schunck and Marchlewski by the decomposition of chlorophyll. It is very similar in properties and in its spectrum to hæmatoporphyrin but differs from the latter substance in containing two oxygen atoms less. Nencki and Zaleski attempted to change hæmatoporphyrin directly into phylloporphyrin but were unsuccessful, obtaining instead mesoporphyrin. Both substances on reduction give the same decomposition product, hæmopyrrole and on oxidation the same hæmatinic acids.

Oxidation Products of Hæmin, Hæmatin and Hæmatoporphyrin.

By the oxidation of hæmatin and also of hæmatoporphyrin by means of sodium dichromate and acetic acid, Küster obtained two crystalline acids, which he called hæmatinic acids. The one was dibasic and had the composition $C_8H_9O_4N$ and the other was the anhydride of a tribasic acid, $C_8H_8O_5$. The dibasic acid was shown to be monobasic by titration with ammonia, but when the ammonium salt was treated with sodium hydroxide two molecules of ammonia were eliminated and a salt of a tribasic hæmatic acid $C_8H_{10}O_6$ was formed. The anhydride of the tribasic acid has the constitution



as it yields a trimethyl ester, boiling at 300°, and a trisilver salt, but no acetyl or benzoyl derivative. Dibasic hæmatinic acid is the imide of the tribasic acid  The silver salt forms a methyl ester when treated with methyl iodide, from which methylamine is obtained by hydrolysis. The acid is also obtained from the anhydride of the tribasic hæmatinic acid by heating with alcoholic ammonia at 110°; at a higher temperature carbon dioxide is eliminated and a substance with the properties of an imide of the maleic acid series results—  The conversion of this imide (dibasic hæmatinic acid) into the anhydride of tribasic hæmatinic acid can be effected by means of sodium hydroxide, sodium carbonate, 50% sulphuric acid or hydrobromic acid at 130°. The anhydride probably has the constitution  for when oxidised it yields succinic acid, and when reduced by hydrogen iodide a mixture of two isomeric tricarboxylic acids having the constitution of pentane $\alpha\beta\gamma$ tricarboxylic acids, which can be converted one into the other.

For a further account of the constitution and properties of these oxidation products the original papers must be consulted.[1]

[1] Küster, *Annalen*, 1906, 345, 1–54; Küster and others, *Zeit. physiol. chem.*, 1908, 54, 501–547.

Hæmopyrrole, $C_8H_{12}N$.

Nencki and Zaleski in 1901 by the reduction of hæmin with hydriodic acid and phosphonium iodide obtained as a by-product a volatile oil, which yielded compounds with mercuric chloride and picric acid. This oil gave the pyrrole reactions and was called *hæmopyrrole.* It is obtained among the products of the action of heat on hæmatin. It is really a mixture of several pyrroles.

More recently Piloty found that hæmatoporphyrin on reduction with zinc and hydrochloric acid yielded a pigment of the formula $C_{34}H_{38}O_5N_4$, which broke up on treatment with more energetic reducing agents into hæmopyrrole and two acids—*phonopyrrolecarboxylic acid,* $C_9H_{13}O_2N$ and *hæmatopyrrolidinic acid,* a compound of hæmopyrrol and phonopyrrolecarboxylic acid. He succeeded in purifying the hæmopyrrole and by the dry distillation of phonopyrrolecarboxylic acid obtained a pyrrole isomeric with hæmopyrrole—*phonopyrrole.*

Crude hæmopyrrole is a colourless oil with a faint blue fluorescence and boils at 86–87°/23 mm.

Piloty and Stock (*Annalen,* 1912, **392,** 215–244) have recently submitted crude hæmopyrrole to successive processes of fractional distillation, fractional precipitation with picric acid in ethereal solution, and fractional crystallization of the picrates. They have isolated the following constituents: (1) *Hæmopyrrole* α, $C_7H_{11}N$. This boils at 81°/18 mm. and does not form a crystalline picrate. It is probably 3-methyl 4-ethylpyrrole, as on oxidation by chromic acid it yields methylethyl-maleimide. (2) *Hæmopyrrole* β (2:3 dimethyl-4-ethylpyrrole); this melts at 16°, boils at 87–88° under 12.5 mm., and forms a picrate melting at 122.5°. It is identical with the isohæmopyrrole isolated by Willstätter and Asahina. (3) *Hæmopyrrole* γ (3:5-dimethyl-4-ethylpyrrole). It boils at 84–85° under 13 mm., and yields a picrate melting at 137.5°, identical with Knorr and Hess's synthetic compound. These three hæmopyrrols yield on oxidation methyl-ethyl-maleimide. (4) *Hæmopyrrole* δ (2:3:5-trimethyl-4-ethylpyrrole); this is identical with Willstätter and Asahiria's *phyllopyrrole.* (5) *Bishæmopyrrole* (bis-2:3-dimethyl-1-ethylpyrrole). It is an oil and yields a picrate melting at 148°. Phonopyrrole has b. p. of 96–98°/19 mm. and is readily distinguished from its isomerides by the formation of an oily picrate, which does not solidify even in a freezing mixture. With nitrous acid it yields a small amount of a syrupy maleinimide derivative.

For further information as to the properties of these derivatives and a discussion of the constitution of the blood-colouring matters the reader is referred to the more recent original papers of Küster and of Piloty.[1]

Hæmocyanin was first investigated by Fredericq and later by Halliburton. It is a copper-containing protein found in the blood of various invertebrates such as the *Octopus vulgaris*, the lobster, the edible snail, crabs and crayfish. It appears to play the same part as hæmoglobin in the blood of mammals. When reduced the pigment is colourless, but when associated with oxygen becomes pale blue.

Hæmocyanin has been obtained in a crystalline form by Henze (*Zeit. physiol. Chemie*, 1901, **33**, 370 and Dhéré, *Compt. Rend.*, 1908, **146**, 784–786). According to Henze, it contains 0.38% of copper. It is generally described as possessing no characteristic spectrum, but Dhéré states that in a stratum 3 mm. thick containing 7.59 grm. per litre, a band in the ultra-violet exists ($\lambda = 292.2\mu\mu$–$\lambda = 262.8\mu\mu$). On increasing the thickness of the layer a fresh band appears and with a stratum 10 mm. thick it extends to $\lambda = 364\mu\mu$–$\lambda = 328\mu\mu$. This band is characteristic, but the more refrangible band is common to all albuminous substances.

Turacin.—This is another copper-containing pigment found in the feathers of musophagidæ (plantain-eaters). Turacin according to Sir A. H. Church is turacoporphyrin and contains 7.1% of copper. P. P. Laidlaw (*J. Physiol.*, 1904, **31**, 469) has succeeded in making from hæmatoporphyrin, prepared from hæmin by Nencki and Sieber's method, a copper hæmatoporphyrin indistinguishable from the naturally occurring pigments. For its formation all that is required is to add to a solution of pure hæmatoporphyrin in ammonia some cupraammonium solution and boil; turacin is formed at once.

Turacin recalls in its spectrum the blood-colouring matter. It has never been crystallised. The spectrum is two-banded—$\lambda = 555$–$575\mu\mu$ and $\lambda = 520$–$\lambda = 545\mu\mu$.

Quantitative Estimation of Hæmoglobin.—For this a great many methods have been devised, based upon the various chemical and physical properties of blood-pigment. We give only a few of those which are most commonly used.

[1] Küster, *Zeit. physiol. Chem.*, 1909, 61, 164–176; *Ber.*, 1910, 43, 370–375; 43, 2960. Oscar Piloty, *Annalen*, 1909, 366, 237–276; *Annalen*, 1910, 377, 314–369; Küster, *Zeit. physiol. Chem.*, 1911, 71, 100–104. Piloty and Thannhauser, *Annalen*, 1912, 390, 191–209; Piloty and Dormann, *Ber.*, 1912, 45, 2592–2595, and 2595–2600; *Annalen*, 1912, 388, 313–329; Fucher. Bartholomäus, *Ber.*, 1912, 45, 1315–1316

Chemical Methods.

1. The amount of oxygen in a sample of blood may be determined by expelling the entire gas. This can be done with the blood-gas pump (*J. Physiol.*, 1910, **40**, 374) or, for very small quantities of blood, by the ferricyanide of potassium method of Haldane, with the blood-gas apparatus suggested by Barcroft (*J. Physiol.*, 1908, **37**, 12; 1910, 429; 1911, **42**, 512). On the assumption that 1.34 c.c. of oxygen at 0° and 760 mm. are combined with 1 grm. of hæmoglobin, the percentage of this can be calculated.

2. By the determination of iron contained in the ash of blood. As it is known that pure hæmoglobin contains 0.336% of iron, and as all the iron in the blood exists in hæmoglobin, we may calculate the amount of this pigment, if the iron in the ash of a known weight or volume of blood is estimated.

About 100 grm. are evaporated to dryness in a platinum basin and then ignited. The incineration of blood is somewhat troublesome. The method in use in Bunge's laboratory is to mix the organic substance with sodium carbonate solution until distinctly alkaline, and then add further an equal amount of this solution. If the material is alkaline to begin with, about 0.5 to 1 grm. of sodium carbonate is added to every 100 grm. The mixture is placed in a platinum dish, evaporated to dryness in a water-bath with constant stirring. It is finally dried at 120°. It is then carefully incinerated over a Bunsen. It is essential that the carbon should be burnt off at the *lowest possible temperature*. If the carbon is not completely burnt away iron will remain which cannot be removed from carbon by boiling with acids, but if the ash is heated to too high a temperature the oxide of iron becomes very insoluble. It is better to burn off the bulk of the carbon at a low red heat, then extract with strong hydrochloric acid and a filter through an iron-free filter. The filter-paper and unburnt carbon are then completely burnt. A. Neumann recommends, instead of incinerating in the dry way, to oxidise the organic matter by gentle warming with a mixture of equal volume of concentrated nitric and sulphuric acids. This may be done in a long-necked flask closed with a loose stopper or funnel, as is adopted in the estimation of nitrogen by Kjeldahl's method. The authors prefer to oxidise the organic matter with excess of 100% volume hydrogen peroxide (Merck) (*Proc. Physiol. Soc.*, 1906, **35**, 32). The action on substances like hæmin is often intense, much heat being evolved and the liquid raised to the boiling-point. For the estimation of the iron in the ash, any gravimetric method, having due

regard to the composition of the ash, may be employed. The most suitable method is that of titration with titanium chloride devised by Knecht and Hibbert (*Ber.*, 1903, **36**, 1551).

As it is not always possible to obtain a sufficiency of material for the above procedure, various methods have been suggested by which the iron can be determined colorimetrically in minute quantities of blood.

The best known is that of A. Jolles. (*Pflüger's Arch. Physiol.*, 1897, **65**, 579; *Deutsches med. Wochenschr.*, 1898, 104; *Berl. klin. Wochenschr.*, 1899, 965; *Münch. med. Wochenschr.*, 1901; see also F. Müller, *Virch. Arch.*, 1901, **164**, 436.) A known amount, 0.05 c.c., of blood is incinerated in a porcelain or platinum dish, and the remaining oxide of iron dissolved by fusion with potassium hydrogen sulphate. The contents are then dissolved in water and treated with potassium or ammonium thiocyanate. The quantity of iron is determined colorimetrically in the colorimeter. As 0.05 c.c. of blood contains only about 0.00002 grm. of iron it is not possible to determine this with such accuracy as to be of much real use for ascertaining the amount of hæmoglobin, for, in addition to the inherent errors of the manipulations of small quantities of blood the colour of the ferric thiocyanate is not stable.

Colorimetric Methods.—In 1865 Hoppe-Seyler prepared a crystallised oxyhæmoglobin solution of known percentage strength. This is placed in a hæmatinometer (page 514). A weighed amount of blood made feebly alkaline with potassium hydroxide is placed in a second hæmatinometer and distilled water added from a burette, and the whole mixed with a long fish-bone until the tints examined against white paper are equal. The percentage by weight of oxyhæmoglobin in the tested blood is given by the following formula. If the weight in grm. of hæmoglobin in 100 grm. of the standard solution = h, the weight of blood examined = b and the grm. of water added to produce equality = w, then the blood solution contains

$$\frac{(b+w)h}{100} \text{ grm. } HbO_2$$

and the weight of blood taken contains

$$\frac{(b+w)h}{wb} \% \text{ by weight of } HbO_2.$$

Rajewsky has shown that this method gives excellent results but is open to the objection that the oxyhæmoglobin solutions are not stable.

He used coloured solutions of picrocarmine standardised against pure oxyhæmoglobin. These picrocarmine standards, however, also undergo a change in colour within a few months.

The Gowers-Haldane *Hæmoglobinometer* (Fig. 49) (*J. Physiol.*, 1900–1, **26**, 496). The standard tube is a 1% solution of defibrinated ox blood saturated with coal gas, sealed up in an atmosphere of coal gas. The mean percentage oxygen capacity of this blood, as determined with Haldane's ferricyanide method, was 18.5%. The percentage of hæmoglobin corresponding to 18.5% oxygen capacity is 13.8, and the percentage carbon monoxide capacity is identical, 18.5. A tube similar in bore to the standard is graduated, and if 20 mm.^{3} of normal human or other blood, saturated with carbon monoxide and diluted

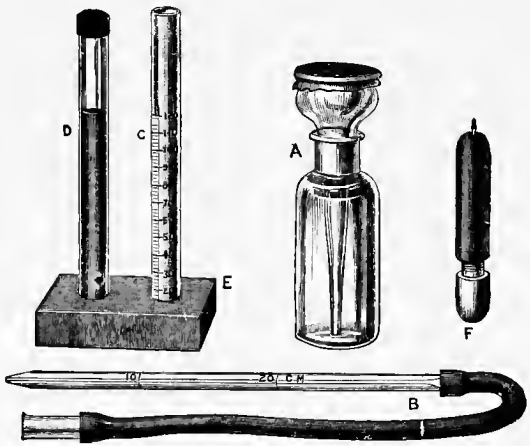


FIG. 49.—A, Bottle with pipette stopper for holding the diluting solution. B, Capillary pipette for measuring the blood. C, Graduated tube for measuring the amount of hæmoglobin. D, Standard tube. E, Support for D and C. F, Puncturing needle.

with water to the mark 100, exactly corresponds with the standard, such a blood has a percentage oxygen capacity of 18.5 c.c. = 13.8% hæmoglobin.

Place distilled water saturated with coal gas or CO in the graduated tube to mark 20. Suck up exactly 20 mm.^{3} of blood in the capillary pipette. This is best done by exceeding the mark, wiping the end of pipette clean and gently dabbing the end on the back of the hand until the blood stands at 20. Now eject this to the bottom of the water, and with careful suction wash out the pipette with the water above the drop of blood. Now carefully add distilled water saturated with coal gas

or CO drop by drop, mixing until the tint appears equal to the standard. Wait a minute, compare the tints again, holding the tubes against the light and frequently changing the tubes from side to side. Add a drop or drops more of water until the tints are just unequal; read again, and take the mean of the first and second readings as correct. The average percentage of hemoglobin of woman's blood is 11%, of children 13%, below the average of adult men.

Therefore, in calculating proportion of hæmoglobin in the blood of women and as percentages of the average normal proportion, it is necessary to add about 1/8 for women and 1/7 for children to the percentage found by the hæmoglobinometer with the standard solution described

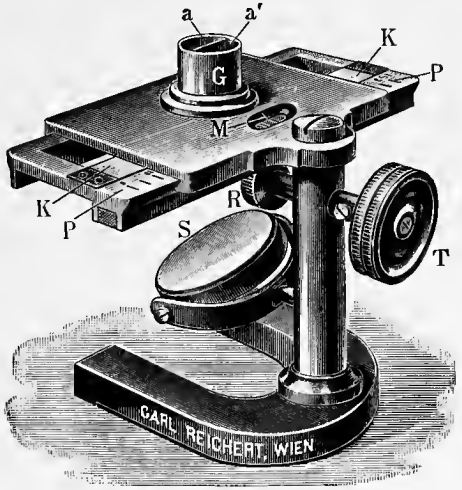


FIG. 50.—Hæmometer of v. Fleischl.

above. The maximal error of this instrument is ±0.8%. Any kind of light, that of the sun, gas or candle, can be employed in estimating the tint. The standard, kept in the dark, remains constant for years.

Sahli's hæmoglobinometer is similar in principle, and also in method of use. The standard is a solution of acid hæmatin. The blood examined is treated with 10 times its volume of $N/10$ hydrochloric acid to convert the hæmoglobin into acid hæmatin, and subsequently diluting to match the standard.

The *hæmometer* of von Fleischl.[1] (Fig. 50.) This apparatus con-

[1] Miescher has modified this hæmometer, which possesses several improvements, in the method of mixing the blood and water, and in devices to vary the actual thickness of the stratum of blood or water which is examined. This instrument—the hæmometer of Fleischl-Miescher—is supplied by H. C. Reichert of Vienna. Full instructions are given for the use of the instrument.

sists of a stand something like the dissecting microscope. The mirror S is a plain white reflecting surface. Under the stage M is a slot carrying a wedge K of coloured glass (gold-purpur), the colour of which shades from deep red to almost pure white. By means of a milled wheel T this slides under the platform. A small metallic cell, G, divided by a vertical partition, stands on the stage, so that one compartment, a′, filled exactly with distilled water, lies over the sliding wedge. The other compartment, a, is filled to a quarter with distilled water. A capillary pipette with a metal handle is filled by dipping one end into a drop of blood, the ends wiped clean, so that the blood is just flush with the surface at each end; it must not present a concave or convex surface. Insert the capillary into the partly filled compartment and move about vigourously until all the blood is out. Finally remove the capillary, eject its contents into the blood and water, thoroughly mix by stirring with the metal handle of pipette, and fill up the compartment exactly with water. The instrument is generally made for use with artificial and not sunlight. Sit sideways, so that the wedge moves toward and away from the observer. By turning the milled screw the wedge is so moved as to exactly match the blood, and on the scale of the metal frame holding the wedge the hæmoglobin percentage is directly read off. In use, as little light as possible should be reflected through the cell from the surface of the plaster mirror. A short black tube of paper over the cell, the use of one eye, and quick judgment in matching the tints, are details for the successful use of the hæmometer. Its error is about 5–10%.[1]

Oliver's Hæmoglobinometer (*Lancet*, 1896, page 1699).—In this instrument a specimen of blood suitably diluted in a shallow white palette is compared with a number of standards prepared by the use of Lovibond's coloured glasses.

The Hæmoglobinometer of Dare (*Philadelphia Med. J.*, 1900, Sept. 22).—In this instrument the blood is examined without any dilution. The apparatus consists of a standardised disc of coloured glass mounted upon a disc of milk-white glass. The tint of this varies, and the depth of colour is standardised in percentages of hæmoglobin, and the disc can be rotated so as to match the sample of blood. On looking down a short brass tube carrying a lens, two circular holes can be seen side by side, illuminated with a candle flame. Behind one hole the coloured disc can be rotated, and behind the other is placed a special blood capillary. This consists of two small flat plates of glass, one

[1] For the comparative value obtained with the hæmoglobinometers of Gowers-Haldane, v. Fleischl and Oliver, see Buckmaster, "*Morphology of Blood*," 1906 (page 204).

clear, the other milk-white. Blood is received into the capillary space, and the capillary at once fixed in the instrument, so that on looking down the lens-tube two circular coloured discs are seen. That of the blood is matched by rotating the standard, and the value read off directly on the graduations of the instrument. The observation must be carried out quickly before the blood coagulates or any deposition of corpuscles takes place in the capillary space.

In *Henocque's hæmoglobinometer* (Fig. 51) the hæmoglobin is estimated by observation of the absorption bands of oxyhæmoglobin.

The apparatus consists of a broad glass plate on which is ruled a millimetre scale 0–60. The plate has two brass ends with slits A and B into which can be dropped two glass plates so mounted as to enclose between their faces a wedge-shaped space; the apex of the wedge *ag*

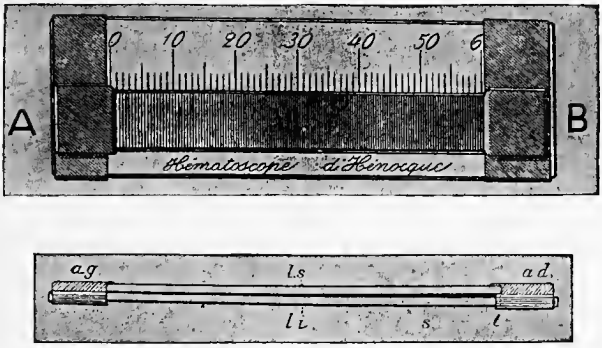


Fig. 51.

lies opposite the 0° of the scale, and at the 60 mark the distance between the plates at *ad* is 0.3 mm., so that as one passes along the scale the distance between the plates increases by 0.005 mm. for each division. Thus at scale mark 20 the distance will be $0.005 \times 20 = 0.1$ mm.

A direct vision spectroscope with a scale of wave lengths is used to examine the blood.

The wedge-shaped space is filled with undiluted blood by capillarity, and the wedge placed in the brass slits of the scale-plate and the spectrum observed with daylight and the slit of the spectroscope set at 1 mm. The spectroscope is moved along against the blood until the two bands of oxyhæmoglobin of $\lambda = 594\mu\mu$, $\lambda = 570\mu\mu$, and $\lambda = 536\mu\mu$–$\lambda = 530\mu\mu$ appear equally wide. As a control the position is also read, in which the two bands just appear and the point at which they just coalesce

into one band. The concentration of oxyhæmoglobin varies with the thickness of the strata. Tables are constructed and supplied with the instrument, giving the percentage of hæmoglobin in terms of the millimetre scale. The graduation of the scale is based on the iron content of the blood and its oxygen capacity.

The spectrophotometer affords the most exact means of determining the amount of hæmoglobin or its derivatives in any fluid.

The ratio of the concentration to the extinction coefficient for a given colouring matter, provided the extinction coefficient is always determined for the same region of the spectrum, is a constant—which Vierordt called the "absorption relation." *Definition of the Term Absorption-relation.*—The more concentrated a coloured liquid is the greater is its absorptive power, and the less thick is the stratum of liquid required to reduce the intensity of light traversing it to 1/10 of its original value. From the definition of the extinction coefficient, it therefore follows that the greater the concentration of solution the greater will be the extinction coefficient. If C is the concentration, *i.e.*, the mass of colouring matter in 1 c.c. of solution, and ϵ is the extinction coefficient for the selected region of the spectrum, $\frac{C}{\epsilon}=A$. If C′, C″, etc., are the concentrations of different solutions of a colouring matter, and ϵ', ϵ'', etc., the corresponding coefficients for the same spectral region, then $\frac{C}{\epsilon}=\frac{C'}{\epsilon'}=\frac{C''}{\epsilon''}$ etc.$=A$. The procedure for the quantitative estimation of hæmoglobin and its derivatives is as follows: Let us take as an example oxyhæmoglobin. Human blood (20 c.c. is amply sufficient) is diluted 100 to 120 times its volume with 0.1% sodium hydroxide solution. The extinction coefficients of this solution are then determined for the regions $\lambda=534.0\mu\mu$–$\lambda=542.0\mu\mu$ and $\lambda=556.5\mu\mu$–$\lambda=564.5\mu\mu$,[1] and Hüfner's quotient of $\frac{\epsilon'}{\epsilon}$ thus determined should be 1.578 with a possible error of 2.5%. The value of this quotient itself is a proof of the purity of oxyhæmoglobin, or that in the solution only oxyhæmoglobin is present.

The absorption relations of oxyhæmoglobin, given by Hüfner in the two regions of the spectrum are given below.

For oxyhæmoglobin, $A_1=0.001312$ and $A=0.002070$. Supposing the values for ϵ' and ϵ for a given solution under examination are 0.895 and 0.565, then $\frac{\epsilon'}{\epsilon}=\frac{0.895}{0.565}=1.58$; this indicates that only oxyhæmoglobin is present. The concentration will be given by,

[1] Instead of these regions, $\lambda=531.5\mu\mu$–$\lambda=542.5\mu\mu$ and $\lambda=554\mu\mu$–$565\mu\mu$ can be used.

$C = \varepsilon.'A' = 0.895 \times 0.001312 = 0.00117$
or $C = \varepsilon.A = 0.565 \times 0.00207 = 0.00117$,
that is to say, 1 c.c. of the solution under examination contains 0.00117 grm. of oxyhæmoglobin.

If the two values are not quite the same, it is perhaps not altogether justifiable to take the mean. The reading for ε' is generally more exact than for ε. If the absolute concentration is not required, but the relative value for the two solutions, then the extinction coefficients are determined for these two solutions in the two spectral regions, and as the extinction coefficients are proportional to the concentrations, the relative concentrations are found.

The blood-colouring matter must be entirely in the state of HbO_2 or HbCO or Hb or met.-Hb. In the following Table IV (Hüfner) *The Absorption Relations of Hæmoglobin and its Various Derivatives* are given, and these must be known in order to carry out determinations of the concentration of solutions.

TABLE IV.

Colouring matter	Absorption relations $\lambda = 531.5 - \lambda = 542.5$	$\lambda = 554 - \lambda = 565$
HbO_2	A^1 0.001312	A = 0.002070
Met.-Hb	A^1 0.001754	A = 0.002078
Hb-CO	A^1 0.001516	A = 0.001829
Hb (red)	A^1 0.001778	A = 0.001354

Butterfield (*Zeitsch. f. physiol. Chem.*, 1909, **62**, 173) gives for HbO_2, A^1 for region 533.5–542 as 0.00118, and for A in the region $\lambda = 556.1$–564.6 as 0.00187.

It is also possible to determine quantitatively the proportion of two colouring matters, provided they do not act upon one another in solution. Vierordt has given the proof that in a solution which contains two or more colouring matters, such colouring matter individually absorbs light in proportion to its concentration and its absorption relation, so that the extinction coefficient of a mixture is the sum of the extinction coefficients of its constituents; so that if $\varepsilon_{1.2}$ is the extinction coefficient of the mixture and ε_1 and ε_2 those of the constituents, then

$$\varepsilon_{1.2} = \varepsilon_1 + \varepsilon_2 \qquad (1)$$

and let x be the unknown concentration of a solution of one colouring matter and ε_1 the extinction coefficient and A_1 its absorption relation for the specified spectral region, and also let y be the unknown concentration of the other colouring matter, ε_2 the extinction coefficient of

its solutions and A_2 its absorption relation for the same spectral region, then

$$\frac{x}{\varepsilon_1}=A_1 \text{ and } \frac{y}{\varepsilon_2}=A_2 \text{ so that } \varepsilon_1=\frac{x}{A_1} \text{ and } \varepsilon_2=\frac{y}{A_2}.$$

Substituting these values of ε_1 and ε_2 in equation (1), then

$$\varepsilon_{1.2}=\frac{x}{A_1}+\frac{y}{A_2}$$

In order to calculate x and y, we must have another relation between these two quantities. This can be determined by ascertaining the extinction coefficient of the same mixture in another spectral region. So, that if $\varepsilon'_{1.2}$ is the observed extinction coefficient of this mixture and A'_1 and A'_2 the corresponding absorption relations for the new spectral region, then

$$\varepsilon'_{1.2}=\frac{x}{A'_1}+\frac{y}{A'_2}$$

and from these equations x and y are obtained,

$$x=\frac{(\varepsilon'_{1.2}\cdot A'_2-\varepsilon_{1.2}\cdot A_2)A_1\cdot A'_1}{A_1\cdot A'_2-A_2\cdot A'_1}$$

$$y=\frac{\varepsilon_{1.2}A_1-\varepsilon'_{1.2}\cdot A_2\cdot A'_2}{A_1\cdot A'_2-A_2\cdot A'_1}$$

and x and y will be the weights of the two colouring matters in 1 c.c. of the mixture.

Theoretically, it is possible to determine three constituents in one solution by working in three different spectral regions; but G. and H. Kruss have shown that in practice the results are unreliable.

Medico-legal Examination of Blood.

The appearance of blood-stains is very variable. The shape should, however, be carefully observed, so that an opinion can be formed as to whether the spot is a smear, a drop, or a splash. In the case of a fabric a knowledge of the side on which the blood fell may be of importance. Blood-stains on fabrics are generally dull, but on polished metal they have the appearance of dark shining spots, and are readily removed.

On making a preliminary inspection of any article supposed to be

stained with blood, careful record should be made of the position in which suspicious marks occur and it should be noted whether any attempt has been made to wash away the stain. In the case of articles of clothing, the positions of these marks should be indicated by inserting safety-pins. Spots on wood or metal may be surrounded with a circular mark made with a black-lead pencil, and similar means should be adopted in other cases to facilitate the subsequent operations. A convenient plan in many cases is to make a sketch of the article and mark on it with red ink the positions of the stains on the original article. Means must also be taken to allow of the certain recognition of the article on subsequent occasions, as in the witness-box, where the following questions may be asked:

1. Is the stain blood?
2. Is it human blood?
3. From what part of the body did the blood flow?
4. What is the age of the stain?

To the last two questions it is rarely possible to give a definite answer.

In the examination of a stain on any surface, material such as hair or substances other than blood should be removed and preserved. For example, it is not difficult to distinguish between the hairs of man and other animals by the use of the ordinary and polarising microscope since it is easy to obtain fresh hairs for comparison. Blood from the stomach often contains epithelial cells, and that from abscesses fat, cholesterol and pus-cells. The presence in suspected stains of spermatozoa, vaginal epithelium, fæcal or biliary matter, brain-tissue, may often have great significance.

The colour of blood-stains varies according to the age of the stain, the quantity of blood, the nature and dye of the stained fabric. As a rule, recent stains are red, old ones brown in colour. The appearance of a stain varies with daylight or artificial light, according to the nature and colour of the material on which the blood has been effused. On metals such as iron or steel, the stain appears as dark shiny spots or smears which, when dry, are often fissured. Many substances stain like blood, for instance jams, solutions of vegetable colouring matters, highly coloured urines, fæcal matter, rust-spots, organic iron salts, etc., and on certain fabrics even grease or paint, pitch or rubber-solutions. The whole examination of a blood-stain should be carried out systematically, and the stains carefully examined with a hand-lens before any further procedure. This would differ according as the stain is recent or old. If the former, the blood-pigment will be more or less soluble,

and the object of the treatment should be such as to afford a solution which can be both examined under the microscope for corpuscles and by other methods for the hæmoglobin. In an old stain the corpuscles are disintegrated and the hæmoglobin in some form such as hæmatin. In recent stains, the piece of material is cut out and treated with some solution which has a similar osmotic pressure as blood plasma, in order that the corpuscles may retain their normal size and not be swollen or crenated. A good solution to use is Pacini's fluid—a 10% solution of chloral hydrate, or 1 part of glycerine in 7 of water, or 0.85% sodium chloride; but even for recent stains, if the garment has been ironed or the dye of the fabric has acted as a mordant, or if the material has been washed with soap and water the extraction by these solvents is very difficult or impossible. In the case of leather or woods rich in tannic acid, the hæmoglobin may have been so fixed as to become insoluble. For old stains various solvents, such as warm water, weak solutions of citric acid or ammonia may be used, or 2% hydrochloric acid. Schaer (*Archiv. Pharm.*, 1898, **236**, 571–579) advises 65 to 85% of chloral hydrate. In a very refractory stain a solution yielding the hæmatin spectrum may be obtained with strong potassium hydroxide. Before actually treating a suspected stain on a dyed fabric, it is desirable to submit a portion of the material, free from stain, to the action of water, citric acid, and ammonia, with a view of ascertaining whether colouring matter is dissolved which will interfere with or modify the blood-spectra subsequently observed. In the event of blood not being recognised in a suspected stain on such fabric, a portion of the material should be treated with blood, and the process applied to the authentic blood-stain so produced.

On exposure to air in a damp place, a blood-stain may become mouldy, with complete destruction of the colouring matter; but if kept dry, the hæmoglobin gradually changes into a complex mixture of methæmoglobin, hæmatin, and hæmatoporphyrin. If moderately recent, such a stain will be brown, and will dissolve with difficulty and probably incompletely in cold water, yielding a dirty brown solution. The alteration of blood-stains takes place far more rapidly in the atmosphere of a town, and especially in a room where gas is burnt, than in the pure air of the country. Contact with sweat may cause the hæmoglobin of a blood-stain on a dirty garment to undergo very rapid alteration into hæmatin. Such facts should be taken into consideration in forming any opinion as to the age of a blood-stain from its colour, solubility, and spectroscopic examination.

The object of all solvents is to remove material from the stain which

shall give evidence of the presence of blood by one or more of the following methods:

1. Microscopical recognition of corpuscles.
2. The chemical test for blood, which depends upon certain colour-tests, *e.g.*, guaiacum or benzidine, etc. (see page 522).
3. The production of hæmin and hæmochromogen crystals (page 574),
4. The spectroscopic recognition of the pigment.
5. The biological test for blood for specific kinds of blood (page 577).

In order to examine a supposed blood-stain to the best advantage, it is desirable to remove as much of the surrounding material as possible. Thus if the stain be on cotton, linen, silk, wool, etc., a portion of the fabric on which the spot exists should be cut out. If on a porous material, such as wood, brick, or stone, the stained substance should be scraped away for some depth, and reduced to fine powder. Stains on metal, especially on iron or steel, are most difficult of treatment, since the colouring matter is readily mordanted and rendered insoluble by the iron. Sometimes, on drying the article thoroughly, the incrustation will peel off, but if not, it must be removed by scraping.

On whatever material the stain may have been, when the suspected portion has been more or less separated in the above manner from the surrounding material, a portion of the coloured substance should be treated in a watch-glass with a few drops of 0.85% sodium chloride and allowed to stand at rest for a short time. If the stain is at all recent, and the colouring matter has not been mordanted in any way, the liquid will acquire a more or less distinct red or brown colour. In this case it is probable that the colouring matter of the blood is, in part at least, in a soluble form, and the subsequent examination will be simple. By preference, the liquid should be decanted from any insoluble residue. If necessary, the liquid may be filtered, but this operation should be avoided if possible. If the decanted or filtered liquid be perfectly colourless, evidence of blood may still be obtained from the insoluble portion. In such a case, as already stated, special means must be employed to effect the solution of the colouring matter. If a blood-stain has been strongly dried before a fire, or otherwise heated, so as to coagulate the albumin, the colouring matter is rendered insoluble, and cannot be extracted by treatment with water, citric acid, or cold ammonia. On heating the stain in dilute ammonia or sodium hydroxide, the hæmatin is readily dissolved, and may be detected by reducing it to hæmochromogen, either with or without previous concentration of the solution.

The same method may be employed for extracting the colouring

matter from a blood-stain which has been washed, the liquid being subsequently concentrated by evaporation or neutralised and precipitated with zinc acetate.

Von Fürth (*Zeitsh. Angew. Chem.*, 1911, **24**, 1625) recommends a combination of Leer's pyridine test with leuco-malachite green. The object to be tested is boiled for a few minutes with a few drops of 50% potassium hydroxide to which a few drops of alcohol have been added. After cooling, the liquid is extracted with pyridine. The pyridine layer is separated and the solution again shaken with potassium hydroxide after which about 1 c.c. of the solution is run on to a filter paper on a glass plate. This is then treated with a solution of leuco-malachite green to which 1% H_2O_2 has been added.

In cases where the colouring matter remains obstinately fixed on a fabric, probably from combination with the mordant, Sorby recommended that the stain should be digested in dilute ammonia (in a watch-glass), and the liquid squeezed out repeatedly by a pair of forceps, and ultimately between the finger and thumb. The thick, turbid, unfiltered liquid is then reduced in the ordinary manner, and examined for the hæmochromogen bands, using lime-light or concentrated sunlight if necessary. By operating in this manner, Sorby succeeded in detecting blood in a stain 6 years old on brown cloth. When the tube containing the turbid liquid was kept in such a position as to allow the suspended matter to settle out, no absorption-band was produced by the supernatant fluid, the colouring matter evidently existing in the insoluble deposit. Hence, in such cases any process of deposition or filtration is inadmissible. The effect of the insolubility must be overcome by increasing the intensity of the light, and not by removing the deposit.

The spectroscopic recognition of blood on *rusty iron* is somewhat difficult. For reasons not thoroughly understood, ammonia and citric acid sometimes fail to dissolve the colouring matter. In such cases glacial acetic acid aided by heat will sometimes be found successful, but a more certain plan is to heat the spot to 50° with a cold-saturated solution of borax. The solution obtained should be treated with acetic or citric acid, filtered if necessary, reduced, rendered alkaline, and examined for the spectrum of hæmochromogen.

The recognition of blood in suspected stains on *leather* presents peculiar difficulties, since the presence of tannic acid so mordants the blood that neither water nor citric acid will dissolve the colouring matter, and ammonia produces an inconveniently dark solution. If the stain is on the surface of the leather, and has never been wetted, a

thin shaving should be cut off, so as to obtain as much blood and as little leather as possible. The shaving should then be bent with the stained side outward, and placed on the mouth of a cell-tube[1] filled with water, in such a manner that the suspected blood-stain shall be in contact with the solvent without the rest of the leather becoming wetted. In this manner the dissolved colouring matter sinks to the bottom of the cell without coming in contact with the rest of the leather. After removing the shaving, the liquid in the cell may be examined in the usual manner.

If a blood-stain on leather has been wetted, the foregoing method is inapplicable. In such cases, Sorby recommended that the stained leather should be digested for a considerable time in water containing 2% by volume of hydrochloric acid. The liquid is then poured off (not filtered) and treated with ammonia in excess, by which it acquires a purple or neutral tint. This colour is intensified by the iron reagent which Sorby used for reduction, but it is evident that the presence of tannin renders this treatment unsuitable. Stannous chloride or ammonium sulphide can be substituted with advantage, and there will then be little difficulty in recognising the absorption-band of reduced hæmochromogen. (For methods of reduction see page 527.)

Graham-Smith and Sanger (*J. Hygiene*, 1903, 258) found that leathers are mostly acid, and extracts of acid reaction give a precipitate with blood. After neutralisation, the precipitin test (page 577) can be applied. If blood is dried on thick polished yellow leather, it is quite impossible to obtain the precipitin test.

Earth and soil-stained clothes render blood completely insoluble. Sorby recommended extraction with ammonia, and examination of the turbid solution with an intense light. A saturated solution of borax, as recommended by Dragendorff, may be advantageously substituted for ammonia. 1 part of blood absorbed in 200 of peat can thus be detected.

Very few colouring matters yield absorption-spectra which can be mistaken for those of blood, even by the inexperienced observer, and these can be absolutely distinguished by the application of reagents.

The petals of the red variety of *Cineraria* contain a colouring matter the spectrum of which exhibits two absorption-bands in the green, similar in position to, but differing in relative width from, those of oxyhæmoglobin. The bands of *cineraria* are completely altered on adding ammonia, while those of oxyhæmoglobin are unaltered.

[1] This is made by cementing a piece of glass tubing 1–2 cm. long and 1 cm. diameter on to a microscope slide so as to form a capsule.

Turacin, the copper-containing colouring matter present in the feathers of the turaco or plantain-eater exhibits a spectrum similar to that of blood, but it does not yield the spectrum of hæmochromogen on reduction.

A solution of *cochineal* in alum shows absorption-bands somewhat resembling those of oxyhæmoglobin, which are rendered more intense on adding ammonia. On subsequently adding excess of boric acid, the cochineal bands shift to the blue end of the spectrum, whereas those due to blood are unaffected by this treatment. The points of maximum absorption for the bands of carminate of ammonia are:

$$\lambda = 560\mu\mu$$
$$\lambda = 518\mu\mu.$$

A solution of *soluble indigo* when *hot* exhibits a spectrum not unlike that of blood, but it is decolorised by treatment with alkali and a reducing agent.

Lac-dye, *alkanet*, *madder*, and *alizarin*, when dissolved in alum, exhibit absorption-bands which distantly resemble those produced by the colouring matter of blood. Alizarin Red W, in alkaline alcohol shows three bands and the position of maximum absorption is

$$\lambda = 610\mu\mu$$
$$\lambda = 559\mu\mu$$
$$\lambda = 518\mu\mu.$$

The spectra are all modified by treatment with ammonia, and the bands are destroyed on adding sodium sulphite, whereas the absorption-bands of blood are relatively unaffected by such treatment. *Rosaniline* (magenta, fuchsin), also, is completely decolorised by sulphites, and the colour is not restored by exposure to air.

Hæmin Test.—In 1853, Teichmann showed that the demonstration of blood crystals, known often as "Teichmann's crystals," was possible with minute traces of blood. The crystals from the blood of all animals are identical, brown rhombic prisms (Fig. 52). The test is performed as follows: the dried suspected material is rubbed up on a microscope slide into a powder. To this glacial acetic acid is added, so as to make a paste, then add a few drops more acid, and cover with a cover-glass. The quantity of acid should be sufficient to form a thin layer between the cover-glass and slide. The slide is now heated with a small Bunsen burner till it just boils. (Do not continue to boil.) On cooling, in about 2 minutes, the dark brown crystals are seen with

the microscope (magnification 1–300). If not present, raise again to the boiling-point, taking care there is a sufficiency of acetic acid.

If the blood-stain is some years old, a minute crystal of sodium chloride or potassium chloride may be rubbed up with the paste. The addition of chloride was originally suggested by Virchow. It is not necessary for recent blood, as there is sufficient of this salt in the plasma. Too much salt interferes with the test.

According to Lewin and Rosenstein (*Virch. Arch.*, 1895, 173), the following are the conditions under which the hæmin test succeeds:

1. The blood must be dry. If liquid, it should dry in the air, not over a flame.

2. Oxyhæmoglobin, hæmoglobin, methæmoglobin, carboxyhæmoglobin, and hæmatin all yield crystals.

3. Hæmochromogen and hæmatoporphyrin yield no crystals by

FIG. 52.

this method. In some meat juices the blood colouring matter is in the state of hæmochromogen.

Rose showed that when blood is mixed with iron-rust the test fails. Stains on rusty iron may, therefore, fail to give the test, or only the most superficial layers may do so. For an explanation, it is probable that in the presence of iron oxide no hydrochloric acid is formed from sodium chloride by the action of acetic acid.

(1) $Fe(OH)_3 + 3C_2H_4O_2 = Fe(C_2H_3O_2)_3 + 3H_2O$.

(2) $Fe(C_2H_3O_2)_3 + 3NaCl = FeCl_3 + 3NaC_2H_3O_2$.

The hæmin test is said to be negative for stains treated with soap and water. However, Lewin and Rosenstein find that blood-stains treated with boiling potassium hydroxide for 20–25 minutes, or washing the stain with soap and water, may yield hæmin crystals.

Many observers consider that the formation of hæmochromogen crystals by the pyridine method given on page 550, and their examination with the micro-spectroscope affords a delicate and certain test for blood-colouring matter. The ruby-red colour of the crystals under the microscope is very characteristic and a minute amount of blood suffices for their demonstration. It is difficult to obtain the crystals as sharply defined as is the case for hæmin.

The test is carried out as follows: (Donogany, *Maly's Jahresber. f. Tierchem.*, 1894, **23**, 126, and *Virch. Archiv.*, 1897, **148**, 234). Some drops of defibrinated blood are mixed on a slide with a few drops of pyridine, and then some drops of ammonium sulphide. The mixture is covered with a cover-glass. The blood of all animals yieids crystals. Carbon-monoxide blood yields crystals of carboxy-hæmochromogen. The crystals of hæmochromogen are red bundles or masses of needles. In their preparation no air-bubbles should be allowed to appear. The test is of diagnostic value for blood or hæmoglobin.

The examination of blood in order to throw light on the cause of death by asphyxia, by carbon-monoxide poisoning, by cyanides, sulphuretted and arseniuretted hydrogen, by potassium pyrogallate and chlorates, may be required.

In asphyxia, most of the blood-colouring matter, both in the arteries and veins, is in the state of reduced hæmoglobin. In poisoning with carbon monoxide, the blood contains a mixture of oxyhæmoglobin, hæmoglobin and carboxyhæmoglobin.

In death from hydrogen sulphide the compound with hæmoglobin is of an evanescent character, but may show the spectrum described on page 539.

In prussic-acid poisoning, the blood is often scarlet in colour, and the spectroscopic features of the pigment are described on page 538.

In poisoning with fluorides or hydrogen fluoride, the spectrum may exhibit the features described on page 544.

In poisoning with chlorates, pyrogallic acid or arseniuretted hydrogen, the blood may contain a variable amount of methæmoglobin.

To differentiate the blood of man from that of lower animals is a matter of practical impossibility, except in very favourable cases where the blood corpuscles can be examined and measured (see page 502).

Mammalian blood is non-nucleated, in that of birds, fishes, reptiles and amphibia, oval nucleated corpuscles are present. These facts are of slight medico-legal importance; it is, however, possible to state whether blood is that of a mammal or not.

Various observers have attempted to differentiate blood by means of the crystals of hæmoglobin or by differences in the solubility of hæmoglobin in different animals, but the methods are unsatisfactory from a medico-legal point of view.

The researches of Uhlenhuth, Schütze, Wassermann, Nuttall and others have shown that the blood of man can be distinguished from that of other animals.

The Precipitin Test for Blood.—The serum of blood must be regarded as resulting from the death of blood; it has the same relation to plasma that killed has to living protoplasm. The serum obtained from the blood of normal animals and that from animals which have been subjected to various procedures, such as the injection of the blood of other animals, foreign proteins, or solutions of ferments, yield a normal or modified serum respectively. In 1899 Tschistovitch, working with eel's serum, showed that rabbits which were injected with this possessed a blood the serum of which produced a remarkable cloudiness on admixture with eel's serum. In other words, an injection of eel's serum produced in the rabbit's serum a substance which, reacting outside the body in a test-tube with eel's serum, caused a precipitate or cloudiness. This observation was confirmatory of work by Bordet, and the reaction is often spoken of as that of Bordet-Tschistovitch. This is the basis of the precipitin or biological test for blood (Uhlenhuth, Ziemke) and, as used by Nuttall, is one which has been employed for determining the relationships which exist between different animals. (Nuttall, "*Blood Immunity and Relationship*," 1904; Cambridge.) When blood is injected into an animal—the rabbit is generally chosen for this purpose—the substances developed in its blood serum are known as precipitins. These are not known by any definite chemical or physical tests, but they are specific. If dog's blood be injected, then the rabbit serum contains a precipitin specific for dog's blood. If fowl's blood, then a precipitin specific for the fowl; human blood, a precipitin specific for man. In all these cases a cloudiness or precipitum results only when the actual blood-containing precipitin is mixed with the blood of the animal which originally supplied the blood for injection. The various sera are termed anti-dog, anti-fowl or anti-human for the above examples, but it is the case that a number of other anti-sera may be obtained by a similar method of experiment. An example may make this clear.

Anti-human serum was obtained by injecting into the peritoneal cavity of rabbits, every 6 or 8 days, 10 c.c. of defibrinated human blood. At the end of a month the rabbit was killed, its blood col-

lected, and allowed to clot. The serum contained a specific precipitin as the following results show:

Using 3 c.c. of blood, diluted 1 : 20 or 1 : 40, adding 1–2 drops of the anti-human serum, a white cloud slowly forms and increases, finally settling at the bottom of the small test-tube (capacity about 6 c.c.). The reaction is accelerated by a temperature of 37° C., but apparently in no other way. (Graham-Smith and Sanger. *J. Hygiene*, 1903.)

Diluted Blood.	
1. Human (fresh)	Precipitum (positive)
2. Human (dried)	Precipitum (positive).
3. Frog	Negative.
4. Fowl	Negative.
5. Guinea-pig	Negative.
6. Ca	Negative.
7. Human Serum	Positive.
8. Horse	Negative.

In Uhlenhuth's original list (*Deutsch. med. Wochensch.*, Feb., 1901; Sept. 1902) 19 different bloods were tested with anti-human serum. A positive reaction (formation of precipitum) occurred with human blood, but not with that of the dog, cat, pig, sheep, deer, fallow deer, horse, donkey, hare, rabbit, guinea-pig, rat, mouse and four different birds. Wassermann and Schütze (*Berl. klin. Wochensch.*, Feb., 1901) give details of 23 observations; only the blood of man and baboon gave positive results with anti-human serum. (See also Ziemke, *Deutsche med. Wochensch.*, Jan. and Oct., 1901.)

Preparation of Anti-sera.—A rabbit is placed on its back and the abdomen lathered over with liquid soap containing lysol. A small area on the left side at the lower end of the abdominal region is then shaved. This area is disinfected with acetone. The skin and muscle are gathered together with the disinfected fingers, taking care that the gut is not included, and the sterile needle of a hypodermic syringe holding about 10 c.c. of sterile blood, or serum, or defibrinated blood, is thrust through the skin and muscle into the peritoneal cavity taking care to keep the needle horizontal and making sure that this enters the abdominal cavity and does not wound the gut. After injection the needle is withdrawn, the skin dried, and the wound treated with some disinfectant dressing such as petol salve or solid boracic acid powder. 5 or 6 injections are made at intervals of 2–4 days, and the animal killed 7–12 days after the last injection. The animal is chloroformed, the thoracic cavity opened under aseptic precautions, the heart is incised

and the blood allowed to flow into the cavity whence it is removed with sterile pipettes into a sterile dish. The blood is allowed to clot and the serum is pipetted up into sterile test-tubes and then transferred to sealed bulbs of small size. Any serum which separates on further shrinking of the clot should be passed through a Chamberland filter to prevent bacterial development. Opalescent sera are of little use in carrying out the precipitin test.

Medico-legal Test for Blood.—The precipitin test was first employed by Uhlenhuth. Having prepared anti-serum by the repeated injections of human blood into rabbits, and diluted the suspected human blood with an equal volume of 1.6% sodium chloride he added 6 to 8 drops of anti-human serum. The reaction is carried out in small test-tubes holding about 5 c.c. After mixing the liquids the tubes are placed in the incubator at 37°. For old dried blood-stains he first applies the guaiacum and hæmin tests, and then, by using various anti-sera which contain the specific precipitins for the blood of different animals, the kind of blood is identified.

According to Nuttall, who used dilutions of human blood 1:100 of normal saline 0.85% and equal volumes of 1:100 diluted antihuman serum, the positive reaction is obtained with old blood (2 months), putrified blood, or blood mixtures of human and other bloods. The test is extraordinarily delicate, dilutions of human blood 1:16,000 reacting positively.

Human anti-serum is not absolutely specific, thus it is maximal for human blood, nearly maximal for the anthropoid apes, less for other monkeys, negative for all other mammals, fish and birds. (For the precipitin test in medico-legal work, see Uhlenhuth, *Das biologische Verfahren zur Erkennung und Unterscheidung im Menschen-und Tierblut*, Jena, 1905, and Buckmaster, "*Morphology of Normal and Pathological Blood*, 1906, pages 147–157.) In the present state of our knowledge, the positive evidence given by the precipitin test is of value, but cannot be regarded as conclusive. Except in the hands of those having constant practice in the procedure, or engaged in serological investigations, the test presents numerous pitfalls.

ALBUMINOIDS OR SCLEROPROTEINS.

By JEROME ALEXANDER.

According to the classification adopted by the American Physiological Society and the American Society of Biological Chemists, the term *albuminoid* should be restricted to simple proteins[1] exhibiting pronounced insolubility in all neutral solvents. Since this group includes elastin (from tendon), collagen (from bone and hide), and keratin (from hoof and horn), substances forming the chief organic constituents of the skeleton of animals, as well as of the skin and its appendages, the Chemical and Physiological Societies of England prefer the term *scleroprotein* to albuminoid.

The term *albuminoid* (literally albumin-like) has in the past been used as synonomous with "proteid," and thus included not only albumin and its congeners, but also gelatin and allied substances. Later it was proposed to disregard the term "albuminoid," and to apply the term "proteoids" to "proteids" of the gelatin group, which includes ossein, keratin, fibroin, chitin, etc.

That a classification of albuminoids on the basis of their chemical constitution is extremely difficult, if not impossible, is evident from the table on page 583[2] which shows the products of hydrolysis of some of the more important members of this heterogeneous group. Furthermore, it is practically impossible to obtain a chemically pure protein, for in colloidal substances of this kind the formation of physicochemical, adsorption, or colloid-compounds has prevented their complete separation from foreign substances, from associated proteins, and from each other. Thus "chondrigen," which has been considered to be an albuminoid, is in reality a glycoprotein (see page 624); and "chondrin," the product of its hydrolysis also belongs to the same group. Chondrigen is apparently a combination of some sort between ossein and mucin, probably an adsorption derivative.

[1] Simple proteins are those which yield on hydrolysis only α-amino-acids or their derivatives. Compare page 17.

[2] Taken mainly from "The Chemical Constitution of the Proteins" by R. H. Aders Plimmer, Part I, page 24.

While the albuminoids are all amorphous and insoluble in cold water, some members of the class are dissolved by boiling with water (with or without pressure), to solutions which gelatinise on cooling. These substances may be conveniently grouped together as *collagens* or *jelly-forming albuminoids* (collagenes or gelatoids). A distinction between proteins in general and collagens which has been very generally insisted on, is that when compounds of the former class are split up by the action of somewhat diluted mineral acids, tyrosine, tryptophane, and other compounds of the aromatic series are prominent among the products of their decomposition; whereas gelatin and its congeners yield no appreciable quantities of aromatic products on similar treatment.[1] This distinction does not hold good for substances of the keratin and fibroïn classes, tyrosine being present to a sensible extent in the products of their decomposition.

In the absence of a better classification, the albuminoids may be tentatively grouped as follows:

A. *Collagens* or *Jelly-forming Albuminoids:* *Collagen* and *gelatin;* from bones, skin, etc. Page 582. *Chondrigen* and *Chondrin;*[2] from permanent cartilages. Page 624 *et seq.* *Isinglass;* from swimming bladders of fishes. Page 618. *Sericin* (silk-gum) from silk. Page 634.	Dissolved more or less readily by boiling water. The solutions gelatinise on cooling. Contain little or no sulphur.
B. *Fibroids:* *Elastin;* from elastic ligaments. Page 631. *Fibroin;* from silk and spiders' webs. Page 635.	Not acted on by boiling water or very dilute boiling alkali. Dissolved by stronger alkali. Unaffected by dilute acids. Contain no sulphur.
C. *Chitinoids:* *Chitin;* from external coatings of invertebra. Page 670 *Chonchiolin;* from shells of mollusca. Page 672. *Spongin;* from sponges. Page 672	Not acted on by boiling water or alkalis. Contain no sulphur.
D. *Keratins:* *Keratin;* from hoofs, horns, feathers, hair, wool, etc. Page 673. *Neurokeratin;* from brains. Page 673.	Not acted on by boiling water. Dissolved by boiling with dilute alkali hydroxide. Contain sulphur.

Collagens or Jelly-forming Albuminoids.

The substances of this group, though insoluble in cold water, are by boiling with water converted into soluble products whose solutions gelatinize upon cooling. Thus collagen or ossein yields gelatin, "chondrigen" yields "chondrin," and sericin, the collagen of silk ("silk-glue") yields, when boiled with water, a solution which if sufficiently concentrated, sets to a jelly on cooling.

[1] For this reason gelatin has been found valuable as a partial substitute for other proteins in cases of intestinal indigestion, especially in infants. See "On Infantilism from Chronic Intestinal Infection," by Prof. Christian A. Herter.

[2] Chondrigen and chondrin properly belong with the glycoproteins, but are considered here because they occur in glue and the materials from which it is made.

ALBUMINOIDS OR SCLEROPROTEINS.

	Gelatin (Fischer, Levene and Aders; Fischer, Hart, Kossel and Kutscher)		Gelatin (Skraup and von Biehler)	Silk-gelatin (Fischer and Skita; Fischer)	Silk-fibroïn (Fischer and Skita; Fischer)	Spider-silk-fibroïn (Fischer)	Elastin (Abderhalden and Schittenhelm; Schwarz; Kossel and Kutscher)	Spongin (Abderhalden and Strauss; Kossel and Kutscher	Koilin (Knaffl-Lenz)	Egg-membrane of Scyllium Stellare (Pregl)
Glycine		16.5	12.4	0.2	36.0	35.2	25.8	13.9		2.6
Alanine		0.8	0.6	5.0	21.0	23.4	6.6			3.2
Valine		1.0			0		1.0			
Leucine		2.1	9.2		1.5	1.8	21.4	7.5		5.8
Isoleucine										
Phenylalanine		0.4	1.0		1.5		3.9			3.3
Tyrosine		0		5.0	10.5	8.2	0.4	0		10.6
Serine		0.4		6.6	1.6					
Cystine										
Proline		5.2	10.4		+	3.7	1.7	6.3		4.4
Oxyproline		3.0	3.0							
Aspartic acid		0.6	1.2		+		+	4.7		2.3
Glutamic acid		0.9	16.8		0	11.7	0.8	18.1		7.2
Tryptophane		0								
Arginine	9.3	7.6	9.3	+	1.0		0.3		3.6	3.2
Lysine	5	2.8	6.0		+	5.24			1.7	3.7
Histidine		0.4	0.4	4.0	+				0.1	1.7
Diamino-trioxydodecanic acid										
Ammonia		0.4				1.2				
Total		41.1	70.3	20.8	73.1	90.44	61.9	50.5	5.4	48.0

ALBUMINOIDS OR SCLEROPROTEINS.

	Keratin from ox horn (Fischer and Dorpinghaus; Morner)	Keratin from sheep's horn (Abderhalden and Voitinovici)	Keratin, from sheep's wool (Abderhalden and Voitinovici)	Keratin, from horse hair (Abderhalden and Wells)	Keratin, from goose feathers (Abderhalden and Le Count)	Keratin, from egg-membrane(Abderhalden and Ebstein; Morner)	Keratin, from egg-membrane of Testudo Graeca (Abderhalden and Strauss)	Ichthylepidin, from fish scales (Abderhalden and Voitinovici)
Glycine	0.4	0.5	0.6	4.7	2.6	3.9	+	5.7
Alanine	1.2	1.6	4.4	1.5	1.8	3.5	+ ?	3.1
Valine	5.7	4.5	2.8	0.9	0.5	1.1	.....	.....
Leucine	18.3	15.3	11.5	7.1	8.0	7.4	.....	15.1
Isoleucine	.....	.....	.....	.....	.....	.....	.....	.....
Phenylalanine	3.0	1.9	.....	0	0	.....	+ ?	.....
Tyrosine	4.6	3.6	2.9	3.2	3.6	.....	.....	1.0
Serine	0.7	1.1	0.1	0.6	0.4	.....	.....	.....
Cystine	6.8	7.5	7.3	above 10.0	.....	7.6	.....	.....
Proline	3.6	3.7	4.4	3.4	3.5	4.0	11.8 ?	6.7
Oxyproline	.....	.....	.....	.....	.....	.....	.....	.....
Aspartic acid	2.5	2.5	2.3	0.3	1.1	1.1	1.8 ?	1.2
Glutamic acid	3.0	17.2	12.9	3.7	2.3	8.1	3.0 ?	9.2
Tryptophane	.....	.....	.....	.....	.....	.....	.....	.....
Arginine	2.3	2.7	.....	.....	.....	.....	.....	.....
Lysine	.....	0.2	.....	.....	.....	.....	.....	.....
Histidine	.....	.....	.....	.....	.....	.....	.....	.....
Diaminotrioxydodecanic acid	.....	.....	.....	.....	.....	.....	.....	.....
Total	52.1	62.3	49.2	35.4	23.8	36.7	16.6 ?	42.0

Collagen. Ossein.

This albuminoid may be conveniently prepared from white fibrous connective tissue or tendons, of which it forms the chief solid constituent. The *tendo Achillis* of the ox which according to Buerger and Gies[1] contains about one-third of its weight of collagen, serves very well. After freeing the tendon from its sheath, it is cut into small pieces and thoroughly washed with frequent changes of cold water to remove soluble protein and inorganic salts as far as possible. It is then digested for several days in cold lime water to dissolve the mucoid cementing-substance between the fibres. The insoluble residue is washed with water, dilute acetic or hydrochloric acid, and lastly with water again. The collagen thus obtained contains small quantities of elastin.

A better product may be obtained by digesting carefully cleansed tendons with trypsin, which dissolve all the tissue elements except the true collagenous fibrils.

Bones also yield a collagen which enters largely into commerce under the name of *ossein*. It may be obtained by digesting bones (preferably crushed or comminuted) in cold dilute hydrochloric acid, which leaches out the calcium phosphate, etc., without affecting the ossein. After soaking in cold water to remove residual acid and calcium salts, the ossein remains as a swollen elastic mass which retains the shape of the original bone.

According to J. J. Andeer (*Compt. rend.*, 1895, **126**, 1295), an aqueous solution of phloroglucinol acts as a powerful decalcifying agent on the bones of animals, but is without action on the most delicate organic tissue. If, in addition to this, the bones are treated with hydrochloric acid, the residual ossein will contain no trace of calcium phosphate or carbonate.

Collagen must not be considered as a definite chemical substance, for its composition varies with its origin.[2] It is insoluble in water, saline solutions and dilute alkalis. In dilute hydrochloric acid it swells up to a transparent gelatinous mass without undergoing solution, and on exact neutralisation of the acid returns to its original condition. The marked insolubility of collagen in all ordinary protein solvents

[1] These authors give the following composition of the fresh tissue:

Water.....	62.87 %			Fatty substance (ether soluble).. 1.04
		Inorganic matter.... 0.47		Coagulable protein..... 0.22
Solids.....	37.13 %			Tendomucoid..... 1.28
		Organic matter.....36.66		Elastin..... 1.63
				Collagen.....31.59
				Extractives, etc..... 0.90

[2] *Abderhalden* states regarding collagen (page 149, Ed. 1906) "Es ist nach mehreren Beobachtungen keine einheitliche Substanz und dürfte bei verschiedenen Tierarten und in verschiedenen Organen ein verschiedenes sein."

is analogous to the behaviour of cellulose among vegetable substances; collagen and closely allied substances are the principal constituents of the rigid frame of animals, while cellulose and its allied substances form the chief structural tissues of plants.

Upon heating with water, collagen or ossein slowly dissolves with conversion into gelatin, the solution of which gelatinises on cooling. The conversion is facilitated by pressure, dilute acids, and proper soaking, swelling, or "plumping" the collagen, especially if it be dry. It is a mistake to speak of this process as "boiling," for if full boiling temperature is reached the gelatin first formed undergoes further change into *gelatin-peptones* or *gelatones*, the solutions of which do not gelatinise on cooling. This change also occurs at lower temperatures, but more slowly, so that in converting collagen into gelatin the operation must not be unduly protracted by too low a temperature. The most desirable temperature may vary from 70 to 90° depending upon the condition of the collagen.

The change of collagen successively into gelatin and gelatin-peptones has been described as a process of hydrolysis, and according to Hofmeister is represented by the following equations:

(1) $\underset{\text{Collagen.}}{C_{102}H_{149}O_{38}N_{31}} + H_2O = \underset{\text{Gelatin.}}{C_{102}H_{151}O_{39}N_{31}}$

(2) $\underset{\text{Gelatin.}}{C_{102}H_{151}O_{39}N_{31}} + 2H_2O = \underset{\text{Semiglutin.}}{C_{55}H_{85}O_{22}N_{17}} + \underset{\text{Hemicollin.}}{C_{47}H_{70}O_{19}N_{14}}$

In view of our present knowledge of the final disintegration products of gelatin, it is quite evident that the convenient term "hydrolysis" simply serves to conceal our real ignorance of what actually does occur. Indeed Emmett and Gies claim that the process is one of molecular rearrangement, and no hydrolysis at all. But even this gives us no definite idea of what happens. With colloidal substances like gelatin the chemical changes are so closely associated with physical changes that it is doubtful if any line of separation can be drawn between the two.

Hofmeister states that gelatin can be reconverted into collagen by heating it to 130°, which is supposed to indicate that collagen is an anhydride of gelatin. It is extremely doubtful if collagen is regenerated under these conditions, the more probable explanation being that, upon driving off the water, the constituent particles of the gelatin approach so close as to form an irreversible gel, thus rendering it insoluble.

Gelatin.[1]

Gelatin does not appear to exist ready-formed in nature, but is produced by the action of boiling water on collagen or ossein.

For the preparation of pure gelatin the best quality of the commercial article should be soaked for some days in successive quantities of cold distilled water to remove salts and soluble substances, warmed until it dissolves in the water it has absorbed, and the hot solution filtered into strong alcohol (90%). The gelatin is precipitated in white stringy masses, which are collected, redissolved in hot water, and reprecipitated by alcohol. The product contains about 0.6% of ash.

The composition of gelatin is approximately: Carbon 50.2, hydrogen 6.7, nitrogen 17.9, oxygen and sulphur 25.0. The following empirical formulæ have been ascribed to gelatin:

Schutzenberger and Bourgeois	$C_{76}H_{124}O_{39}N_{24}$
Hofmeister	$C_{102}H_{151}O_{39}N_{31}$

When, however, we consider the nature of the products of the hydrolysis of gelatin (see page 594) and also the fact that gelatins from different sources vary in composition, it will be seen that these formulæ tell us very little. For example, the "Hausmann numbers" of gelatin show its nitrogen to be distributed about as follows:[2]

Amide nitrogen	1.61
Amino nitrogen	62.56
Basic nitrogen Humin nitrogen	35.83

The wide variation in the percentage of sulphur found in gelatin, would seem to indicate that the presence of this element is due largely, if not entirely, to the presence of adsorbed impurities. Schlieper found from 0.12 to 0.14% of sulphur in gelatin from bones and ivory, and von Bibra always found very appreciable quantities of sulphur in bone gelatin. Hammarsten found 0.7% of sulphur in fine commerical gelatin yielding 1.74% of ash, and a like amount was found by Chittenden and Solley. It should be borne in mind that commercial gelatin is frequently bleached with sulphurous acid, and then contains sulphites, sometimes in considerable quantity.

The constitution of gelatin is discussed on page 596.

Pure gelatin is an amorphous, more or less transparent substance of vitreous appearance, and brittle when dry. It is free from colour, taste and smell.

When heated, gelatin softens without actually melting, swells up and

[1] Gelatin has sometimes been called *glutin*, an undesirable synonym, owing to its close resemblance to *gluten*, one of the proteins of wheat.

[2] See "The General Characters of the Proteins," S. B. Schryver, pages 34–35.

decomposes with an odour resembling that of burning hair or feathers, leaving a difficulty combustible charcoal.

When in the dry solid state, gelatin is unalterable in the air, but when moist or in solution it putrefies with extreme facility, the solution first becoming acid and subsequently ammoniacal. The formation of putrefaction-products of acid reaction is characteristic of gelatin.

When immersed in cold water, gelatin gradually swells up and softens, taking up from 5 to 10 times its weight of water without undergoing solution to any sensible extent. The amount of water absorbed is sufficient to dissolve the gelatin completely upon warming it to 30°.

In boiling water, pure gelatin dissolves readily, yielding a solution neutral to litmus. If the hot aqueous solution be very dilute, it remains unchanged upon cooling, but if somewhat stronger it becomes viscous. When the proportion of gelatin exceeds about 1%, the solution sets to a jelly upon cooling, the resiliency or consistency of the jelly formed increasing with the concentration of the liquid, but being also dependent on the origin, test, and purity of the gelatin. The jelly liquefies again completely below the boiling-point of water; repeated heating and cooling destroy the property of gelatinising, and long-continued boiling at ordinary pressure has the same effect. The gelatinising power is lost speedily by heating the solution under increased pressure at 140°.

If potassium dichromate be added to a solution of gelatin in hot water, the jelly which forms on cooling becomes insoluble in warm water after exposure to light. This fact is largely used in photo-lithography.[1]

Solutions of gelatin are eminently undiffusible; in fact, the term "colloid"—literally "glue-like"—was for this reason invented by Thomas Graham to describe such substances. From the modern colloid-chemical point of view, a warm liquid solution of gelatin in water would be classed as a *hydrosol*, or for brevity a *sol*. As pointed out by Proctor, the cold solution or jelly can hardly be called a *gel;* in fact, the term "solid hydrosol" proposed by Lottermoser appears to be more applicable. In the ultramicroscope, a hot solution of pure gelatin shows a clear field, the few ultramicrons and slight Tyndall effect being probably due to traces of impurities. According to R. Zsigmondy a

[1] The exact *modus operandi* varies materially according to circumstances. Broadly speaking, it consists in obtaining a sensitive film by pouring a solution of dichromated gelatin on to a glass plate. This is exposed under a photographic negative, and then treated with cold water. The gelatin in those parts of the film protected from the light by the opaque parts of the negative, absorb the most water and swell up above those parts affected by the light, which thus form depressions, whose depth increases with the amount of light transmitted. When inked over, the ink enters the depressions, but is repelled by the moist high lights, and on printing a reproduction of the photograph is obtained. Another method is to treat the exposed plate with warm water, which dissolves the soluble protected parts, thus forming a reproduction of the photograph in relief. This may be hardened with alum and used in a printing press, or the impression may be transferred to a lithographic stone.

0.2% solution of gelatin 2 days old appears heterogeneous. The fluid is entirely filled with small whitish particles at the limit of visibility; (about $5\mu\mu$), and with these were visible larger hydrogel particles. In a 0.01% solution the distance between the free moving particles was less than 1μ. A solution of gelatin prepared at the boiling-point showed, however, a homogeneous light-cone in the Siedentopf-Zsigmondy ultramicroscope.

Gelatin is a typical reversible, (Hardy, Zsigmondy), emulsion, (Wolfgang Ostwald), or hydrophile-colloid (Perrin). When dried out at temperatures under 100°, it can be redissolved, and this operation repeated again and again. It is also a most powerful protective colloid (Schutz-kolloid) showing the lowest gold-figure on Zsigmondy's table.[1] For this reason even traces of gelatin may act as a "colloidiser," and cause great difficulty for the analyst by entirely inhibiting the formation of precipitates, or else by causing the precipitate to form in such a finely subdivided or colloidal condition, that it will pass through the finest filter paper.[2]

Gelatin exhibits a strong lævorotation. The value of $[\alpha]_D$ at 30° is stated by Hoppe-Seyler to be $-130°$.

Reactions of Gelatin.

Gelatin is practically insoluble in ice-cold water containing 10% of alcohol. This fact is employed for its determination. In absolute alcohol, gelatin is insoluble at any temperature, and on adding excess of alcohol to its aqueous solution, the gelatin is thrown down as a white, coherent, elastic mass. No true coagulation occurs, for the precipitate has all the characters of the original gelatin, swelling up in cold water and dissolving on heating.

Gelatin is wholly insoluble in absolute alcohol, ether, chloroform, benzene, carbon disulphide, and fixed and volatile oils.

Strong acetic acid dissolves gelatin to form a solution which does not gelatinise on cooling, but yet possesses powerful adhesive properties. "Coagulin" and similar commercial preparations are of this nature. Dilute nitric acid forms a similar product soluble in cold water ("soluble glue"; compare page 621). When heated with strong nitric acid, gelatin is destroyed with formation of oxalic acid and other products.

Boiling with hydrolysing agents converts gelatin into a mixture of gelatin-peptones or gelatones (page 594).

[1] See R. Zsigmondy, "Colloids and the Ultramicroscope," page 81.
[2] See J. Alexander, *J. Soc. Chem. Ind.*, 1909, **28**, 280.

On dry distillation, gelatin gives a highly offensive oil (Dippel's oil; bone oil), containing a considerable proportion of pyridine bases, together with ammonia, permanent gases, and other ordinary products of the decomposition by heat of nitrogenised organic matters.

On adding gallotannic acid to an aqueous solution of gelatin, a white or buff-coloured precipitate is formed. A solution of gelatin in 5,000 parts of water is at once rendered turbid by gallotannic acid or other variety of tannin. Nevertheless, the precipitate is not wholly insoluble in pure water, especially if hot, but is quite insoluble in presence of excess of tannin.

The reaction of gelatin with tannin is utilised for the detection of both substances, and is employed for the assay of tannin matters. (See Vol. V, page 58). It is also utilised for tanning, the gelatinoids of the skin being thereby converted into leather.

It is misleading to speak of the combination between tannin and gelatin at *tannate of gelatin*, for the ratio in which these substances combine varies greatly with the conditions of the experiment. The precipitation of gelatin with tannin is a typical instance of the mutual coagulation of oppositely charged colloids. In aqueous solution tannin is a positively charged colloid (negatively conducted). Gelatin on the other hand is amphoteric; and as Ricevuto has pointed out (*Koll. Zeit.*, 1908, **3**, 114), it is not precipitated by tannin unless it is in the negative condition (positively conducted), *i.e.*, carefully dialysed gelatin is not precipitated by tannin. The so-called *tannate of gelatin* is therefore a variable colloid or adsorption compound, and as von Schroeder has shown the precipitation of gelatin by tannin follows the adsorption isotherm. (*Kolloidchem. Beihefte*, **1**, No. 1.) See also note on page 592.

Gelatin is not thrown down from its aqueous solution by the ordinary mineral or organic acids, by alkalies,[1] or by most metallic salts. Phosphomolybdic and phosphotungstic acids precipitate it, as does mercuric chloride (if used in excess); but no precipitate is produced by alum, ferric chloride or sulphate, salts of copper, or by neutral or basic lead acetate. Saturation of its aqueous solution by ammonium sulphate, magnesium sulphate, or zinc sulphate completely precipitates gelatin.

On adding a saturated aqueous solution of picric acid to a cold aqueous solution of gelatin, a precipitate is produced which redissolves on shaking. On gradually continuing the addition of picric acid, the

[1] Addition of sodium hydroxide or ammonia to a solution of commercial gelatin often produces a considerable precipitate of calcium phosphate.

precipitate becomes permanent; but dissolves on heating, and reappears as the solution cools. The precipitate coagulates on shaking, producing a yellow sticky mass, and leaving the liquid nearly clear (Allen and Tankard).

Solutions of silver and gold are stated not to precipitate gelatin, but by the action of sunlight some of the metal becomes reduced. Upon exposure to light a solution of gelatin containing auric chloride develops a ruby red colour, due to the formation of colloidal gold.

Platinic chloride solution reacts with gelatin in much the same way as picric acid, except that the precipitate does not appear to be so readily soluble in excess of gelatin.

Platinic sulphate solution also gives a precipitate with solutions of gelatin, which rapidly coagulates on standing, even without shaking. In other respects, the reaction of gelatin with platinic sulphate is similar to its behaviour with the chloride.[1]

L. Crismer recommends an acid solution of chromic acid as a precipitant for gelatin (compare page 588).

With the biuret test (page 39) gelatin gives a violet colouration. No precipitation of cuprous oxide occurs on boiling the liquid.

When a current of chlorine is passed through a solution of gelatin of about 1% strength, the liquid remains clear for a time, but subsequently froths strongly, each bubble becoming encased in a white pellicle. When the chlorine is in excess, as indicated by the yellow colour of the liquid, the frothing subsides, the liquid becomes clear, and the whole of the gelatin is thrown down as a white granular precipitate. When this is thoroughly washed with cold water and dried *in vacuo* over sulphuric acid, the substance is obtained as a pale yellowish-white powder, which is odourless, tasteless, and imputrescible, and insoluble in water or alcohol, but soluble in alkalies.[2]

According to Cross, Bevan and Briggs (*J. Soc. Chem. Ind.*, 1908, **27**, 260), moist gelatin when spread out very thin by immersing cotton yarn in its solution, combines with 15.4% of chlorine gas, forming what they call a gelatin chloramine. Different qualities of gelatin give approxi-

[1] According to E. Davy, platinic sulphate precipitates gelatin in the form of brown, viscous flakes, which blacken on the filter and are afterward easily pulverised. He regards the reaction as an infallible test for gelatin, applicable even in pressence of albumin and in solutions so dilute as to give no indication with tannin. The author has been unable to confirm Davy's observation.

[2] The observations described in the text are those of Rideal and Stewart (Analyst, 1897, page 228). The reaction of chlorine on solutions of gelatin was first described by Mülder in 1840, and was regarded by him as an argument in favour of his protein theory, the precipitate itself being termed protein-chlorous acid (*Berzelius' Jahresber.*, 19, 734; *Jour. f. Chem.*, 44, 489). Considerable controversy occurred as to the chemical nature of the compound, but all agreed as to its properties, its constancy of composition, and its insolubility. The proportion of nitrogen found in the compound by the early observers is in close agreement with the percentage found by Rideal and Stewart in specimens prepared by them and analysed after drying at 80°.

mately identical results. This chloramine is sensitive to "antichlors," and when treated with sulphurous acid reverts to the original condition. These authors have used the chloramine reaction as the basis of a method for estimating gelatin in tub-sized papers.

Contrary to the general statement, that bromine and iodine give no compound with gelatin similar to that yielded with chlorine, Allen in collaboration with A. B. Searle (*Analyst*, 1887, page 258) proved bromine to act in a very similar manner to chlorine, and this observation has been independently made and extended to iodine by Hopkins and Brook (*J. Physiol.*, **22**, 184). The method of operating employed by Allen is that described on page 422, but the process as applied to gelatin is not at present capable of successful employment.[1]

Gelatin forms an insoluble compound with formaldehyde, the maximum amount permanently fixed, according to Lumière and Seyewetz (*Bull. Soc. Chim.*, 1906, **35**, 872; *J. Soc. Chem. Ind.*, 1906, **25**, 1058) being between 4.0 and 4.8%, when 10% formaldehyde solution acts upon dry gelatin. Formo-gelatin is decomposed by repeated treatment with boiling water, by cold 15% hydrochloric acid, and by heating to 110°. Lumière and Seyewetz have also investigated the insoluble compound formed between gelatin and quinone (*Bull. Soc. Chim.* 1907, [iv], **1**, 428–431; *J. Soc. Chem. Ind.*, 1907, **26**, 703). They find that 100 grm. of gelatin are rendered insoluble by 1 grm. of quinone, the time varying with the concentration of the solutions. The gelatin product thus obtained has a pink colour and swells in cold water becoming elastic; but it becomes brittle again upon drying. Prolonged treatment with boiling water, or with solutions of ammonia or alkali carbonates do not affect it, but it is gradually decomposed by solutions of acids or alkali hydroxides.

The recognition of gelatin in animal fluids is attended with some difficulty. The reaction with tannin is fairly delicate, but is also given by all the proteins. The behaviour of gelatin with picric acid (page 590) is peculiar, and may be occasionally useful for its recognition. As a rule, the property of gelatinising on cooling is the only test from which the presence of gelatin in a complex animal liquid can be safely inferred.

Trillat (*Comptes Rend.*, 1898, **127**, 724; *J. Soc. Chem. Ind.*, 1898, **18**, 78) has proposed to estimate gelatin in gums and alimentary

[1] Experiments made under Allen's direction by A. R. Tankard, on various specimens of commercial gelatin and glue, yielded results which at present are incapable of interpretation. The completeness of the precipitation of gelatin by bromine-water is affected by conditions not at present understood. In some cases the precipitation was very complete, while in other experiments, in which the conditions were but very slightly varied, much nitrogen remained unprecipitated. "Previous history" of colloids affects their structure and hence their ability to form adsorption compounds.

substances by weighing the precipitate produced by formaldehyde. Henzold (*Zeits. offentl. Chem.*, 1900, **6**, 292; *J. Soc. Chem. Ind.*, 1900, **19**, 1042) proposed the following method: The material is boiled with water, and the filtrate boiled with an excess of 10% potassium dichromate solution. After cooling, if gelatin be present, a few drops of concentrated sulphuric acid produce a white flocculent precipitate, which gradually agglomerates at the bottom of the vessel.

The U. S. Department of Agriculture[1] use Stokes' picric-acid method (*Analyst*, 1907, page 320) for detecting the presence of gelatin in cream or ice-cream. The procedure is as follows: Dissolve 5 grm. of mercury in 10 grm. of nitric acid (sp. gr. 1.42) and dilute to 25 times its bulk. To 10 c.c. of this solution add 10 c.c. of cream and 20 c.c. of water, in order to precipitate all proteins except gelatin. If gelatin be present the filtrate will give an immediate yellow precipitate with an equal part of a saturated aqueous solution of picric acid.

For the detection of gelatin in preserves, A. Desmoulière (*Ann. Chim. anal. appl.*, **7**, 201; *J. Soc. Chem. Ind.*, 1902, **21**, 1157) treats 20 grm. of the preserve with 100 c.c. of 90% alcohol, added gradually, and allows the mixture to stand for 2–3 hours.[2] The liquid is carefully decanted and the residue dissolved in hot water. In the presence of gelatin, the solution gives precipitates with tannin and picric acid, and evolves ammonia when treated with calcium oxide (quicklime).

Gelatin is distinguished from albumin and its allies by not yielding a precipitate with potassium ferrocyanide or ferricyanide. Reactions distinguishing gelatin from chondrin and mucin are described on page 627.

Gelatin is very readily digested; and the fact that calves foot and similar jellies have for years been recommended by physicians as a food for invalids and convalescents, indicates that it has considerable food value. Guérard and other French investigators believed gelatin to be a very nourishing food. Voit, however, showed by feeding experiments that gelatin does not build up the body, but is broken up and used in place of the circulating albumin which is thereby economised. It thus spares both the circulating as well as the organised albumin, and accomplishes the same results as the carbohydrates and fats, only to a more intense degree.[3]

Gelatin also is a great aid in the digestion and absorption of other foods. An important instance of its value in this respect may be seen

[1] *Dept. Agri. Bur. Chem. Bull.*, 107, rev., page 121, 1910.
[2] Since gelatin is slightly soluble in 90% alcohol (*J. Soc. Chem. Ind.*, 1904, **23**, 380), somewhat stronger alcohol should be used.
[3] A. Jacobi, "Intestinal Diseases of Infancy and Childhood," 1889, Vol. I, page 64. See also J. R. Murlin, *Am. J. Physiol.*, 1907, **19**, 285; *Chem. Abs.*, 1907, 2392.

in the case of milk. The addition of gelatin to cows' milk inhibits the coagulation of the casein by acid or by rennin. In coagulating, the casein mechanically entraps the milk fat, forming a greasy curd very difficult to digest, and in preventing its coagulation, the gelatin (which acts here as a protective colloid) furthers the digestion of both the casein and the fat.[1] Recently C. A. Herter pointed out that gelatin s especially valuable in cases of bacterial infection of the intestinal tract, because it contains neither the trytophane nor the tyrosine nuclei and cannot yield indole, skatole and other objectionable products.[2]

Cleavage Products of Gelatin.—Gelatoses, Gelatin-peptones or Gelatones, Peptides, Amino-acids.

In common with other proteins, gelatin is split up or decomposed by acids, alkalis, proteoclastic enzymes and bacteria, the nature of the degradation products varying with the conditions of the experiment. Under the action of these agents gelatin loses its characteristic property of forming a jelly when its hot, tolerably concentrated, aqueous solution is cooled. The products formed by the hydrolysis of gelatin are analogous to those produced by the hydrolysis of other proteins (see page 467) and may be classified as follows:

1. Metaproteins = { acid gelatin / alkali gelatin }
2. Proteoses[3] = gelatoses
3. Peptones = gelatones
4. Polypeptides or peptides
5. Amino-acids.

} possess protein properties.

These groups are not at present very sharply defined, but in general gelatones differ from gelatoses in that they are more diffusible, not precipitated by saturation with ammonium sulphate, and give no reaction with potassium ferrocyanide and acetic acid, potassio-mercuric odide and hydrochloric acid, picric acid, and trichloracetic acid. Peptones are now known to consist of peptides or mixtures of peptides, but the term peptide is at present reserved for those possessing a recognised structure.

Gelatoses are divided into *protogelatoses*, which are precipitated upon the addition of an equal volume of cold-saturated ammonium sulphate, and *deuterogelatoses* which are precipitated only upon saturation with

[1] See J. Alexander, *Z. Chem. Ind. Kolloide*, 1909, 5, 101; 1910, 6, 197; *J. Am. Chem. Soc.*, 1910, 32, 680.
[2] See C. A. Herter, "Infantilism," 1908, page 96.
[3] Proteoses are sometimes called "albumoses," a term that should be reserved for the proteose of albumin.

this salt. Two distinct gelatones are recognised by Hofmeister, who terms them respectively semiglutin and hemicollin (see page 473). Semiglutin is sparingly soluble in 70 to 80% alcohol, and is precipitated by platinic chloride, whereas hemicollin is soluble in 70 to 80% alcohol, and is not precipitated by platinic chloride.

C. Paal (*Ber.*, **25**, 1202) has shown that when gelatones[1] are formed by the action of dilute mineral acids on gelatin they unite with the acid to form salts, which are not only soluble in water, but, unlike the free gelatones, in ethyl and methyl alcohols. For their preparation, Paal warms 100 parts of the purest commercial gelatin on the water-bath with 160 parts of water and 40 of concentrated hydrochloric acid, until a sample of the product is completely soluble in a large quantity of absolute alcohol. The whole is then poured into 4 or 5 volumes of absolute alcohol, the precipitated salts filtered off, the filtrate treated with ether, the precipitate redissolved in alcohol, and the resultant solution evaporated under reduced pressure. The gelatone hydrochloride thus obtained forms a brittle, white, vesicular mass, which is readily soluble in water, methyl alcohol, ethyl alcohol, and acetic acid; but is insoluble in ether, benzene, or carbon disulphide. It is very hygroscopic, is unaltered at 130°, and gives a violet colouration with the biuret test. It is lævorotatory, the value of $[a]_D$ in aqueous solution being about $-60°$. The proportion of hydrochloric acid found in different preparations ranged from 10.5 to 12.5%, while the ash was only about 0.5%. When stronger hydrochloric acid was employed, or the heating continued longer, compounds containing a higher percentage of acid were obtained. This fact tends to show that the products are a mixture of compounds representing different degrees of peptonisation. The hydrochlorides containing high proportions of

[1] P. A. Levene (*Zeit. für Physiol. Chem.*, 1904, 41, 10) prepared gelatin peptone by dissolving gelatin in 10 times its weight of 0.5% sodium carbonate, adding 0.1% of trypsin with a little toluene as preservative. After several months standing the solution was concentrated, and the traces of proteose still remaining removed with ammonium sulphate, which was then dialyzed out. The gelatone was precipitated from the acidified solution by phosphotungstic acid, and the precipitate washed with boiling water to remove hexone bases, until the wash water gave no precipitate upon standing. The residue, which formed a hard stone-like mass on cooling, was dissolved by rubbing in a little ammonia, the sulphuric and phosphotungstic acids removed, and the ammonia evaporated. The gelatone was further purified by washing with absolute alcohol and ether, the former of which dissolved considerable of it. The peptone insoluble in alcohol was a white very hygroscopic powder.

The results of the analysis of Levene's peptone, as compared with those prepared by Siegfried, and by P. Tartarinoff (*Compt. rend.*, 97, 713) are given in the following table:

	C	H	N	Difference
Levene	45.96	6.71	17.93	29.40
Siegfried	46.76	6.23	17.32	29.69
Tartarinoff (average)	49.76	7.13	12.63	30.48

acid are more diffusible and soluble than those containing small percentages of hydrochloric acid. Thus, by dialysing a salt containing 10.56% of acid, Paal obtained from the diffusate a compound containing 14.19% of hydrochloric acid, whereas the residue contained only 5.79% and was insoluble in absolute alcohol.[1]

A similar separation was effected by treating the alcoholic solution with mercuric chloride, whereby two mercurichlorides were obtained. One of these separated out at once, and the second on addition of ether.

On warming gelatin with a weaker acid than was used in the foregoing experiments a salt was obtained which contained 6.85% of hydrochloric acid, and was insoluble in ethyl alcohol, but soluble in methyl alcohol; while the product obtained by means of pepsin and very dilute hydrochloric acid was not completely soluble in cold methyl alcohol, and could be separated by dialysis into two fractions, one of which contained 2.97% of hydrochloric acid and was almost insoluble in methyl alcohol, while the other contained 11.13% of hydrochloric acid.[2]

In order to prepare the free gelatones from their hydrochlorides, a slight excess of silver sulphate should be added to the solution, the excess of silver removed from the filtrate by hydrogen sulphide, and the sulphuric acid by an equivalent amount of baryta-water. The gelatones thus obtained are soluble in all proportions in water, but are insoluble in alcohol or ether. Their aqueous solutions are acid to litmus, but do not turn congo-red to blue. The percentage of carbon in gelatones is somewhat less and that of hydrogen rather higher than in gelatin, which fact is in agreement with the view that they are products of the hydrolysis of the latter substance. Paal considers that the gelatin-molecule is resolved by hydrolysis into gelatones of gradually decreasing molecular weight, till a point is reached at which they are split up with formation of amino-acids, lysine, lysatine, etc., (page 467); and that, as the molecule of gelatin, like that of albumin, consists of two protein atom-complexes which suffer hydrolysis with different degrees of facility (the salts containing a high proportion of acid being the more readily converted into amino-acids and other non-protein products), the sim-

[1] It was found that only those salts containing 10% or upward of hydrochloric acid were soluble in absolute alcohol, and those fractions which were only just dissolved by ethyl alcohol were much less soluble in propyl alcohol, and insoluble in amyl alcohol; while the salts with less than 10% of acid were all readily soluble in methyl alcohol, and dissolved also in the ethyl alcohol solutions of the salts containing more acid.

[2] From determinations by the cryoscopic method in aqueous solution, and by the boiling-point method on solutions of gelatone hydrochlorides in water, methyl alcohol, and ethyl alcohol, Paal concludes that the molecule is less as the percentage of acid increases, the molecular weight of the free gelatones being about 300 in 3 cases, and 215 in a fourth; while gelatin itself has a molecular weight of about 900. (Hofmeister's formula for gelatin, $C_{102}H_{161}O_{39}N_{31}$, corresponds to a molecular weight of 2433.) Paal's results further show that the gelatone hydrochlorides are stable in solution in ethyl alcohol, but that when in solution in water or methyl alcohol they are dissociated into equal molecules of gelatone and hydrochloric acid.

pler products of decomposition are always mixed with unaltered gelatones.

In a later paper (*Ber.*, **31**, 956), Paal states that the hydrochlorides containing the lower proportions of acid are precipitated by saturating their solutions with ammonium sulphate, and hence are salts of *gelatoses*, analogous to the proteoses (page 594). The portions not precipitated by ammonium sulphate consist of salts of gelatin-peptone or *gelatone*. On the average, these latter salts contain, when anhydrous: ash, 0.18 to 0.58%; hydrochloric acid, 10.38 to 13.14; C, 43.28 to 45.95; and H, 6.43 to 7.13%. By dialysis, Paal effected a further fractionation of these salts. Thus from the diffusate of a hydrochloride which was hardly soluble in ethylic alcohol, but which dissolved readily in cold methyl alcohol, Paal obtained a yield of more than 60% of the original substance, containing: ash, 0.89; HCl, 13.44; and carbon and hydrogen corresponding to C, 48.86, and H, 7.31% in the free gelatone. On dialysing this last product into water, a further separation was effected, the portion diffusing during the first 24 hours being an extremely hygroscopic yellow mass, which was very soluble in alcohol, contained 16.06% of hydrochloric acid, and yielded 1.21% of ash on ignition. The portion which diffused during the second period of 24 hours was much less soluble in alcohol, less hygroscopic, and contained only 9.38% of hydrochloric acid.

Paal finds these salts to be partially precipitated by phosphotungstic acid, with liberation of the gelatone, while the unprecipitated portion gives a distinct biuret reaction.[1] By treating the phosphotungstic precipitate with barium hydroxide in excess, a *barium-gelatone* was obtained; and this reacted with ferrous sulphate, yielding an aqueous solution of *ferro-gelatone*. This latter substance absorbed atmospheric oxygen, especially on warming, forming free gelatone, which remained in solution, and ferric hydroxide, which was quantitatively precipitated.

Paal finds that when gelatone salts are dissolved in absolute alcohol and the solution saturated with hydrochloric acid gas, while warmed on the water-bath under a reflux-condenser, merely a trifling increase occurs in the amount of combined hydrochloric acid, further peptonisation only taking place in presence of water.[2]

Chittenden and Solley (*J. Physiol.*, **12**, 23; abst. *J. Chem. Soc.*, 1891, 949) have investigated the products of the digestion of gelatin by the gastric and pancreatic enzymes. They obtained three distinct

[1] Paal attributes this result to the formation of products intermediate in nature between peptones and amino-acids.

[2] C. Paal has continued his researches on the *salts of gelatone* (*Ber.*, 31, 956; abst. *J. Chem. Soc.*, 1898, I, 456).

products, two of which are related to the albumoses, and the third to Kühne's albumin-peptone. The two former substances, *protogelatose* and *deuterogelatose*, are formed both in gastric and in pancreatic digestion, and are distinguished from the third product, which is a true gelatin-peptone or *gelatone*, by being (like albumoses) precipitated on saturating the liquid with ammonium sulphate. Protogelatose is partially precipitated by saturating its neutral solution with sodium chloride, and is completely precipitated on subsequently adding a little acetic acid. Protogelatose yields a heavy precipitate with hydrogen platinichloride. Deuterogelatose is not precipitated by either of these reagents. By further ferment action, protogelatose is converted into deuterogelatose, and finally into gelatin-peptone or gelatone. No trace of heterogelatose was obtained. Chittenden and Solley give the following analytical figures representing the percentage composition of the gelatin employed, and of the gelatoses obtained:

	Gelatin used	Products of gastric digestion		Products of pancreatic digestion	
		Proto-gelatose	Deutero-gelatose	Proto-gelatose	Deutero-gelatose
Carbon	49.38	49.98	49.23	49.45	49.07
Hydrogen	6.81	6.78	6.84	6.61	6.66
Nitrogen	17.97	17.86	17.40	17.81	17.52
Sulphur	0.71	0.52	0.51	0.57	0.65
Oxygen	25.13	24.86	26.02	25.56	26.10
	100.00	100.00	100.00	100.00	100.00
Ash	1.26	1.98	1.08	1.75	1.08

P. A. Levene (*Zeit. f. physiol. Chem.*, 1902, **37**, 83) obtained, by digesting gelatin with papain, gelatoses whose composition was substantially the same as those given above. Levene further found the gelatoses contained more glycine (amino-acetic acid) than the original gelatin, and what was still more surprising (*ibid.*, 1904, **41**, 8) more than the gelatin peptone. This was, however, due to the fact that the glycine remains in solution upon the precipitation of the peptone by phosphotungstic acid.

Bacterial Decomposition of Gelatin.

The products of the decomposition of gelatin under the action of bacteria have been studied by Rideal and Stewart (*Analyst*, 1897, **22**,

255). The following is a tabulated summary of the results obtained, the figures being grams per 100 c.c. of the liquid:[1]

	Total nitrogen	Gelatin and albumoses	Albumoses	Ammonia and volatile bases	Bases and extractives	Peptone N.unaccounted for	Gelatin	Albumin and cellulose
Original gelatin.....	1.242	1.109	0.514	0.003	0.061	0.069	0.595	.....
Original gelatin.....	1.246	.....	0.528	0.004	.....	.....	0.581	.....
After incubation at 20 to 21° with—								
B. prodigiosus, 14 days	1.232	1.024	0.409	0.049	.....	.....	0.615	.....
B. fluor. liquefaciens, 1 day.....	1.33	1.141	0.402	0.028	.....	.....	0.739	.....
B. fluor. liquefaciens. 2 days.....	1.256	0.682	0.297	0.045	0.105	0.424	0.385	.....
B. fluor. liquefaciens, 3 1/2 days.....	1.267	0.242	0.135	0.063	0.486	0.476	0.107	.....
B. fluor. liquefaciens, 16 days.....	1.26	0.216	0.081	0.168	0.742	0.124	0.135	0.01

It will be noticed that the original dry gelatin contained roughly 50% of matter precipitable by Stutzer's reagent (so-called albumoses). In the early stages these albumoses seem to have been first attacked by the organism; but subsequently their decomposition proceeded at an equal rate with that of the true gelatin.

A notable feature is the small production of ammonia and volatile bases, in view of the fact that substances such as trimethylamine, indole, and skatole have been generally stated in analyses by other observers to be constant accompaniments of the liquefaction of proteins.[2]

The figures for the nitrogen unaccounted for, which would be almost entirely peptones, are interesting. The original gelatin solution contained 0.069 grm. per 100 c.c., but this gradually increased as the liquefaction proceeded, until, with *B. fluorescens liquefaciens*, it reached the maximum figure of 0.476, when the medium was just entirely liquefied, and then diminished, after 16 days, to 0.124, showing that the bacillus, after bringing about the decomposition of nearly all the gelatin and albumoses, subsequently attacked the peptone, which had meanwhile

[1] The gelatin and albumoses were determined by precipitating the solution with saturated ammonium sulphate solution, dissolving the precipitate in warm water, and dividing. The total nitrogen was determined in one-half of the solution, and in the other, very exactly, the SO_3, the nitrogen due to the corresponding ammonium sulphate being deducted from the total ammonium sulphate.

The albumoses were determined separately by precipitating a portion of the original solution with Stutzer's reagent, washing, and estimating the nitrogen in the precipitate.

The volatile bases were determined in a portion of the original liquid by distillation with caustic soda and titration of the distillate. A further portion of the original was precipitated with alcohol, filtered, and the nitrogen-content of the filtrate regarded as existing as bases and extractives.

[2] That this was not due to the volatilisation of such bodies during the incubations was proved by the comparative absence of odour and of strong alkaline reaction in the air of the flasks, and in such loss not being indicated by the total nitrogen. Previous investigations by Rideal and Stewart with proteins showed that the quantity of ammonia produced was insignificant, amounting, even after 16 days' incubation with *B. fluorescens liquefaciens*, to only 0.168 grm. of nitrogen per 100 c.c., corresponding to 0.204 grm. of ammonia.

been produced, with a corresponding increase of bases and extractives soluble in alcohol.

It is known that certain bacteria produce peptonising enzymes, and in the case of the *B. fluorescens liquefaciens* the organism appears, as shown above, to have subsequently fed on the peptone produced by its enzyme.

Formogelatin.

On adding a solution of formaldehyde—such as commercial formalin—to an aqueous solution of gelatin, no change occurs unless the gelatin solution be very concentrated or contains free alkali, but on evaporating the liquid to dryness, the gelatin is converted into an insoluble substance known as formogelatin. To effect complete conversion of the gelatin into this substance, any free acid should be previously neutralised by agitation with precipitated calcium carbonate or other means. On treating the residue left on evaporation with boiling water any trioxymethylene formed from the formaldehyde is dissolved, while the formogelatin is unaffected.

From the small proportion of formaldehyde required to produce formogelatin, it is very doubtful whether that substance is a definite compound. Acrylic aldehyde is stated to yield a similar product, but acetic aldehyde only reacts in the absence of water.

Formogelatin is quite insoluble in either cold or boiling water, but is completely dissolved by treatment with diluted sulphuric acid (1.34 sp. gr.) for 12 hours, whereas the corresponding compound from casein (page 125) remains undissolved.

According to E. O. Beckmann (*Chem. Centr.*, 1896, **2**, 930) the same insoluble substance is obtained by the action of formaldehyde on gelatin which has lost its power of gelatinising by prolonged heating,[1] but he states that gelatin-peptone is not similarly acted on. "Albumin-peptone" and "tryptone" are also said to be unaffected. Beckmann based on these facts a method for the determination of gelatin in meat-extracts.

According to A. Zimmermann, Methylene-blue colours formogelatin but not ordinary gelatin.

Formalingelatin has received several practical applications. Schrö-

[1] To prepare an insoluble film of formogelatin from altered gelatin, A. Zimmermann (*Eng. Patent*, 1894, No. 23585) dissolves 75 grm. of gelatin in 500 c.c. of water, and boils the liquid for 2 days, replacing the water as it evaporates. He then adds 4.2 grm. of commercial formalin, containing 40% of formaldehyde. The liquid remains clear for a prolonged time, but on evaporation yields insoluble formogelatin. Gelatin altered by boiling with dilute acids or alkalis acts in the same manner.

der prepares it (abst. *Pharm. Jour.*, 1896, **2**, 63) by adding 2% of commercial "formalin" (40% aqueous solution of formaldehyde) to a warm solution of gelatin in its own weight of water. The liquid is stirred, the resulting mass covered with "formalin," and allowed to stand for some time. The product is then powdered, well washed with water, and dried. H. K. van Vloten (*J. Soc. Chem. Ind.*, 1896, page 553), who prepared the compound in a somewhat similar manner, states that powdered formogelatin can be introduced into wounds without producing irritation.

The following method has been proposed by G. Romijn (abst. *J. Soc. Chem. Ind.*, 1896, **15**, 679) for the detection of unaltered gelatin in formogelatin. About 0.5 grm. of the sample is mixed with 10 c.c. of water and heated for 10 minutes in the water-bath with frequent shaking. The mass swells considerably, but particularly so in the presence of unaltered gelatin, which goes into solution. If the liquid is now passed while hot through a dry filter, it will gelatinise on cooling if unaltered gelatin be present. The separation of the gelatin may be accelerated by placing the filtrate in a freezing mixture and allowing it to melt slowly.

0.5 grm. of the sample is mixed with 5 c.c. of water and 1 c.c. of sodium hydroxide solution. The mass swells considerably, but much more strongly in the presence of unaltered gelatin. If now a mixture of 2 c.c. of normal silver nitrate solution and 0.5 c.c. of ammonia be added, the mass should begin to darken within 1 or 2 minutes, and after 5 or 10 minutes the liquid should have acquired a violet-brown colour. If much free gelatin is present the colour makes its appearance more slowly, and assumes a pure brown shade, without any violet. Pure gelatin does not produce any colouration until after the lapse of a few hours.

Glue and Gelatin.[1]

It is not easy to draw a sharp line of demarcation between glue and gelatin. Broadly speaking, glue is impure, dark coloured, or low test gelatin, and usually contains considerable quantities of "chondrin," gelatoses, and gelatones. Gelatin is distinguished from glue by its purity, light colour, clearness, and high jelly strength.[2] The same raw material may yield glue or gelatin, depending upon the skill and

[1] An extensive bibliography of glue and gelatin, including more than 150 references, was given by Dr. Rudolph Ditmar, in the *Zeits. für Chemie und Industrie der Kolloide*, 1906, **1**, 80.

[2] The French recognise a class of gelatin-glues (*colles gelatines*) intermediate between gelatin and glue.

care bestowed upon its manufacture.[1] A sharp distinction should be made between *technical gelatins* intended for use in manufacture and the arts, and *food gelatin* intended for alimentary purposes. (See page 616.)

In the manufacture of glue and gelatin, the raw material or "stock," after being cleaned and "plumped" by appropriate treatment (see below), is subjected to the action of boiling water or steam. The resulting liquor[2] or "soup" is skimmed to remove fatty matters, bleached, clarified, filtered and evaporated if desired, and then run into galvanised pans, or on water-cooled slabs, where it sets to a jelly. The jelly is cut into sheets, which are spread on nets of galvanised wire or fish cord, and dried in a current of warm dry air circulated by fans.

The chief sources of gelatin and glue are bones, sinews, and skins or hides.[3] It is frequently but erroneously stated that glue is made from hoofs and horns, but these consist of keratin and yield no glue. The bony support of the horn, or horn-pith, yields a particularly fine gelatin.

Most hide stock consists of trimmings, skivings and fleshings from tanneries; besides these, commercial hide stocks include rawhide cuttings, rabbit skin shreds from makers of hatters felt, calves pates, and occasionally worn out bits of rawhide, such as loom-pickers and Turkish moccasins. Various parts from the calf, cattle, goat, sheep, rabbit, pig, and horse each yield characteristic glues, and are valued about in the order given.

The treatment of the stock depends upon the technique of the particular factory, and the use for which the finished glue is intended. Bones may be boiled without even washing, but usually the grease is first steamed out, or else removed by volatile solvents. The clearest and best bone glues are obtained by leaching the bones with dilute acid,[4] which dissolves out the lime salts and leaves the collagenous matter. Such crushed leached bone enters commerce under the name of *osseine*. Hide stock, sinews, and leached bone are soaked in dilute

[1] Stout consommé, which sets to a jelly when cold, although a food product, would yield on drying only a low-grade glue.

[2] The liquor thus obtained is known as a "run" or "boiling." Since prolonged exposure to heat causes hydrolysis, and injures the quality, it is customary to make a number of successive runs or boilings, which may be combined or kept separate as desired. As a rule, first runs are best both in colour and strength, second runs next, and so on. (See H. J. Watson, *J. Soc. Chem. Ind.*, 1904, 23,, 1189.)

[3] Glues made from bones are called bone glues. That made from hide should be called hide or skin glue, though it is sometimes called *leather glue*, which is a misnomer, because leather yields no glue. Patents have however been taken out for de-tanning leather and making glue from the recovered hide. Besides the two classes referred to, the Germans recognise a class of mixed glue (Misch-leim), made from a mixture of bone and hide stock.

[4] Hydrochloric acid is most generally used. In the Grillo and Schroeder process (*Eng. Pat.* 2175 (1894). *J. Soc. Chem. Ind.*, 1894, 13, 408), sulphur dioxide is passed over the bones, which absorb 11–12 % with the formation of insoluble $CaHPO_4$ and $CaSO_3$. Bones so treated are readily disintegrated by water at 100°, the gelatin passing into solution.

lime water until sufficiently swollen or "plump," and after washing, the last traces of alkali are neutralised with acid. Sulphurous acid is frequently used as a bleach.

Glue and gelatin occur in commerce in a great variety of forms (sheet, flake, shred, ground, powdered, etc.). While gelatin is usually colourless or else clear pale-yellow or brown, glue varies in colour from milk-white to almost black, and all possible shades of brown and yellow, transparent or opaque.

Although appearance[1] is no certain criterion of quality, glue is often erroneously judged by its colour, clearness, fracture, etc. Since glue is used for a great variety of widely differing purposes, the *use for which the glue is intended* should always be borne in mind when subjecting it to test or technical examination. *A number of tests*[2] will, therefore, be given, *which may be conveniently run in consecutive series*,[3] and their practical significance will be pointed out in a few instances.

Thin blown glasses about 3 1/2 in. high and 2 3/8 in. in diameter are convenient for making tests. 25 grm. of each glue to be tested is broken into small pieces and soaked in 100 c.c. of cold water until thoroughly softened. Thick sheet or flake glues must be soaked over night and should be allowed to stand in a cool place. With the glues under examination, there are at the same time soaked up a number of glues of known strength (standards) for tests of glue should always be comparative. It is desirable and convenient to use the standards hereafter described. (See page 608.) With high test glues, or in cold weather, less glue may be used, providing the unknown glues and the standards are treated exactly alike. In warm weather low test glues must sometimes be tested 30 to 100. Gelatins are usually tested 10 to 100, or even 3 to 100.

When the glues are thoroughly softened, the glasses are immersed in a water-bath, and their temperature raised to 80° with constant stirring to insure complete solution. With thick cut glues, care must be taken

[1] Glue in small oblong cakes (3 in. × 6 in.) is known as *Cologne glue*. *Scotch glue* is dark reddish-brown, in large oblong sheets. *French glue* is generally about 9 in. square, and is frequently stamped with a trade-mark (*medal glue*). Glue opaqued with oxide of zinc is often called *Russian glue*, although in America thin-cut opaques are commonly known as white-shell glues. *Ribbon glue* comes in thin strips about 2 in. × 6 in., and *noodle glues* in thicker and narrower pieces. The dark, heavy noodle glue, popular in Eastern countries, is called *Bazaar glue*. The form in which a glue is dried or cut, does not of course affect its quality.

[2] E. G. Clayton (*J. Soc. Chem. Ind.*, 1902, 21, 670) at the conclusion of a paper on the technical examination of glue, says "while it would be rash to form a judgment on glue from a single test, the evidence afforded by a number may be irresistible. The expert's wisest system appears to be, not to rely upon single short-cut tests of general quality, but to employ a number of methods, including any having especial bearing on the prospective or present uses of the glue, and then base his conclusions on a consideration of all the results together."

[3] See J. Alexander, *J. Soc. Chem. Ind.*, 1906, 25, 158. A typical test sheet, showing results of this series of tests, is given on page 604.

No. 1496
Dec. 15th.

LABORATORY TEST SHEET.[1]

20 Grm. Glue to 100 c.c. Water. Soaked 16 hours.

Marks	Visc.	Swt.	Grse.	Foam	Alk.	Acid	C. Set.	Sngth.	Jelly	Reaction
1. Low-grade hide glue	17 3/4	1	2	1	2 1/2	1	14	58	Separates.	
2. Medium-grade hide glue	19 3/4	1	3	1	2	1	10	82	Opaque.	
3. Medium-grade hide glue	20 1/4	1	2	1	1	1	5	102		
4. High-grade hide glue	24	1	1 1/2	1	1	1	3	140		
5. High-grade hide glue	27	1	1	1	1	1	1	160		
6. Low-grade bone glue	16 1/2	1	1	3	1	1 1/2	18	34		
7. Medium-grade bone glue	17	1	1 1/2	1	1	2	11	76		
8. Medium-grade bone glue	18 1/2	1	2	1	1	1	8	90		
9. High-grade bone glue	17 3/4	1	1	1	1	2	4	110		
10. Second run Osseine stock	21	1	1	1	1	1	2	150		
11. Third run Osseine stock	18 3/4	1	1	1	1	1	6	104		
12. German oblong sheet (bone)	16	1	1	2 1/2	1	1	16	48		
13. English oblong sheet (bone)	16 1/2	1	1	1 1/2	1	1	12	60		
14. Dutch oblong sheet (bone)	16 3/4	1	1	2	1	1 1/2	13	56		
15. French square sheet (bone)	16	1	1	2	1	1 1/2	17	50		
16. Italian square sheet (bone)	17	1	1	1 1/2	1	2	15	52		
17. German "Lederleim"	18 3/4	1	2	1	1	1	9	80		
18. English "skin glue"	18 1/2	1	1 1/2	1	1 1/2	1	7	90		

This column used to describe the jellies when desired

This column used to receive litmus-paper strips

[1] For the sake of brevity, figures are adopted to describe the various characteristics:
1 = perfect or free; 2 = slight; 3 = considerable; 4 = much; 5 = very much.

With each test must be run a sufficient number of standard glues to permit the proper grading or interpolation of the unknown glues.

This test-sheet form is the one that has for many years been used in the laboratory of the National Gum and Mica Co., of New York (owners of National Glue and Gelatine Works). Thanks are due them for permission to publish this and other information contained in this section.

to see that no undissolved pieces stick to the bottom of the glass. The following tests are then made in the order given.

Reaction.—This is determined with strips of litmus paper.

Where the degree of acidity or alkalinity is desired, a separate titration is made.

Viscosity or Running Test.[1]—The viscosity is taken by running the hot glue solution at 80° from a pipette, and noting the time of efflux by a stop-watch. The relative viscosities are thus fixed in seconds.

The pipette adopted as standard has the following dimensions:

Capacity	45 c.c. of water at 80°
Internal diameter of effluent tube	6 mm.
External diameter of effluent tube	9 mm.
Length over all of effluent tube	7 cm.
Smallest diameter of outlet (about)	1.5 mm.
Outside diameter of bulb	3 cm.
Length of bulb	9.5 cm.
Length of upper tube	22 cm.

This pipette should permit the efflux of 45 c.c. of hot water at 80° from the glue bath in exactly 15 seconds. The viscosities of glues vary widely, as may be seen from the table of standards on page 609.

Great care must be taken to make 2 pipettes that will give concordant results. The size and shape of the outlet hole, and the length and diameter of the effluent tube are the chief factors controlling the time of delivery. The efflux hole is made by cutting the effluent tube square across, and holding it vertically in a Bunsen flame with constant rotation. As the glass softens, the hole gradually draws together, and after a few trials can be brought to the desired size. It is desirable to have the lower graduation point just where the effluent tube joins the bulb, for otherwise in glues of high viscosity there is much uncertainty caused by dribbling of the last few drops.

While running the pipette may be kept in a simple thermostat, consisting of a water-bath specially made for the purpose. The projecting effluent tube is protected by a mica cylinder through which the end point is observed. When the thermostat is used a small piece of rubber tubing controlled by a pinch cock is slipped over the upper end of the pipette, or a glass stopcock may be fused on.

After each determination, the pipette is washed out with hot water from the glue bath, and care must be taken that no undissolved glue, glue "skin," slime, or other obstruction clogs the outlet, even momentarily during use.

[1] This is sometimes known as Fels' test. As a rule, bone glues show lower viscosity than hide glues of equal jelly strength.

More complicated viscosimeters, as Engler's[1] (see *J. Soc. Chem. Ind.*, 1890, **9**, 654) or the Rideal-Slotte (see *J. Soc. Chem. Ind.*, 1891, **10**, 615) may be used, but they are cumbersome and slow of operation, and therefore impractical in routine work, where many determinations are made.

Odour.—To an experienced nose, the odour of the hot glue solution usually gives some indication of the raw material from which it is made. Decomposition is readily detected, although it is frequently masked by antiseptics or ethereal oils. Glues are rated as "sweet" or "off" as the case may be. With food gelatins, freedom from odour is highly desirable.

Grease.—A brush-full of the glue solution is mixed with little aniline or other colour, and painted out on a piece of white paper, when spots or "eyes" appear roughly proportionate to the amount of grease present. The grease is recorded arbitrarily on a comparative basis. For an exact estimation of fat in glue, etc., Kissling[2] dissolves 20 grm. of the sample in 150 c.c. of water containing 10 c.c. of hydrochloric acid of 1.19 sp. gr. The liquid is heated for 3 or 4 hours on a water-bath under a reflux-condenser. The solution is cooled, 50 c.c. of petroleum-ether added, the liquid well shaken, and, after standing until clear, a known measure of the solvent is drawn off, evaporated, and the residue weighed.

Foam.—Foam is determined by agitating the glue solution with a rod or mechanical agitator, and like grease, is recorded comparatively. Some of the conditions affecting the foaming of glues are discussed by Trotman and Hackford (*J. Soc. Chem. Ind.*, 1906, **25**, 104) and H. J. Watson (*ibid.*, page 209).

Comparative Set.—The glasses are taken from the water-bath, and note is made of the comparative set or order in which the solutions gelatinise. In warm weather, especially with glues of low jelly strength, the glasses must be allowed to stand in ice water. As a rule, glues and gelatins gelatinise in order of their jelly strength.

Jelly Strength or "Finger" Test.[3]—When the glue solutions have gelatinised, the glasses are arranged in order of the strength or resiliency of the jellies, as determined by pressure with the finger tips. Notwithstanding the personal equation, expert operators obtain much more uniform results than are given by the various mechanical devices.

[1] J. Fels (*Chem. Zeit.*, 1897, **21**, 56 and 70; *J. Soc. Chem. Ind.*, 1897, **16**, 264) proposed to take the viscosity of a 15% solution at 30° with Englers' viscosimeter; later (*J. Soc. Chem. Ind.*, 1901, **20**, 139) he advised using 35°. In practical use, however, glue solutions are usually much warmer and more concentrated.

[2] R. Kissling, *Chem. Zeit.*, **11**, 691, 719; abst. in *J. Soc. Chem. Ind.*, 1887, **6**, 565.

[3] This is sometimes known as Kissling's test.

The unknown glues naturally group themselves as stronger or weaker than the several standards, and if the proper selection of standards has been made, the unknown glues can, if necessary, be graded in between the standards (see table on page 609).

Lipowitz (*Neue Chem. tech. Unters.*, Berlin, 1861, page 37) first proposed the relative consistency of the jelly and its capacity for bearing a weight, as a test for the value of a glue. J. Fels (*Chem. Zeit.*, 1897, 56 and 70) described this as "correct as a comparative method."

The value of Lipowitz' method as a practical test of the quality of glue has also been confirmed by Heinze. The instrument used is made as follows: A saucer-shaped piece of tinned iron, 1 in. in diameter, is soldered in its concave side to a stout iron wire having a small tin funnel fixed to the other end. The arrangement is placed vertically in the beaker so that the saucer rests on the jelly, and the whole is supported by a slip of metal placed across the top of the beaker. By placing *shot* in the funnel a gradual increase of weight on the jelly is produced, until the saucer breaks through. The weight it is found necessary to use to rupture the jelly is a measure of the tenacity of the glue.

Instruments of this type are inaccurate for reasons that will be given below. They have, however, been largely used, and since shot has generally served for the added weight, the test has been known as the "*shot test.*" One modification of the "shot test" is based upon the time required for rods of different weight to sink a certain distance in the jelly. Another modification, perhaps the best, consists in determining the weight required to produce a certain compression of the jelly in the glass, without breaking it. In this modification, the upright rod in moving, actuates a rotary pointer, and when this reaches a certain point on its scale, the weight resting on the glue indicates the jelly strength; or the motion produced by a definite weight is recorded.

On a somewhat similar principle to this last modification is the apparatus devised by E. S. Smith (U. S. Pat. 911277; see *J. Soc. Chem. Ind.*, 1909, **28**, 252) for measuring jelly strength. It consists of a pressure chamber one side of which is closed with a thin elastic rubber diaphragm, a rubber bulb to produce pressure, and a manometer to measure the pressure produced. Upon compression of the bulb, the diaphragm displaces the jelly, and the pressure required to produce a certain displacement is taken as the jelly strength.

All forms of apparatus which depend upon the breaking or compression of the jelly in glasses, are subject to two sources of error. The glasses usually vary in diameter, thus forming a surface of variable area, and there is always formed a skin of greater or less thickness,

which interferes with the accuracy of the test. To escape these difficulties, J. Alexander (see U. S. pat. 882731; *J. Soc. Chem. Ind.*, 1908 **27**, 459) devised a jelly tester to determine the resiliency of jelly blocks free from containing walls, the skin being placed at the bottom. It consists of a brass cylindrical vessel supported like a gas tank by four vertical rods, against which it slides with almost frictionless roller bearings. This brass cup is allowed to rest on a truncated cone of jelly of definite size and composition, and at a definite temperature; and shot are gradually poured into the cup until a definite compression of the jelly is observed. Beneath the cup are two vertical adjustable brass uprights 3.5 cm. high, connected with an electric bell circuit; so that when the cup reaches their level, it completes the circuit and the bell rings.

The weight of the brass cup plus the weight of the added shot, gives a figure that expresses the jelly strength.

The jellies were cast in round brass cups 6 cm. high, 5.5 cm. in diameter at the open top, and 5 cm. at the bottom, which is closed with a tight-fitting external friction cap. If the jellies do not push out readily on removing the cap, the covered cup may be dipped for an instant in hot water, taking care that no material loss results. The liberated truncated cones should be exactly 4.5 cm. high, the cups being filled only to that level. They are placed in a thermostat until they reach the desired temperature.

This instrument was used in fixing the following standards, which cover the range of jelly strengths found in commercial glues and gelatins.

Standards.—The selection of standards is of great importance, for once they are taken, all unknown glues are measured by them. Most published results of glue tests cannot be compared with each other, because of the great diversity of methods and glues used, and the absence of standards. That definite standards of jelly strength will simplify and harmonize the grading of glue is self-evident.

Sixteen arbitrarily established, nearly equidistant *grades*, cover the usual range of jelly strengths. The grades are given values from 10 to 160, thus allowing 10 points between each grade. The following table gives the viscosities (as determined by the standard pipette, see page 605), and the jelly strengths (as determined by Alexander's jelly tester, see above) of these 16 standards. The determinations were made on solutions of the glues containing 25 grm. of glue to 100 c.c. of water. For the standard viscosities there are taken the average of laboratory tests extending over many years, and inasmuch as standards of the same

jelly strength are apt to vary in viscosity, there is indicated in the table the reasonable limit of such variation. Glues often greatly exceed these limits, but should not be chosen as standards.

Standards	Viscosities (in seconds) at 80° solution 25–100	Allowable variation of viscosities (in seconds)	Jelly strength (in oz.) at 10°	Jelly strength (in grm.) at 10°
10	15½	± ¼	.....	.....
20	16	± ¼	.....	.....
30	16½	± ¼	.....	.....
40	17	± ¼	60	1,701
50	18	± ½	82	2,324
60	19	± ½	104	2,948
70	20	± ½	126	3,572
80	21	± ½	148	4,196
90	22	± ¾	170	4,820
100	23	± ¾	192	5,443
110	24	± ¾	214	6,067
120	25	± 1	236	6,691
130	26	± 3	258	7,314
140	28	± 5	.....	.....
150	34	± 8	.....	.....
160	40	± 12	.....	.....

The highest standards from 60 and upward, should be neutral hide glues, clear, clean, well made, free from any odour of decomposition, and practically free from foam and grease. For the lower standards, bone glues should be chosen, because most low-test glues are bone glues, and besides low-test hide glues usually have abnormally high viscosities.[1]

Keeping Properties.—After determining the jelly strength, the glasses are allowed to stand uncovered for several days at room temperature to observe the relative keeping properties of the jellies. If it is desired to know the keeping properties under special conditions, these conditions must be simulated.

After observing the keeping properties, the glasses are washed out, and the routine tests referred to on page 605 end. There will now be given a number of additional tests which are often referred to, with comments upon their value.

[1] Many American manufacturers and dealers use for comparative testing the so-called "Cooper grades" which were established many years ago by Peter Cooper, a pioneer American manufacturer, as grades under which his glues were sold.

A Extra	= Standard 130
1 Extra	= Standard 120
1,	= Standard 110
1 X (called one cross),	= Standard 100
1¼	= Standard 90
1⅓	= Standard 80
1½	= Standard 70
1⅔	= Standard 60
1¾	= Standard 50
1⅞	= Standard 40
2	= Standard 30

In all probability, these were originally based upon the distance a certain weighted rule would compress a certain dish or bowl of jelly. The "No. 2" allowed it to sink 2 in., the "No. 1," only 1 in., while the intermediate grades were measured in eighths of an inch.

Water.—From 2 to 3 grm. of glue are roughly granulated and dried at 110 to 115° until constant in weight. The estimation of water is without much practical significance if the glue is commercially *dry;* for the more moisture a glue contains, the weaker it will show on the viscosity and jelly tests.

Ash.—The ash of glue varies considerably, both in amount and composition. In the case of some bone glues the ash consists largely of phosphates, while in hide glues considerable calcium sulphate or other calcium salts (formed upon neutralisation of the lime used in preparing the stock), are apt to be present. Chalk, clay, zinc oxide, and lead carbonate and sulphate which are sometimes added as whiteners, and zinc sulphate which is used as a preservative, are often founp.

For the estimation of ash, a weighed quantity (2 or 3 grm.) of glue is incinerated in a large covered platinum crucible, using a few drops of nitric acid if necessary, to ensure the oxidation of the carbon. The heating must be conducted with care, as the glue at first intumesces violently. The ash of bone glue is supposed[1] to be fused by the heat of the bunsen burner, to be neutral, and to contain phosphoric acid and chlorides; whereas the ash from hide glue is supposed to be infusible owing to the presence of lime, to be alkaline, and generally free from phosphoric acid and chlorides.

The composition of the ash, in reality depends more upon the nature of the process than upon the raw material used in making the glue, and from a practical standpoint its estimation is generally unnecessary, unless to detect some added adulterant or for the purpose of comparison. Hide and bone glues are frequently mixed, both in the liquor and in the finished form, and it is unsafe to attempt to judge the raw material of a glue on the basis of its ash.

M. P.—Strictly speaking, a glue jelly has no absolute m. p., for it softens up gradually and shows no sharp line of demarcation between solid and liquid. In general, the m. p. is proportionate to the jelly strength, although glues of low viscosity *appear* to melt more readily than those of heavier body. The m. p. may be ascertained by the simple apparatus described by N. Chercheffsky (*Chem. Zeit.*, 1901, **25**, 413; *J. Soc. Chem. Ind.*, 1901, **25**, 731); this consists of a 250 c.c. beaker filled with refined paraffin oil, into which is hung a wire with a horizontal end upon which are threaded several small blocks of jelly. When these lose their rectangular form upon warming, the m. p. is read on a thermometer which hangs as near them as possible. For exact work, the beaker may be water jacketed in a larger one.

[1] See Kissling, *Chem. Zeit.*, 11, 691 and 719; *J. Soc. Chem. Ind.*, 1887, 6, 565.

Another apparatus for ascertaining m. p. is Cambon's "fusiometer" (see *J. Soc. Chem. Ind.*, 1907, **26**, 703). Perhaps the best way is to reheat the jellies in the test-glasses (see page 603) and note comparatively how they melt.

The m. p. is uncertain, and takes more time than it is worth, for the jelly test is preferable.[1]

Hygrometric Test (*Cadet's Test*).—This is based on the quantity of water absorbed by a glue on exposure to damp air, and is even more unreliable than the succeeding.

Water Absorption Test (*Schattenmann's Test*).—A known weight of glue is immersed in water at ordinary temperature for 24 hours. In this time, the high-test glues absorb from 10 to 15 times their weight of water, whereas weaker glues absorb only 3 to 5 parts.[2] Very low-test glues form a slime rather than a jelly.

Although the best glues take up more water than inferior qualities when immersed in the liquid, well-made and well-dried glues are much less hygroscopic than badly made specimens, or than those prepared from inferior materials. The latter are also liable to undergo putrefaction exposure to damp.

The water absorption is roughly proportionate to the jelly strength, but is so inaccurate that it is hardly worth the trouble involved, unless as a rough approximation.

"Chemical Tests."—The exact estimation of acidity or alkalinity (see page 605) by titration might be included under this category. For the estimation of free acid, Kissling (*loc. cit.* on page 610) suspends 30 grm. of glue in 80 c.c. of water and allows it to stand for several hours. The volatile acids are then driven over by a current of steam. As soon as the distillate amounts to 200 c.c., the distillation is arrested and the contents of the receiver titrated with standard alkali. Sometimes the distillate contains sulphurous acid, in which case a known volume of standard alkali should be previously placed in the receiver.

For the estimation of free organic acid, Kalmann (*J. Soc. Chem. Ind.*, 1890, **9**, 113) dissolves 1 grm. of the coarsely-powdered sample of glue in water on the water-bath, tests the solution with blue litmus-paper,

[1] The determination of the temperature of gelatinisation, as proposed by K. Winkelblech (*Zeit. f. angew. Chem.*, 1906, **19**, 1260; *J. Soc. Chem. Ind.*, 1906, **25**, 769) is also uncertain and troublesome to determine.

[2] S. Rideal soaks 10 grm. of the coarsely-powdered sample of glue in water at 15° in a weighed beaker for 48 hours. At the end of this time the water is carefully decanted, and the increase of weight ascertained, the character and odour of the jelly being also noted. Good glues give a firm jelly and absorb from 5 to 9 times their weight of water. In other cases, the product is not jelly but a slimy liquid. According to Kissling the amount of water absorbed by glue affords no indication of its cohesive power, and Fels describes it as an uncertain test. If the sample is *finely* ground or *powdered*, this test cannot be made.

H. R. Proctor (*Kolloidchem. Beihefte*, 1911, **2**, 243) found experimentally that the swelling maximum of gelatin is influenced by the volume of the original jelly from which it was dried. The presence of highly ionised acids enormously increase the water absorption of gelatin.

and then titrates the liquid with $N/10$ alkali, using phenolphthaleïn as the indicator. The liquid is then cooled, a little starch solution added, and a $N/10$ solution of iodine added until the liquid becomes blue, the amount of iodine required being equivalent to the sulphurous acid of the sample. The blue colour should then be discharged by adding a drop of a solution of sodium hydrogen sulphite, and then again titrated with $N/10$ alkali and phenolphthaleïn. The volume required indicates the amount of hydriodic acid formed, according to the equation: $M_2SO_3 + I_2 + H_2O = M_2SO_4 + 2HI$. The result should agree with that of the iodine titration, and affords a proof that the organic matter of the glue has not affected the accuracy of that determination.

The exact estimation of sulphurous acid and sulphites in food gelatin is of especial importance in connection with pure food laws, and will be referred to later.

Since the value of glue depends upon its adhesive power, an estimation of the non-gelatinous substances present is of some value. C. Stelling[1] (*Chem. Zeit.*, **20**, 461; abst. *Analyst*, 1896, **21**, 239) estimates these constituents by the following method: A weight of 15 grm. of the sample is soaked overnight in 60 c.c. of water in a 250 c.c. flask. Next day the jelly is dissolved on the water-bath, and any loss by evaporation made up. The flask is then filled to the mark with 96% alcohol and the liquid thoroughly shaken. After standing for 6 hours, 25 or 50 c.c. of the liquid is filtered off, evaporated to dryness, and the residue consisting of the non-gelatinous substances, dried at 100° and weighed. Stelling gives the following figures, which, though only of approximate accuracy, are comparable with each other.

Description	Number of samples	Non-gelatinous substances		
		Maximum	Minimum	Average
		%	%	%
Gelatin (various kinds)	5	4.53	2.53	3.39
Glue	5	4.70	2.00	3.49
Hide glue	3	7.60	4.30	5.73
Bone glue from acid bones	4	11.84	9.24	10.33
Bone glue from acid bones	3	16.78	13.16	15.15
Pressed glues from neutral bone powder	17	32.10	14.30	20.66
Whale glue	1	.....	.....	22.00
Substance used for clarifying wine	1	.....	.....	33.20
Substance used for clarifying wine	1	.....	.....	59.30

[1] Clayton (*J. Soc. Chem. Ind.*, 1902, **21**, 670) considers this the best single chemical test. In criticising Stelling's process, Kissling (*Chem. Zeit.*, 1898, **22**, 171) points out that pure gelatin is not completely insoluble in alcohol of 72%; while the non-gelatin consists of fat and other impurities beside the decomposition-products of gelatin. Nevertheless, Kissling considers that the method affords a partial indication of the value of a glue. He found the "non-gelatin" estimated by Stelling's method in 12 samples of commercial glue to range from 7.6% in a skin glue to 23.2% in a very inferior specimen of bone glue.

Several other precipitation tests have been proposed. The one based on the precipitation by tannin is totally unreliable, because of the indefinite nature of the adsorption compound formed.[1] Trotman and Hackford (*J. Soc. Chem. Ind.*, 1904, **24**, 1072) proposed to separate the hydrolysed nitrogenous products from the true gelatin ("albumoses") by precipitation of the gelatin on saturation with zinc sulphate, and its subsequent estimation by the Kjeldahl method. The results of such a test, even if accurate, would be of little use in ascertaining the value of a glue.

As a rule, it is not advisable to waste time in making "chemical tests" on glues, for the physical tests recommended give more reliable and more useful information.[2]

Tensile or Binding Strength.—Among the numerous methods proposed to measure the "strength" of glue, only a few can be mentioned.

Weidenbusch's method consists in observing the breaking strain of a rod made of glue and plaster of Paris. J. Fels (*Chem. Zeit.*, 1897, pages 56, 70) describes this method as "uncertain."

Setterberg (*J. Soc. Chem. Ind.*, 1899, **18**, 55) dips strips of filter paper in definite solutions of glue, and determines their breaking strength after drying. This is also unreliable.

Methods of testing glue have been based on the weight required to forcibly separate two surfaces of nickel-plated steel, wood, or stone which had been glued together by a solution of the samples. Rideal found wood and metal quite unreliable, and the results with stone-china far from constant. He considers that the rigidity of the stone blocks gives them an advantage over wood, the elasticity and compressibility of which often brings about the rupture prematurely.

As a rule tests of this character are extremely variable, depending largely upon the personal equation, state of the weather, time and conditions of drying, etc.

[1] Hans Trunkel (*Biochem. Z.*, 26, 458; *Chem. Abs.*, 1910, 4, 3091) states that 1 grm. of freshly dissolved gelatin is completely precipitated by 0.7 grm. tannin. After the gelatin has been in solution 24 hours, 0.4 grm. tannin will precipitate it, but the original condition returns upon warming. Any excess of tannin up to 3 parts of tannin to 1 part of gelatin is carried down. By prolonged washing of the tannin-gelatin complex with alcohol, 97% but not all of the tannin is removed. He concludes that the gelatin-tannin compound combination is an adsorption.

[2] F. Gantter (*Zeit. Anal. Chem.*, 32, 413; abst. *J. Chem. Soc.*, 1895, 2, 610) employs the following method for the valuation of hide-clippings for glue-making. A weight of 100 grm. of the clippings is boiled with 1 litre of water containing a few drops of sodium hydroxide until dissolved. Any sand or other mineral impurity is allowed to settle out, and fat removed in the usual way. The clear solution is then made up to 2 litres, 20 c.c. evaporated, and the residue dried at 105° weighed and ignited. This gives the total soluble organic matter. Another portion of 10 c.c. is diluted with 30 c.c. of water, neutralised with acetic acid, and treated with a solution of tannin till no further precipitation takes place. The liquid is then made up to 100 c.c., filtered, the excess of tannin removed by hide-powder, and the organic matter not precipitable by tannin determined in the filtrate by evaporating, weighing, and igniting as before. The difference between the total soluble organic matter previously determined and that not precipitable by tannin is regarded as gelatin.

Interpretation of the Results of Tests.—Glue is used for a multitude of purposes, and each line of work has its special requirements. Experience has taught that glue of certain characteristics made from certain kinds of stock will in certain cases, do the best work at the lowest cost. It is, therefore, best to test unknown glues against those which are actually doing the work, and give the ones which show up well, a trial under actual working conditions. If this is impossible, a laboratory test must be devised, in which the actual working conditions are exactly simulated; the more nearly this is done, the more valuable the test will be.

Regarding the selection of glue for particular purposes, a few special instances may be mentioned.

Wood Joints.—Most preferable are hide glues showing a jelly strength of 70 or over. While some bone glues, especially those made from acidified bone, answer admirably, in general they should be avoided. Lower viscosity of the working solution is preferable for hard wood, on account of the better penetration; whereas with soft porous wood, higher viscosity is desirable.

Veneers.—Here viscosity is of great importance, and low test hide or mixtures of bone and hide glue testing from 50 to 80, are most desirable. Higher test glues are apt to set too quick. If worked on a veneering machine, freedom from foam is essential, for foam bubbles cause "blisters."

Sizing.—As most sizing is done with special machinery and mixtures, each case must be considered individually. Generally a free-flowing glue, free from foam, is required. If used to surface paper, the absence of grease, which makes spots or "eyes," is desirable, as is any marked acidity or alkalinity which might turn the shade of the colours with which the size is mixed.[1]

Gelatin.—*Technical gelatin* is used for such purposes as stiffening straw hats, finishing curtains, making hectograph composition, photographic emulsions, etc. As a rule some kind of preservative is used in gelatin of this character, which may be properly described as a very strong, clear, light-coloured glue.

In judging of the suitability of gelatin for photographic purposes, Abney[2] considers: (1) The proportion of ash of minor importance,

[1] For further instances, see Alexander, *J. Soc. Chem. Ind.*, 1906, **25**, 158.

[2] Many manufacturers of photographic plates test out each boiling of gelatin by making small trial emulsions, and rejecting the lots of gelatin which do not give perfect results. In the absence of more definite knowledge on the subject, this appears to be the safest way; and it is interesting to note that boilings unsatisfactory to one manufacturer, may please another who uses a slightly different method of manipulation. Information regarding gelatin for photographic plates may also be found in Eder's Handbuch der Photographie, 5th Ed. 1, 31, 36, 39, 1902.

specimens of excellent quality sometimes containing 2.5%. (2) A good photographic gelatin will take up from 5 to 10 times its weight when soaked in cold water. (3) The solubility of commercial gelatin varies considerably. Thus Nelson's gelatin will dissolve in the ordinary "cold" water in warm weather, and scarcely sets at 24° whereas Coignet's gold-label gelatin only melts at about 43.3°, and sets rapidly. For ordinary photographic emulsions, Abney recommends a mixture of hard and soft gelatins in proportions dependent on the weather, a good mixture containing 1 part of the former to 3 of the latter. (4) Fatty matters may be determined in the usual manner, or removed by skimming the solution of the sample or converting it into jelly and removing the top. (5) The colour of the solution, the tenacity of a jelly of known strength, and the presence of acid (which is sometimes present in sufficient proportion to be recognised by the taste) should also be noted.

Gelatin containing much chondrin is of inferior quality, and unsuitable for the preparation of photographic emulsions, the solution having less gelatinising power than that of pure gelatin. When present in notable proportion, chondrin may be detected by making a solution of the sample of gelatin in 10 parts of hot water. To this, a strong solution of chrome-alum is added, which in presence of much chondrin will cause the liquid to set to a jelly while still hot.[1]

Gelatin photographic emulsions are of very variable composition, and occasionally it is desirable to examine them quantitatively.

For the estimation of the silver, the sample should be dissolved in or diluted with water, and digested with excess of dilute nitric acid at 100° for some hours. The precipitate will probably consist simply of silver bromide, but may also contain chloride and iodide.[2]

Addition of silver nitrate to the filtrate causes a precipitation of silver bromide, etc., corresponding to any potassium bromide present in the original emulsion. For the estimation of the water, the emulsion is dried at 100°. Air-dried gelatin emulsions generally lose from 8 to 15% of water at 100°. The proportion of gelatin in the sample is ascertained by subtracting from the dry residue the amounts of silver and potassium salts previously found.

Haloid salts of potassium and silver may, with gelatin, be regarded

[1] For the detection of traces of chlorides in gelatin ordinary methods fail. Luppo-Crämer (*Z. Chem. u. Ind. Kolloide*, 1909, 5, 249–259) allows a small quantity of 10% gelatin solution to gel on a glass plate. On top is placed a drop of 10% $AgNO_3$, and in the course of a few hours an opalescent ring of gradually increasing density and about 1 or 2 cm. wide, forms about the drop. On exposure to light the whitish ring surrounding the drop becomes greyish-blue, whereas the colourless layer under the drop turns reddish-yellow.

[2] Where great exactness is not required the silver chloride may be dissolved out by ammonium carbonate, and the bromide separated from iodide by strong ammonia.

as the normal constituents of gelatin emulsions. Some samples contain foreign ingredients, for the detection of which a large quantity of the emulsion should be pressed between canvas, and 50 grm. macerated in cold water for 12 hours. The soluble salts diffuse into the water, and may be detected therein by ordinary methods. For the detection of alcohol, the original sample should be distilled, while phenol and thymol may be detected by the odour developed on warming with sulphuric acid. Salicylic acid may be detected by precipitating the aqueous solution with three volumes of alcohol, evaporating the filtrate to a small bulk, agitating with ether, treating the ethereal layer with dilute sodium hydroxide, faintly acidifying with hydrochloric acid, and adding ferric chloride solution, which will give a violet colouration if salicylic acid be present. Excess of silver nitrate may be detected by neutral potassium chromate, and may be determined by titration with standard sodium chloride, employing the chromate as an indicator.

For the analysis of collodio-gelatin emulsions, the sample should be treated with a large quantity of water, which precipitates the silver bromide and pyroxylin. After drying at 100°, the precipitate is weighed, moistened with nitric acid, and ignited, the loss of weight being the pyroxylin. The aqueous filtrate contains the gelatin. The acetic acid is determined by titrating a portion of the liquid, and the alcohol by distillation of the neutralised portion. Ether does not occur in this last form of emulsion.

Food Gelatin.—Only clean, high test gelatin, free from harmful substances, and properly made from carefully selected raw material, under sanitary conditions, should be sold for food purposes. Food gelatin is used in the manufacture of confectionery, charlotte-russe, ice-cream, "jellies," and for numerous other culinary purposes, besides serving to coat pills and make gelatin capsules and globules used in pharmacy.[1]

In food gelatins the purity, colour, clearness and jelly strength are of prime importance, and since they are usually of very high test, the "finger test" of the jellies is made on solutions of from 3 to 10 grm. of gelatin to 100 c.c. of water.

Food gelatin may be tested[2] for the usual metallic impurities, preservatives, etc. W. B. Hart (7th Int. Cong. of Appl. Chem.) reports finding as much as 104 mg. of copper per kilo in gelatin that was used to garnish pressed beef. R. Krzizan (*Zeit. offentl. Chem.*, 1909, **15**, 31;

[1] See U. S. Pharmacopœia and British Pharmacopœia.

[2] Many tests are seriously affected by the presence of gelatin or similar protective colloid, in the presence of which most precipitates are colloidal or fail to form. The gelatin should therefore be burned to an ash, or the impurities sought dialysed out. See J. Alexander, *J. Soc. Chem.*, 1909, **28**, 285.

Chem. Abs., **3**, 1430) also reports finding 0.014–0.026% in two samples of gelatin.

The estimation of sulphurous acid and sulphites in food gelatin is of particular interest because these are the antiseptic substances more frequently sought for and found.

For a qualitative test William Lange (*Arb. kais. Gesundheitsamt*, **23**, 144; *Chem. Abs.*, 1909, **3**, 2989) treats 5–10 grm. of gelatin in a wide-mouthed 150 c.c. flask with 30–40 c.c. of water, warms slightly, and adds 5–10 grm. H_3PO_4 (sp. gr. 1.15). The flask is loosely stoppered with a cork from which is appended a strip of potassium iodide paper. On warming, sulphur dioxide produces a blue colour on the paper.

The gravimetric[1] method is generally used in the estimation of sulphur dioxide in gelatin. 200 c.c. of 20% phosphoric acid are added to a solution of 10 grm. of gelatin in 10 c.c. of water, and 50 c.c. are distilled over, in a current of pure carbon dioxide into a solution of iodine or bromine. The sulphur dioxide that distils over is oxidised to sulphuric acid, which is precipitated and weighed as barium sulphate.

The accuracy of this method, especially for small percentages of sulphur dioxide, has been frequently attacked. Alexander (*J. Am. Chem. Soc.* 1907, **29**, 783) found, that when tested by the gravimetric method, skin, bone and horn pith obtained from animals freshly slaughtered under government supervision, showed apparent sulphur dioxide, evidently derived from the oxidation of volatile sulphur compounds which distil over. His results were confirmed by William Lange (*loc. cit.*), who states that the error due to this source is from 0.0007 to 0.0012%. A Mueller (*ibid.*, **34**, 164; *Chem. Abs.*, 1910, **4**, 3083) found apparent sulphur dioxide in all the gelatin he examined, but observed that the 0.014–0.082% found in the finest white gelatin did not interfere with the growth of bacteria, which would indicate that it is adsorbed or combined in some manner. The Official Association of Agricultural Chemists recommend (*U.S. Dept. Agri. Bur. Chem. Bull.* 107, rev. page 113, 1910) that in the case of meat food products, "mere traces" of SO_2 be ignored.

E. Gudeman, *J. Ind.* and *Eng. Chem.*, 1908, **1**, 81) also criticises the gravimetric method, and describes a modified form, using steam distillation, by which he claims to obtain very accurate results. L. Pade (*Ann. Chem. anal.*, 1908, **13**, 299; *J. Soc. Chem. Ind.*, 1908, **27**, 914) gives details of a modification of the gravimetric method.

[1] The volumetric estimation of SO_2 by receiving the distillate into standardised iodine, and titrating with sodium thiosulphate to determine the amount of iodine consumed, is quite inaccurate owing to the presence in the distillate of reducing substances other than SO_2. Free SO_2 is estimated by distilling without the addition of phosphoric acid.

Isinglass[1] (Ger. *Hausenblase*, literally sturgeon's bladder) is a variety of collagen prepared from the "sound" or swimming bladder of the sturgeon and other fishes. It should be white, with a yellowish tinge, opaline or semi-transparent, fibrous, and tenacious. When pure, isinglass has no odour, and but a faint taste. It contains from 15 to 20% of moisture.

According to Mülder, isinglass contains: Carbon, 50.76; hydrogen, 6.64; nitrogen, 18.32; and oxygen and sulphur, 24.69%.

Isinglass consists of nearly pure collagen. It is not gelatin, but is converted into that substance by boiling with water. It consists of fibres or threads, which when immersed in cold water, swell up but retain their organised structure, and it is to this property that the use of isinglass for clarifying wine, beer, and other liquids is partially due. The best qualities of isinglass dissolve almost entirely in boiling water. The solution gives a remarkably strong jelly on cooling.

Russian isinglass is the kind most valued, its solution having a higher viscosity than any other variety. It is obtained from various varieties of sturgeon, *Acipenser stellatus* (the seuruga), *A. ruthenus* (the sterlet), and *A. güldenstadtii* (the ossetr), which inhabit the Volga, Caspian and Black Seas, and the Arctic Ocean. The finest are Astrakhan and Taganrog (Samovey) isinglass, which is prepared by steeping the sounds in hot water to remove mucus, cutting them open and drying the inner membrane, and then removing the outer membrane by rubbing or beating.

The unopened sound is called "pipe," "purse," or "lump isinglass;" when opened or folded, "leaf or honey comb isinglass"; when folded and dried, "book isinglass"; and when rolled, "ribbon isinglass."

Penang isinglass is made from various unknown fish. Brazilian isinglass, sometimes called "Cayenne isinglass" is obtained from *Silurus Parkerii*, and occurs in leaves an inch or more in thickness. Rat's tail isinglass is made from the swimming bladder of the cod (*Morrhua vulgaris*), the hake (*Phycis Americanus*) and other fishes; it is opaque, and incompletely soluble in water.[2]

Isinglass which has been bleached by sulphurous acid generally retains traces of sulphates, and hence the solution is precipitated by barium chloride.

Isinglass is not infrequently adulterated with bone-gelatin, gut, and inferior kinds of fish-gelatin.

[1] It is a common popular error to call the mineral mica, "isinglass," probably because its sheets are transparent and flexible.

[2] Interesting information on the origin, preparation, and applications of isinglass will be found in the *J. Soc. Chem. Industry*, 1887, 6, 764.

On treating Russian isinglass with hot water the susbtance swells uniformly, producing a whitish opaline jelly, which gradually dissolves entirely. Gelatin, on the contrary, swells irregularly, and gives a nearly transparent solution.

On ignition, Russian isinglass usually leaves from 0.4 to 0.9% of reddish ash, containing a little calcium carbonate. Gelatin yields at least 1.5% of white ash, consisting of calcium phosphate or carbonate, with traces of chlorides and sulphates.

On treating isinglass with hot water, any admixture of ordinary intestinal membrane will be left insoluble, or, at any rate, not more than 30% of the adulterant will pass into solution. Inferior fish-gelatins leave from 20 to 30% of insoluble residue, and give solutions of a very strong and disagreeable odour.

F. Prollius (abst. *J. Chem. Soc.*, 1884, **45**, 647) has examined a number of samples of isinglass and fish-gelatin from different sources. The insoluble matter was determined by weighing the residue obtained on treating the sample with hot water. The gelatinising power was ascertained by dissolving 1 part of the sample in 90 parts of water, filtering, and determining the viscosity of the resultant solution. The following were the results obtained:

Source of isinglass	Ash, %	Water, %	Residue insoluble in hot water, %	Viscosity, seconds
Astrakhan, from Schmidt and Dihlmann, Stüttgart	0.20	16.0	2.8	507
Astrakhan, from a collection	0.37	18.0	0.7	485
Astrakhan, fine iridescent Russian quality, Tübingen collection.	1.20	17.0	1.0	500
Astrakhan, Russian, from Gehe, of Dresden	0.80	19.0	3.0	491
Astrakhan, in laminæ, from Gehe	0.50	19.0	0.4	480
Astrakhan, in threads (Hamburg threads)	0.40	17.0	1.3	477
Hamburg isinglass	1.30	19.0	2.3	470
Hamburg isinglass, another quality	0.13	19.0	5.2	.....
Rolled northern fish-bladder	3.20	.....	10.8	467
Icelandish bladder	0.60	17.0	21.6	463
Indian isinglass	0.78	18.0	8.6	437
Yellow, unknown quality	2.30	17.0	15.6	360

Prollius recommends the microscopic examination of isinglass as an indication of its purity. Isinglass exhibits a very pronounced fibrous structure, which is never observed in the case of ordinary gelatin.

The commoner kinds of isinglass, especially coarse Brazilian, are employed for clarifying wine, etc. *Beer-finings* are usually prepared by treating isinglass or fish-gelatin with sour beer, or with acetic or sulphurous acid. The last reagent is preferred by brewers, since the antiseptic properties of the sulphurous acid are of value. One pint of fin-

ings containing 1/8 oz. of isinglass, "cut" by sulphurous acid, should fine a barrel (36 gallons) of beer. The finings are not actually dissolved but simply softened or "cut" by the acid. The clarifying action appears to be purely mechanical, the particles causing turbidity becoming entangled in the gradually sinking network of gelatinous matter. Hence an actual solution of any kind of gelatin would be useless for this purpose.[1]

Isinglass is employed by cooks for thickening soups, jellies, etc., but in this case its clarifying properties are not called into play, and hence gelatin is often substituted under the name of "patent isinglass."

Isinglass, though free from chondrin, is not suitable for the production of photographic films, on account of its ready solubility and inferior tenacity.

Size is a kind of coarse gelatin solution employed in paper-making, distemper-painting, and for weighting and imparting stiffness and gloss to cotton goods, etc.

Bone-size is pale, clear, and forms solid, semi-transparent cakes or masses.[2] Its adhesive power is inferior to glue-size.

Glue-size is a dark brown, semi-fluid, very adhesive mass. It frequently contains hairs and animal refuse, and it often gives strongly the reactions for chondrin (page 627).

When exposed to warmth and moisture, size is liable to mildew, with disastrous results to the goods on which it has been applied. Salts of magnesium and zinc and other substances of antiseptic properties are largely employed to prevent this change. Common salt is sometimes present in large proportion.

The feel and apparent tenacity are not reliable guides in judging of the quality of size, and the sp. gr. is equally misleading.

In assaying size, a portion should be dissolved in water, and a quantity of the solution representing a known weight of the sample should be evaporated to dryness in a shallow dish to ascertain the proportion of solid matter. Another portion of the solution should be treated with sulphuric acid, evaporated to dryness, and the residue ignited to obtain the sulphated ash, which will represent the mineral additions to the size.

Casein-glue is prepared by coagulating separated milk, as free as possible from fat, with dilute acetic acid. The curd is well washed, pressed, and dissolved in a strong solution of borax or sodium hydrox-

[1] High test gelatin, broken in small pieces, is also used for fining, and is to be recommended from the standpoint of cleanliness and purity.

[2] According to Wagner, the best bone-size is made from the "sloughs" of the horns of oxen. 4 cwt. of these are boiled with water for 10 hours, and the liquid strained. 3 pounds powdered alum and 2 pounds of powdered zinc sulphate are then added to the hot liquid with vigorous stirring. The liquid is run into shallow tubs and allowed to cool. The yield of size is about 10 cwt., and it will keep 18 months.

ide; or commercial casein is used. The resultant viscous fluid has strongly adhesive properties, and is well-suited for use by joiners, bookbinders, etc.

Gluten- or albumin-glue is prepared from partially decayed gluten, obtained in the manufacture of starch from wheaten flour.

Liquid glue is prepared by dissolving 5 parts of glue in its own weight of water, and adding 1 part of nitric acid of 1.31 sp. gr. When the evolution of nitrous fumes is at an end, the liquid is cooled. By the above treatment, the glue loses its character of gelatinising without its adhesive properties being impaired. A preferable preparation is obtained by dissolving gelatin or fine glue in moderately strong acetic acid, with the aid of heat, and then adding to the solution some powdered alum and one-fourth of its volume of alcohol.[1]

Coagulin is a strong solution of gelatin in concentrated acetic acid. It becomes fluid on warming, but gelatinises partially on cooling.

Gelatin Substitutes.

Various lichens and sea-weeds yield a highly gelatinous product on boiling with water, and some of them are employed as articles of food and in medicine.

The following table (from Thorpe's *Dictionary of Applied Chemistry*) shows the composition of various *British algæ:*

Alga	Water	Nitrogen in dry substance
	%	%
Chondrus crispus, bleached	17.92	1.534
Chondrus crispus, bleached	19.79	1.485
Chondrus crispus, unbleached	21.47	2.142
Chondrus crispus, unbleached	19.96	2.510
Gigantina mamillosa	21.55	2.198
Laminaria digitata (dulse tangle)	21.38	1.588
Rhodymania palmata	16.56	3.465
Porphyra laciniata	17.41	4.650
Sarcophyllis edulis	19.61	3.088
Alaria esculenta	17.91	2.424

From the above results it is evident that the jellies from these sources, as a rule, contain but little nitrogen, and resemble gelatin merely in their physical characters.[2]

[1] Knaffl prepares a superior liquid glue by treating 3 parts of glue with 8 parts of water, 0.5 of hydrochloric acid, and 0.75 of zinc sulphate. The whole is heated for 12 hours to a temperature not exceeding 85°. The product keeps for a long time and is largely employed for joining horn, wood, and mother-of-pearl.

[2] **Cetrarin** or **Lichenin** is the gelatinous substance of *Cetraria Islandica*, a native of the north of Europe, commonly known as Iceland moss or Iceland lichen. The lichen was official in the British Pharmacopœia of 1885, which described it as almost odourless when dry, but having a feeble sea-weed-like odour when moistened with water. The taste is mucilaginous and bitter. A strong decoction gelatinises on cooling. By prolonged

Gelose is a gelatinous substance contained in *Gelideum corneum*, an alga known as Chinese moss or Japanese isinglass, Japanese gelatin, or vegetable isinglass. The sea-weed contains only traces of soluble matters, but swells up in cold water and dissolves wholly in boiling water with the exception of 2 to 3% of nitrogenised corpuscles. The solution coagulates on cooling to a colourless translucent jelly. Payen prepared gelose by treating *Gelideum corneum* (or certain other algæ) with cold dilute acetic acid, water, and dilute ammonia; washing thoroughly, dissolving the substance thus purified in boiling water, and drying the jelly which forms on cooling.

Gelose occurs in commerce in bundles of long, very slender threads, resembling isinglass. Gelose has 10 times the gelatinising power of isinglass, and will set to a jelly with 500 times its weight of water. It is not, however, a suitable substitute for isinglass, since the m. p. of the jelly is above the temperature of the mouth. The aqueous solution of gelose is precipitated by alcohol. After drying, gelose is insoluble in cold water, dilute acids or alkalies, and Schweitzer's ammoniocupric solution. Gelose loses its property of gelatinising when heated with water under a pressure of six atmospheres, or when boiled with dilute acids (including acetic acid), and the product reduces Fehling's solution on boiling. When heated with nitric acid, gelose yields mucic and oxalic acids. A 1o% aqueous solution of gelose exhibits a specific rotatory power of $-4.25°$; but by boiling the acidified solution, the liquid gradually acquires a nearly equal dextro-rotation, and is no longer precipitated by alcohol. On the other hand, by treating gelose with water at 100°, Porumbaru (*Comp. rend.*, **90**, 1081) obtained a *lævo*-rotatory sugar containing $C_6H_{12}O_6,H_2O$.

The gelatinous principle of *agar-agar*, an edible sea-weed found in Malacca, Borneo, Ceylon, etc., is probably identical with gelose.

Alginic Acid or **Algin** is a gelatinous substance present in certain sea-weeds, especially *Laminaria*. It was first isolated by E. C. C. Stanford (*J. Soc. Chem. Ind.*, **3**, 297; **4**, 518, 595; *J. Soc. Arts*,

boiling with dilute sulphuric acid, the jelly from Iceland moss yields a crystallisable sugar, having an optical activity of $[\alpha]_D = +46.85°$. *Lichenstearic acid*, $C_{43}H_{76}O_{13}$, and *cetraric acid*, $C_{39}H_{30}O_{12}$, have been obtained from Iceland moss (abst. *J. Chem. Soc.*, 1890, page 600). Cetraria is omitted from the B. Pharmacopœia of 1898, but is official in most foreign Pharmacopœias. It is described as demulcent, nutritious, and slightly tonic. In Iceland, the bitter principle is removed by mashing in cold water and sodium carbonate, and the lichen then made into jelly, or dried, ground, and made into bread.

Carrageenin is the gelatinous substance contained in *Chondrus crispus* or Irish moss, a sea-weed which is only exposed at low spring tides. It contains about 80% of dry matter, containing 1 1/2 to 2% of nitrogen. The greater part of the moss consists of carrageenin, a gelatinous substance apparently allied to pectin. The decoction or jelly is employed as a demulcent and emollient in pulmonary affections, etc., and is also used as a substitute for isinglass. It also finds employment as a size, and, to a limited extent, for thickening colours in calico-printing and for stiffening silk. A thick mucilage of Irish moss, suitably scented, is employed as "bandoline" or "fixature." On the west coast of Ireland, where it abounds, the moss is used as an article of food. Carrageen moss is official in many foreign Pharmacopœias.

1884), who has observed that the whole of the alkaline salts, together with a considerable quantity of extractive matter containing dextrin and mannitol, can be extracted from the Laminaria or "dulse tangles" by simple maceration in cold water. This treatment removes about 33 % of the air-dry weed, of which from 20 to 22% consists of salts of potassium, sodium, and magnesium, including the whole of the iodine. The residue insoluble in cold water consists substantially of the above-mentioned nitrogenised substance alginic acid, together with the algic cellulose or algulose which represents the cellular fabric of the plant. The alginic acid or algin is removed by digestion in a hot dilute solution of sodium carbonate, which dissolves it as sodium alginate, leaving the algulose in an extremely fine state of division, which renders it very difficult to remove by filtration. On treating the filtrate with hydrochloric acid, the alginic acid is precipitated as an amorphous substance of light amber colour, which is washed and bleached.[1]

Several patents have been obtained for obtaining "*tang-acid*" and other organic products from *sea-weed* (see *English Patents*, 1896, No. 11538; 1898, Nos. 12275 and 12277).

Alginic acid is stated to have the formula $C_{78}H_{80}O_{22}N_2$. When precipitated by adding a mineral acid to the solution of its salts, alginic acid forms a very gelatinous precipitate, which when dry resembles albumin or horn. It has a sp. gr. of 1.5. Alginic acid is insoluble in either hot or cold water.

Alginic acid acts on sodium carbonate with evolution of carbon dioxide and formation of *sodium alginate* or soluble algin.

Soluble algin dissolves in water to form a very viscous solution. Stanford states the viscosity of algin at 14 times that of starch, and 37 times that of gum-arabic. Solutions of sodium alginate are precipitated or coagulated by alcohol, acetone, and collodion, but not by amylic alcohol, ether, glycerin, or sugar. Alginic acid is precipitated from the solution of its sodium salt by most mineral acids, and by picric, oxalic, tartaric, and citric acids. A 2% aqueous solution is rendered semi-solid by acidifying it with hydrochloric acid. On the other

[1] Stanford gives the following analyses of *Laminaria*. In addition to the three main constituents, the aqueous solution contains salts, mucilage, and mannitol, while in the sodium carbonate solution a modified dextrin is present.

	Water	Alginic acid	Cellulose	Salts and undetermined matters
Laminaria digitata, stem	37.04	21.00	28.20	13.76
Laminaria digitata, frond	44.00	17.85	11.00	27.15
Laminaria stenophylla, stem	34.50	25.70	11.27	28.53
Laminaria stenophylla, frond	40.02	24.06	15.06	20.86

hand, no precipitation occurs on treating a solution of sodium alginate with acetic, formic, benzoic, succinic, carbolic, tannic, arsenious, or boric acid. Soluble algin yields no precipitate with silicates of alkali-metals, chromates, permanganates, tungstates, or molybdates. It is not precipitated by hydrogen peroxide, chlorine, bromine, or iodine.

Sodium alginate gives no precipitate with the alkali hydroxides or with ammonia, but insoluble alginates are thrown down by lime-water and baryta-water. Precipitates are also produced by most metallic salts, some of the alginates formed being of curiously complex composition. No precipitates are produced by salts of magnesium, the alginate of this metal being soluble. By bringing magnesia or magnesium carbonate in contact with alginic acid and water, the two insoluble bodies react to form soluble *magnesium alginate.*

Solutions of sodium alginate are distinguished from those of albumin by not coagulating on heating and by yielding no precipitate with mercuric chloride, potassium ferrocyanide, or tannic acid; from mucin, by not being precipitated by acetic acid; from gelatin, by giving no precipitate with tannin; from starch, by yielding no blue colour with iodine; from gelose, by containing nitrogen and not gelatinising on cooling. Sodium alginate differs from dextrin, gum-arabic, gum-tragacanth, and pectin, by its insolubility in dilute alcohol and dilute mineral acids.

A number of ingenious applications of alginic acid and its salts have been proposed by Stanford (*J. Soc. Chem. Ind.*, 1885, page 518). Some of these salts are likely to prove valuable as envelopes for certain medicines—such as mercury, bismuth, etc.—since they pass through the stomach unaltered, and hence delay the absorption of the medicine till it has reached the duodenum.

Chondrigen and Chondrin.

Chondrigen is the principal albuminoid constituent of the matrix of hyaline cartilage.[1] It is an elastic, semi-transparent substance, which

[1] The following results by Hoppe-Seyler show the composition of two typical kinds of human hyaline cartilage:

	Costal cartilage	Articular cartilage
Water	67.67 %	73.59 %
Organic solids	30.13 %	24.87 %
Inorganic solids	2.20 %	1.54 %
	100.00 %	100.00 %

is insoluble in hot or cold water and does not swell up materially by treatment either with water or with dilute acetic acid. By prolonged treatment for some hours with water under pressure (at 120°), chondrigen is gradually dissolved with formation of *chondrin*, the solution of which gelatinises on cooling. Hence the hyaline matrix of cartilage appears to bear the same relation to chondrin that the ground-substance of connective tissue bears to gelatin.

For the preparation of chondrin in a state of approximate purity, costal cartilage should be boiled with water for a few minutes, and the perichondrium removed by scraping. It is then cut into fine slices and boiled with water at the ordinary pressure for 24 hours; or preferably heated with water under pressure for 3 to 4 hours to 120°. The solution is filtered while hot—to remove elastin, cellular elements, etc.—and treated with a large excess of alcohol. The precipitated chondrin is washed successively with alcohol and ether, and if necessary purified by re-solution in hot water and precipitation by alcohol.

Chondrin so prepared forms a hard, transparent mass, free from taste or odour, and insoluble in cold water. It dissolves in hot water, and the solution gelatinises on cooling, but less strongly than a solution of gelatin of the same strength.

The following is the percentage composition of chondrin according to various authorities:

	Carbon	Hydrogen	Nitrogen	Sulphur	Oxygen
Mulder	49.3	6.6	14.4	0.4	29.3
Fischer and Bödeker	50.0	6.6	14.4	0.4	28.6
Schützenberger and Bourgeois	50.16	6.58	14.18	none	29.08
Von Mering	47.74	6.76	13.87	0.6	31.04

It will be seen from the above figures that the elementary analyses of chondrin present considerable discrepancies, and suggest that the substance dealt with is not a definite substance but liable to variations in composition. The results obtained by Morochowetz, and confirmed by Landwehr, Krukenberg, and Mörner, strongly support this view. Morochowetz found that on treating cartilage from various sources with lime- or baryta-water, a 0.5% solution of sodium hydroxide, or a

The *inorganic solids* of costal cartilage in 100 parts of ash consisted of: K_2SO_4, 26.66: Na_2SO_4, 44.81; NaCl, 6.11; Na_2PO_4, 8.42; $Ca_3(PO_4)_2$, 7.88; and $Mg_3(PO_4)_2$, 4.55 %.

The *organic solids* in the cells are mostly of protein nature. The ground-substance of the matrix, which forms the greater part of the organic material of cartilage, consists substantially of chondrigen. According to P. B. Hawk, the principal solid constituents of the matrix of cartilaginous tissue are chondromucoid, chondroitin-sulphuric acid, chondro-albuminoid and collagen.

10% solution of common salt, mucin is dissolved out, and may be thrown down from the solution by acetic acid; while the substance left undissolved is readily convertible by boiling with water into perfectly normal gelatin. According to these observations, chondrin is a mixture of gelatin and mucin, while chondrigen is a mixture of collagen with mucin or hyalogen, the latter component masking its true nature.[1] By micro-chemical examination of the cartilage of the trachea, and the use of staining agents, Mörner detected the existence of a network of collagen, enclosing spherical masses termed "chondrin balls." By treating sections of cartilage with very dilute hydrochloric acid (0.1 to 0.2%), and then with very dilute solution of potassium hydroxide (0.1%), he succeeded in dissolving out the chondrin balls and leaving the network, which by treatment with dilute acids or superheated water was in great part converted into typical gelatin. The chondrin balls were found to consist of a mixture of free chondroitic acid and a mucin called chondromucoid, which on decomposition yielded protein matter and chondroitic acid.

Chondroitic acid, or *chondroitin-sulphuric acid*, $C_{28}H_{51}O_{30}N_3S$, has the characters of a hyaline (page 629), though Mörner was unable to verify the existence of the corresponding hypothetical hyalogen. Chondroitic acid yields a reducing sugar when boiled with dilute sulphuric acid. It is interesting on account of its low percentage of nitrogen, and from the fact that the sulphur of the molecule is wholly in the form of an ethereal sulphate.

When acted upon with acids, chondroitin-sulphuric acid splits off sulphuric acid, and yields *chondroitin*, a nitrogenous substance, which is then converted into another nitrogenous substance called *chondrosin*, acetic acid being formed in the process. This latter substance reduces Fehling's solution even more strongly than dextrose (Hawk).

According to Schützenberger and Bourgeois, the products of the decomposition of chondrin by boiling with baryta-water differ from those yielded by gelatin under the same treatment in a much larger (three times) production of acetic acid, and in the entire absence of glycocine. The latter of these results, if correct, is difficult to reconcile with Morochowetz' view of the complex nature of chondrin.

The behaviour of chondrin with solvents and reagents corresponds exactly with a mixture of gelatin and mucin. Thus:

[1] Chondrin is probably an adsorption compound of simpler substances, *i.e.*, gelatin and mucin.

	Gelatin	Chondrin	Mucin
Solubility	Insoluble in cold water, alcohol, or ether. Soluble in hot water; such solutions set into a jelly when cold.	Insoluble in cold water, alcohol, or ether. Soluble in hot water; such solutions set into a jelly when cold.	Insoluble in cold water, alcohol, or ether. Insoluble in hot water.
Reaction with:			
Acetic acid	No precipitate.	Precipitate; insoluble except in large excess.	Precipitate; insoluble except in large excess.
Mineral acids	No precipitate	Precipitate; readily soluble in excess.	Precipitate; readily soluble in excess.
Tannic acid	Precipitate.	Precipitate.	No precipitate.
Mercuric chloride	Precipitate.	Precipitate.	No precipitate.
Lead acetate	No precipitate.	Precipitate.	Precipitate.
Alum	No precipitate.	Precipitate.	Precipitate.
Boiling dilute mineral acids.	No reducing substance formed.	1. Syntonin. 2. Substance reducing cupric oxide.	1. Syntonin. 2. Substance reducing cupric oxide.

The reducing substance formed by boiling chondrin with dilute acids has been called chondriglucose, but it is said to contain nitrogen, and it is doubtful if it is a true sugar. It is probably identical with the reducing substance resulting from the hydrolysis of mucin by acids.

Mucins.[1]

Under the general name of mucin are grouped various closely allied substances which give to many animal secretions, such as snail-slime, synovia, saliva, etc., their characteristic viscid or ropy consistency. A mucin is the characteristic principle of *mucus*, the thick, slimy liquid which covers mucous membranes. Mucins are widely distributed in the animal kingdom, in many cases probably playing a part at present very imperfectly understood. Mucins are contained in many of the tissues, especially the umbilical cord, the tendons, and the submaxillary gland. Mucins are especially abundant in the snail, the mantle and foot containing two distinct varieties.

The method used for the preparation of mucins varies with the material treated, but is based on the solubility of mucins in dilute alkaline liquids, and their precipitation therefrom by acetic acid. Precipitation by alcohol, or saturation of the solution by a neutral salt, may also be employed for the preparation of mucins.

When freshly precipitated, the mucins are glutinous substances which mix with water without undergoing real solution. They dissolve in dilute solutions of the alkali hydroxides, in lime-water, and in baryta

[1] The mucins are compound proteins of the gluco-protein class, and are classed with the albuminoids or scleroproteins only for the sake of convenience.

water. On addition of an acid to either of these solutions, the mucin is precipitated. If acetic acid be used, the precipitate is practically insoluble in excess, nor does hydrochloric acid of a strength ranging from 0.1 to 1.0% of real hydrochloric dissolve most varieties of mucin, but solution is generally effected by acid of 5% strength.

In their natural condition, the mucins are soluble in a 10% solution of common salt, but they become insoluble after precipitation by an acid. By saturating their solutions with common salt, ammonium sulphate, and certain other neutral salts, the mucins are completely precipitated, but not coagulated. The mucins are not coagulated either by boiling with water or by precipitation with alcohol.

The mucins behave like ordinary proteins with Millon's and Adamkiewicz' reagents (page 40). With the biuret test, mucins yield a rose-red colouration. No reduction occurs on boiling.

Lead acetate completely precipitates mucins from their neutral or faintly alkaline solutions. Alum also precipitates them, but negative reactions are yielded by most other metallic salts (including mercuric chloride); though all mucins do not behave in the same manner with such reagents. The mucins are not precipitated by tannin.

Such varieties of mucin as have been examined in that respect are not dissolved by pepsin-hydrochloric acid, but are digested by alkaline solutions of trypsin.

By prolonged boiling with concentrated sulphuric acid, the mucins yield leucine and tyrosine.

By boiling for 20 to 30 minutes with dilute sulphuric acid, mucins are decomposed into a protein substance which is apparently syntonin, and a pseudo-sugar which reduces Fehling's solution on boiling, is optically inactive, does not undergo the alcoholic fermentation, and is said to contain nitrogen. This decomposition shows the mucins to belong to the class of compound proteins.

The following analyses of mucins from different sources have been recorded:

Origin of mucin	Carbon	Hydrogen	Nitrogen	Sulphur	Oxygen	Observer
1. Mucous contents cyst.	52.17	7.01	12.64	.....	28.18	Scherer.
2. Snails.....	50.30	6.84	13.62	1.74	27.53	Hammarsten.
3. Snails (*Helix pomatiæ*).	48.94	6.81	8.50	.....	35.38	Eichwald.
4. Tendon.....	48.3	6.44	11.75	0.81	.....	Loebisch.
5 Submaxillary gland	52.31	7.22	11.84	.....	28.63	Obolensky.
6 Submaxillary gland	48.84	6.80	12.32	0.84	31.20	Hammarsten.

No. 2 yielded 0.33, and No. 6, 0.35% of ash. Hammarsten has found that the mucin from the foot of the snail is distinct from that present in the mantle.

Hyalogens and Hyalins.—Hyalin is a term originally applied to the leading constitutent of the walls of hydatid cysts, but it was subsequently extended by Krukenberg to allied substances obtainable from other animal structures. These substances, in their natural condition, are insoluble, and are termed hyalogens, but by the action of alkalies or water under pressure, they are converetd into soluble substances which are generically called hyalins. Thus chondrosin is a hyalogen contained in the sponge, *Chondrosia reniformis;* spirographin is obtained from the cartilage and skeletal tissues of the worm, *Spirographis;* and neossin is the chief constituent of the edible bird's nest. The hyalins corresponding to these hyalogens are called respectively chondrosidin, spirographidin, and neossidin. Similar hyalogens have been described as existing in the vitreous humour of the eye and in hyaline-cartilage (page 625). They agree in their leading characters with the mucins, of which they appear to be simply varieties.

FIBROIDS.

By W. P. DREAPER, F. I. C.

The proteins of the fibroid class are insoluble in water, and are not readily acted on by dilute acids. They dissolve in solutions of alkali hydroxides, especially on heating.

The fibroids are represented by elastin and the fibroïn of silk (wild and cultivated) and the spider's web. They contain no sulphur.

Elastin.—Elastin is the elastic fibre of connective tissue. This protein is the characteristic constituent of the fibres which remain after the removal of more soluble compounds (mucin, gelatin, fat, etc.) from the ligaments of the neck of herbivorous animals,[1] by successive treatment with boiling water, alkali hydroxide, dilute acids, alcohol, and ether.

Thus obtained, it forms a pale yellowish substance, in which the shape of portions of the original elastic fibres may be distinguished under the microscope. When moist, it is yellow and elastic, but on drying becomes brittle, and may, with some difficulty, be reduced to powder.

Elastin is insoluble in any solvent which does not act on it chemically. The following analyses of elastin have been published. When

Origin.	C.	H.	N.	S.	O.	Ash.	Authority.
Neck-band (*ligamentum nuchæ*).....	54.24	7.27	16.70	0.30	21.79	0.90	Chittenden and Hartwell.
Neck-band (*ligamentum nuchæ*).....	55.45	7.41	16.19	....	20.89	.....	Müller.
Aorta.....	53.95	7.03	16.67	0.38	21.97	0.72	Schwarz.

treated with strong alkalies or mineral acids at 100°, elastin dissolves and in the latter case, if sulphuric acid be used, leucine (30 to 40%)

[1] For the preparation of elastin, the *ligamentum nuchæ* of an ox should be cut into thin slices and boiled with water for several days, then treated for some hours at 100° with a 1% solution of potassium hydroxide, and afterwards again boiled with water. This is removed and the ligament boiled with 10% acetic acid. It is next treated with cold water containing 5% of hydrochloric acid, washed with water, boiled with strong alcohol, and finally digested for several weeks with ether.

and tyrosine (0.25%) are found among the products of its decomposition. When boiled with strong hydrochloric acid and stannous chloride, the same crystalline products are obtained together with ammonia, glycine, and an aminovaleric acid; but no aspartic or glutamic acid. This behaviour distinguishes elastin from both proteins and gelatin.

Pepsin, hydrochloric acid, or trypsin digests it with formation of albuminoses, but it is very slowly acted on by these ferments. Elastin contains the smallest per cent. of tyrosine of all the albuminoid substances. It does not give the lead sulphide reaction. (Engel, *Zeit. f. Biol.*, 1897, **27**, 274.)

Animal Fibres and Their Recognition.

Silk.—Silk is the filament which the silkworm spins and winds round itself before assuming the condition of a chrysalis.

Many varieties of silkworms exist and the silks they produce are of very different quality and commercial utility. The silkworm which lives principally upon the leaves of the white mulberry tree (*Moria alba*), known as *Bombyx mori*, is the chief producer of silk; but much attention has of late been given to other silk-producing larvæ, and especially to the Tussah worm, *Antheræa mylitta*, and *A. pernyi*, which feeds on the leaves of the oak, etc. (see *J. S. D. & Col.*, 1888, **4**, 155).

The Bombyx worm secretes a thread from twin orifices situated near the head,[1] and communicating with sacs running one on each side the whole length of the body. The glutinous secretion rapidly coagulates on its exit into the air. As it flows from two distinct apertures, the thread produced is double, the twin fibres being enclosed in a thin, perfectly smooth case, known as "silk-glue," "silk-gelatin," "silk-gum," or "bast" (in France, *grès*). This gelatin is readily soluble in hot soap solution, dilute caustic alkalies, and weak chromic acid, the application of these reagents effecting a separation of the enclosed twin threads. The internal fibre, freed from the gelatin covering, consists essentially of *fibroïn*, while the coating is composed substantially of *sericin*. According to Bolley, in the silk-producing and secreting gland of the worm, glutinous semi-fluid fibroïn occurs without admixture with sericin, the latter compound being a product of a subsequent aërial oxidation of fibroïn at the moment of formation of the fibre.

Raw commercial silk from the mulberry silkworm is generally regarded as containing 11% of moisture, 66% of fibroïn, 22% of

[1] Chappe triturated the contents of the glandular organs of silkworms with about one-third their weight of water, and was thus able to blow permanent globes and diversely shaped vessels with the same.

sericin, and 1% of mineral and colouring matter.[1] The mineral matters are chiefly potash, lime, magnesia, and phosphoric acid, the ash containing about 10% of the last-named constituent. The greater part of the mineral matters of raw silk may be removed together with the sericin by prolonged boiling with soap-solution. The residual fibroïn retains only about 0.6% of mineral matter.[2]

Silbermann (*Chem. Zeit.*, 1895, **19**, 1683) gives the following analyses of typical types of *mulberry silk:*

Kind of silk	Fibroïn	Ash of fibroïn	Sericin	Wax and fat	Salts
White; cocoons	73.59	0.09	22.28	3.02	1.06
White; raw	76.20	0.09	22.01	1.36	0.30
Yellow; cocoons	70.02	0.16	24.29	3.46	1.92
Yellow; raw	72.35	0.16	23.13	2.75	1.60

A sample of *mulberry silk* was found by Richardson to contain:

	Air-dry	Dried at 100°
	%	%
Water	12.50	.....
Fatty substances	0.14	0.16
Resinous substances	0.56	0.64
Sericin	22.58	25.81
Fibroïn	63.10	72.11
Mineral matter	1.12	1.28
	100.00	100.00

Raw Tussah silk has been analysed by Bastow and Appleyard (*J. S. D. & Col.*, 1888, **4**, 88) with the following results, the sample being reeled and previously dried at 100°.

Soluble in hot water	21.33%
Subsequently dissolved by alcohol (chiefly fatty acid)	0.91%
Subsequently dissolved by ether	0.08%
Total loss by boiling off with 1% solution of curd soap	26.49%
Mineral matters	5.34[3]%

[1] The colouring matter of yellow silk has been investigated by Dubois (*Compt. rend.*, 1890, 111, 482), who has isolated from it several substances resembling in many respects vegetable carrotin. These are yellowish-red and crystallisable, altered by exposure to light and air, soluble in alcohol, ether, chloroform, and benzene to golden-yellow solutions, and in carbon disulphide to a brownish-red solution. They give absorption-spectra free from bands, and dissolve in strong sulphuric acid, with blue colouration changing to green, destroyed by addition of water. In addition to these colouring matters, Dubois isolated a deep greenish-blue pigment, which is present in very small quantity.

[2] Carter Bell (*J. Soc. Chem. Ind.*, 1897, 16, 304) states that the ash of a genuine silk varies according to the district in which it is produced, and averages 0.5%. He found 0.35% of ash in a pure white silk, while a Chinese salmon-coloured silk contained only 0.25%. In the raw state, the ash of silk varies very much. In good Italian silk it is about 1%. Yama-mai silk sometimes yields as much as 8% of ash, which is greatly reduced by successive treatments of the fibre with alcohol, dilute sulphuric acid, and boiling soap solutions.

[3] The *ash* of Tussah silk was found by Bastow and Appleyard to have the following composition: K_2O, 31.68; Na_2O, 12.45; CaO, 13.32; MgO, 2.56; Al_2O_3, 1.46; SiO_2, 9.79; P_2O_5, 6.90; SO_3, 8.16; CO_2, 11.14; Cl, 2.89; less O equivalent to Cl, 0.65; total, 99.70. As sulphur is not a constituent of the organic compounds of mulberry silk, the occurrence of sulphates in the ash of Tussah silk is characteristic. The presence of a notable proportion of alumina is to be noted, as aluminium is not a generally recognised constituent of animal products, and is contained in so few plants that its presence in the secretion of the silkworm is difficult to account for. It is probable that the greater part of the ash found by Bastow and Appleyard in Tussah silk was derived from adhering impurities. The fibroïn prepared from the same silk contained only 0.226% of ash.

Silk Conditioning.—In a damp atmosphere, raw silk will take up a large proportion of moisture (25 to 30%) without any indication of the fact, other than increase in weight. To ascertain the proportion of moisture in raw silk, a number of hanks of the sample are dried at 105° in a current of air until constant in weight. To the weight thus obtained it is usual to add 11%, the result being regarded as the weight of the silk in a normal condition of humidity.

This per cent. is recognised as a trade standard and the different Conditioning Houses in the silk districts are established for testing the same.

Silk Scouring.—In the process of scouring silk, the silk-gum or sericin is removed more or less completely. This is generally effected by heating the hanks of raw silk in a solution of soap at 90 to 95°, the process being sometimes repeated with a fresh soap-bath. The fibre at first swells up, but as the gum dissolves, it gradually acquires a soft and silky feel. The resulting liquid, known as "boiled-off liquor," is a useful addition to the dye-bath in dyeing silk with coal-tar colours. The last traces of sericin are removed by washing the silk in water at 60°, to which some soap and sodium carbonate have been added. The silk is sometimes again boiled with soap solution, rinsed with warm sodium-carbonate solution, and lastly washed with cold water. If the silk is to remain undyed or to be dyed a pale colour, it is sometimes exposed while moist to sulphur dioxide gas for 5 or 6 hours, or to the action of hydrogen peroxide. The former operation, which has for its object the bleaching of the silk, is, in many cases, repeated several times.

Degumming has been carried on at lower temperatures in more strongly acting alkaline solutions (NaHO, etc.) in the presence of a protective substance, such as dextrose.

Besides the above process, which is employed to produce completely ungummed silk, involving a loss of 22 to 33% of the original silk, *souple silk* is produced when only from 8 to 12% of sericin is removed;[1] and when *écru* silk is required, the hanks are simply washed with hot water containing a little soap, whereby a loss of only 3 to 4% results.

Sericin, obtained by treating silk with water with or without pressure, is a yellowish, transparent substance, resembling gelatin. It

[1] Soupling seems to alter the nature of the silk-gum as well as reduce its percentage in the raw silk fibre, whereby the sericin is rendered soft, pliant, and lustrous. Souple silk is stated by Silbermann to possess the property of taking up vegetable and mineral weighing materials in a greater degree than either raw or boiled-off silk. For coloured silks, the soupling is often carried out in a boiling bath of cream of tartar or acid sodium sulphate. For black silk, the soupling may take place in the weighting and dyeing processes as tannic acid acts in this direction at a temperature above 68–70°.

dissolves in hot water, hot soap solutions, and dilute alkali hydroxide. The hot aqueous solution forms a strong jelly on cooling. Aqueous solutions of sericin are precipitated by alcohol, tannin, basic lead acetate, stannous chloride, chlorine, and bromine. Potassium ferrocyanide in presence of acetic acid gives a greenish precipitate.

By the action of dilute sulphuric acid on sericin, Crämer obtained 5% of tyrosine (distinction from gelatin) and 10% of serine, a substance having the constitution of an amino-glyceric acid, $CH_2(OH).CH(NH_2).COOH$.[1]

According to Mülder, sericin has the following empirical formula and ultimate composition. For convenience of comparison, the formula and composition of fibroïn (according to Mülder and Crämer) are also given:

	Sericin, $C_{15}H_{25}O_8N_5$	Fibroïn, $C_{15}H_{23}O_6N_5$	Fibroïn (Crämer)
Carbon	42.60	48.80	48.60
Hydrogen	5.90	6.23	6.40
Nitrogen	16.50	19.00	18.89
Oxygen	35.00	25.00	26.11
	100.00	100.00	100.00

Bolley attributed to sericin the composition: Carbon, 44.32; hydrogen, 6.18; nitrogen, 18.30; and oxygen, 31.20%.

An excess of oxygen in sericin over that in fibroïn is possibly due to the greater oxidation of the surface of the fibre, the conversion of fibroïn into sericin being assumed to take place by reaction with water and oxygen:

$$\underset{\text{Fibroïn.}}{C_{15}H_{23}O_6N_5} + H_2O + O = \underset{\text{Sericin.}}{C_{15}H_{25}O_8N_5}$$

Fibroïn is related in character to elastin (page 631), and is obtained by treating silk successively with boiling water, alcohol, ether, and acetic acid. The resultant fibroïn retains the fibrous character of the original silk, but is generally white, of good lustre, and soft to the

[1] Serine seemingly resembles glycocoll (Vol. VII) in its power of uniting with both bases and acids. It differs from alanine or amino-propionic acid by an atom of oxygen. By the action of nitrous acid, alanine yields lactic acid, while serine gives glyceric acid when similarly treated.

$CH_3.CH(NH_2).CO_2H$ Alanine. — $CH_3.CH(OH).CO_2H$ Lactic acid.

$OH.CH_2.CH(NH_2).CO_2H$ Serine. — $OH.CH_2.CH(OH).CO_2H$ Glyceric acid.

touch.[1] On ignition, fibroïn evolves an odour resembling that of burnt horn. Fibroïn does not dissolve in ammonia or solutions of the carbonates of alkali-metals, and is not appreciably affected by a 1% solution of potassium or sodium hydroxide, but is dissolved by stronger ley, especially at high temperature. It is, however, never treated with such solutions when the lustre of the silk is a consideration. On dilution, a precipitate is obtained which is said to consist of unaltered fibroïn, but this is very doubtful. Fibroïn is soluble in hot glacial acetic acid, and also in strong hydrochloric, nitric, sulphuric, or phosphoric acid. It also dissolves in alkaline solutions of nickel, zinc, or copper salts, etc.[2]

The proportion of fibroïn in the silk from *Bombyx mori* has been variously stated according to the method employed for its determination. Thus Mülder, who prepared it by boiling the raw silk with acetic acid, gives it as 53 to 54%. Städeler, by acting on the silk with a 5% solution of sodium hydroxide in the cold, obtained from 42 to 50% of fibroïn. Crämer, by heating the silk with water at 133°, obtained 66%; and Francézon, by boiling the silk twice with soap, and then treating it with acetic acid, found 75% of fibroïn. Vignon, as the result of an improved method of determination, states the average proportion of fibroïn in raw silk at 75%. This agrees with the results obtained in practice. These higher figures doubtless refer to silk previously dried at 100°.

The formula of silk-fibroïn is still uncertain. Mülder attributed to it the composition $C_{15}H_{23}O_6N_5$. Mills and Takamine adopt the expression $C_{24}H_{38}O_8N_8$. Schützenberger and Bourgeois, by the analysis of the fibroïn prepared by Francézon's process arrived at the formula $C_{71}H_{107}O_{25}N_{24}$;[3] Crämer, $C_{15}H_{23}O_6N_5$; while F. W. Richard-

[1] For the preparation of pure fibroïn, Vignon (*Compt. rend.*, 1892, 115, 17, 613) recommends that a skein of raw white silk weighing about 10 grm. should be boiled for 30 minutes in 1,500 c.c. of water containing 15 grm. of neutral soap. The silk is then rinsed successively in hot and in tepid water to remove the soap, hydro-extracted, and submitted to a repetition of this treatment, a fresh soap-bath being used in which the silk is immersed for 20 minutes. The silk is then rinsed with water, passed through dilute hydrochloric acid, again rinsed with water, and then washed twice with 90% alcohol. The fibroïn thus obtained is brilliant, white, and soft, and leaves only 0.01% of ash on ignition. It was found by Vignon to have the elementary composition: C, 48.3; H, 6.5; N, 19.2 and O, 26.0%.

[2] Bastow and Appleyard (*Jour. Soc. D. & C.*, 1888, 4, 89) have pointed out that owing to the fact that the fibroïn of Tussah silk is acted on by solvents far less readily than the fibroïn of mulberry silk, the two products are probably chemically distinct. Tussah fibroïn was prepared by repeatedly boiling the silk with a 1% solution of curd soap, washing with water, extracting with hydrochloric acid, again washing with water, drying, and extracting successively with alcohol and ether. Thus purified, Tussah fibroïn had the following elementary composition (exclusive of 0.226% of ash): Carbon, 47.18; hydrogen, 6.30; nitrogen, 16.85; and oxygen, 29.67%.

[3] When heated with baryta-water under pressure, Silbermann found fibroïn to undergo decomposition with formation of oxalic, carbonic, and acetic acids, and an amino-mixture containing: $C_{68}H_{141}O_{43}N_{21}$.

The amino-mixture is stated to have undergone further decomposition as follows:

$$C_{68}H_{141}O_{43}N_{21} = C_9H_{11}O_3N + 7C_2H_5O_2N + 7C_3H_7O_2N + 2C_4H_9O_2N + 4C_4H_7O_2N$$

Amino mixture. Tyrosine. Glycine. Alanine. Amino-butyric acid. Amino-acid of the acrylic series.

son (*J. Soc. Chem. Ind.*, 1893, **12**, 426) suggests for fibroïn the empirical formula $C_{60}H_{94}O_{25}N_{18}$. The fibroïn prepared by Vignon's process has a composition corresponding to the formula $C_{29}H_{47}O_{12}N_{10}$. It may possibly be of a composite nature.

Richardson has attempted to prove the presence of an amino group in silk by diazotising the fibre with a solution of sodium nitrite containing free hydrochloric acid. After 24 hours' exposure to the nitrous acid the silk acquired a characteristic pale-yellow colour, and on being then washed and plunged into the alkaline solutions of different phenols (phenol, resorcinol, pyrogallol, and α- and β-naphthol) it was dyed differently with each.

The writer has attempted to isolate and examine these colouring matters but they are present in very small quantity.

From the results of the action of alcoholic potash, Richardson considers the fibroïn of mulberry-silk to be more probably an amino-anhydride than an amino-acid.[1]

Weyl (*Ber.*, 1888, **21**, 1529) found that by boiling purified white silk under a reflex condenser for 18 hours with dilute sulphuric acid (1 : 5), it dissolved to a yellowish-brown liquid, leaving a few globules of a fatty acid. From the product Weyl isolated 5.2% of tyrosine, 7.5% of glycocoll, and 15% of a crystalline sublimable compound which was apparently α-alanine. The remaining products (72%) could not be isolated or identified.

The constitution of silk from a chemical standpoint has been considered in detail by Silbermann (*Die Seide*, Band II, 1897). (See also H. Silbermann, *Chem. Zeit.*, 1903, **17**, 1693), and the chemistry of Tussah by Wardle (*Textile Manuf.*, 1891, p. 563).

The decomposition products of fibroïn are given by Fischer and Skita (*Chem. Zeit. Rep.*, 1901, **25**, 274) as follows (per 100 pt.): 10 of *l*-tyrosine, 21 of *d*-alanine (aminopropionic acid), 1.5 *l*-leucine, 1.5 *l*-phenylalanine, 36 of glycine, traces of other amino-acids and a trace of certain diamino-acids.

The same authorities, Fisher and Skita (*Zeit. Physiol. Chem.*, 1902, **35**, 221) state, that serine gives on hydrolysis diamino-acids including arginine and the same monoamino-acids as fibroïn.

[1] Richardson suggests for fibroïn the following constitutional formula, in which X represents a hydrocarbon residue:

$$\begin{matrix} II & NH-CO & II \\ X & & X \\ & CO-NH & \end{matrix}$$

The equation depicting the decomposition which takes place on saponification with potash would then be:

$$\begin{matrix} II & NH-CO & II \\ X & & X \\ & CO-NH & \end{matrix} + 2KOH = 2X \begin{matrix} II & NH_2 \\ & CO.OK \end{matrix}$$

Silk-fibroïn resembles proteins in its behaviour with Millon's and Adamkiewicz' reagents, and with the biuret test.

Richardson found combed and well-purified mulberry-silk to absorb 30% of iodine when treated with Hübl's reagent (Vol. 2). The product retained a deep-yellow colour even after washing with potassium iodide and water. Attempts to acetylate fibroïn gave entirely negative results.

Silk is acted on by dilute nitric acid. When immersed for about a minute in nitric acid of 1.133 sp. gr., at a temperature of about 45°, and then washed thoroughly with water, silk acquires a characteristic yellow colour, fast to light and air. Pure nitric acid (free from nitrous compounds) does not produce the colour, which varies in intensity with the proportion of nitrous compounds present. Nitrous acid, produced by adding hydrochloric acid to a solution of sodium nitrite, dyes silk a pale-yellow colour which is affected by light; but if the silk thus treated be then immersed in dilute nitric acid or in a mixture of hydrochloric acid and potassium permanganate, the yellow colour becomes deeper and permanent to air and light. If this colour be treated with an alkali, the yellow shade is considerably deepened, the shade obtained varying with the base used, being lightest with ammonia and approaching red with baryta. Alkaline carbonates produce the same intensity of colour as the corresponding hydroxides. It is said the alkali cannot be removed by washing. Nitro-silk is slowly decolourised when boiled in a concentrated acid solution of stannous chloride, but alkaline reducing agents appear to have no action. Nitro-silk behaves like ordinary silk with solvents, except that when treated with concentrated sulphuric acid it swells up and gives a sticky mass similar to that given by ordinary egg-albumin. Vignon and Sisley, to whom the above observations on this product are due (*Compt. rend.*, 1891, **113**, 802), analysed two samples of white Canton silk, which had been degummed and purified by successive treatments with boiling soap solution, distilled water, hydrochloric acid, water and alcohol. One of the samples thus purified was "dyed" with nitrous nitric acid, by which treatment it gained 2% in weight. The following figures show the composition of the dyed and undyed fibroïn:

	Undyed silk	Dyed silk
Carbon	48.3%	46.8%
Hydrogen	6.5%	6.5%
Nitrogen	19.2%	21.6%
Oxygen	26.0%	25.1%
	100.0%	100.0%

Action of Solvents on Silk.—Silk is gradually dissolved by cold concentrated sulphuric acid to form a slightly brownish-coloured solution.[1] Hydrochloric acid dissolves it without additional colour to a limpid liquid. Zinc chloride and alkaline solutions of copper and nickel also dissolve silk. Cold concentrated solution of potassium or sodium hydroxide dissolve silk completely, but 2% solutions dissolve the sericin only, leaving the fibroïn. The solutions are not precipitated by dilution with water, but evolve ammonia when heated.

Different varieties of silk differ materially in their behaviour with reagents. Von Höhnel claimed to have effected a quantitative separation of a mixture of real silk, Yama-mai silk, sheep's wool, and cotton into its four constituents, by boiling the mixture with hydrochloric acid for 30 seconds whereby the real silk only was dissolved; while on continuing the boiling for 2 minutes with concentrated hydrochloric acid, the Yama-mai silk passed into solution. On heating the residue with potassium hydroxide solution, the wool was dissolved, while the cotton remained unchanged. The analysis of mixed fibres is described in detail on page 642 *et seq.* Bastow and Appleyard (*J. Soc. D. & C.*, 1888, **4**, 89) observed the following differences between mulberry and Tussah silk:

Treated with	Mulberry silk (*Bombyx mori*)	Tussah silk (*Antheræa mylitta*)
A hot 10% solution of sodium hydroxide.	Completely dissolved in 12 minutes.	Required 50 minutes for complete solution.
Cold concentrated hydrochloric acid (sp. gr. 1.16).	Dissolved almost instantly.	Only partially dissolved in 48 hours.
Cold concentrated nitric acid.	Dissolved in 5 minutes with light-yellow colour.	Dissolved in 10 minutes to a light-brown solution.
Neutral solution of zinc chloride of 1.725 sp. gr.[1]	Dissolved almost instantly.	Required considerable time for complete solution.
Saturated aqueous solution of chromic acid mixed with an equal measure of water.	Dissolved immediately.	Slowly acted on.

It must however be remembered that mulberry silk will dissolve in 10% or even 5% sodium hydroxide solution in a much shorter period (2–3 minutes).

F. Filsinger[2] (*Chem. Zeit.*, 1896, **20**, 324) has shown that mulberry silk is readily soluble in an alkaline solution of copper hydroxide in glycerin, whereas Tussah silk is scarcely affected by this reagent.

[1] By diluting the solution of silk in cold concentrated sulphuric acid with water, boiling, neutralising with milk of lime, and evaporating the filtered solution to dryness, Richardson obtained 15% of glycine, leucine, and tyrosine, and 85% of a substance having the odour and appearance of glue, readily soluble in water, but insoluble in alcohol.

[2] Persoz also states that mulberry silk is almost immediately (in less than 30 seconds) dissolved by a boiling solution of zinc chloride of 1.45 sp. gr., while Tussah silk is only very slowly acted on; and a rough separation of the two silks may be effected by immersing the mixture in the hot reagent for 1 minute, and then removing and washing it, when the residue will consist of Tussah silk only.

Examination of Raw Silk.—In the analysis of raw silks the following results obtained by Gianoli (*Rev. Gen. d. Mat. Col.*, 1906, **10**, 199) are of interest. On the average the matter soluble in 3% soap solution at the boil was from 21.44–25.9. In water at 50–55°, 0.44–1.05 was extracted; in ether from 0.1–0.45%.

This strength of soap solution is in excess of that actually required. In the actual assay of raw silk, for the amount of silk gum present a 1% soap solution is quite satisfactory. This may be followed up by a 0.2% solution if it is thought necessary.

Gnehm (*Farb. Zeit.*, 1903, **14**, 69) has recorded adulteration of raw silk with fat up to 8.5%. It must, however, be remembered that raw silk is very often treated with a strong solution of fatty matters in order that it may spin and weave more easily, but in the case mentioned it seems that the imported raw silk in the original skeins contained the fat.

Optical Activity.—Solutions of silk are lævorotatory. L. Vignon (*Compt. rend.*, 1891, **113**, 802; 1892, **114**, 129) removed the colouring matter from silk (from *Bombyx mori*) by repeated treatment with hot alcohol acidified with hydrochloric acid. The silk thus purified was immersed in a cold 3% aqueous solution of sodium hydroxide, when a yellowish, limpid solution of *sericin* was obtained having an optical activity of $[\alpha]_D = -39.2°$. Silk from the same source was boiled twice with a 10% solution of soap, which was washed away by water after each boiling, the residual silk washed with acidulated water (0.1% of hydrochloric acid), and finally with alcohol. The white *fibroïn* thus obtained was dried and dissolved in moderately strong hydrochloric acid, when a clear viscous solution was obtained having after dilution an optical activity corresponding to $[\alpha]_D = -40°$, which was materially altered by further dilution, or by the addition of excess of ammonia. Solutions obtained by Vignon in a similar manner from other varieties of silk showed a value for $[\alpha]_D$ from $-9°$ to $-43.6°$, mean $-29.6°$, for the alkaline solution of the sericin; and from $-39.4°$ to $-50°$, with a mean of $-44°$, for the hydrochloric-acid solution of the fibroïn. The fibroïn of *Yama-mai* was found to be insoluble in hydrochloric acid; but soluble after some time in cold concentrated sulphuric acid.

Microscopical Characters.—The silk from *Bombyx mori* (mulberry silkworm) and *Saturnia spina* is a homogeneous, hyaline, formless fibroïn thread resembling glass-rod, and rarely exhibits signs of striation. The fibre of real silk is distinguished from its substitutes by the microscope. Its fibre is round, of less diameter than its substitutes with the exception of Thiele artificial silk.

On the other hand, the fibres of Tussah and other "wild" or exotic silks, now much used on account of their low price, consist of bundles of circular threads varying in diameter from 0.0003 to 0.0015 millimetre, (3 to 15μ) which give the main fibre a striated appearance. The fibrillæ are agglutinated by a matter not greatly differing from their own substance, and not capable of being sharply distinguished or easily separated from the fibroïn, as the silk-gum of mulberry silk is from the enclosed fibroïn. Many of the twin fibres of the exotic silks are flattened to a marked extent. The wild silks are darker in colour; Tussah being brown in the natural state (see Fig. 54).

Filsinger (*Chem. Zeit.*, 1909, **20**, 324) describes the cross-section of Tussah fibres as larger and flatter than that of mulberry silk, and showing many fine air-tubes. Characteristic bands frequently cross the fibres in an oblique direction, giving them a miscroscopic appearance similar to that of cotton.

Von Höhnel (*Dingl. polyt. Journ.*, 1885, **146**, 465) has recorded the following characters of raw silk from different sources. The figures refer to the maximum diameter of single threads and are expressed in thousandths of a millimetre:

Kind and origin of silk	Diameter in μ	Colour and microscopic appearance	
		Broad side	Narrow side
Mulberry silk from *Bombyx mori*.	20 to 25.	White or yellowish-white; shining.	White or yellowish-white; shining.
Senegal silk from *Bombyx Faidherbi*.	30 to 35.	Shining yellowish or brownish-white, or pale yellow, grey, brown, and occasionally bluish-white.	Grey, brown, or black, with occasionally lighter shades.
Ailanthus silk from *Bombyx cynthia*.	40 to 50.	Shining yellowish-white, with yellow, brown, brownish-grey spots.	Dirty grey or brown to black, with green, yellow, red, violet, or blue spots.
Yama-mai silk from *Antheræa yama-mai* (Japan).	40 to 50.	Bluish-white, with dark blue, blue, and black shades.	Glaring and fine colours with dark or black shades.
Tussah silk from *Actius selene*(?).	50 to 55.	Irregular in thickness. Thickest parts with grey and blue spots; thinner parts, bluish-white, yellow, or orange-red.	Dark grey, with pink or light-green spots.
Tussah silk from *Antheræa mylitta*.[1]	60 to 65.	Similar to *A. selene*, but spots orange-red, red, or brown.	Similar to *A. selene*.

[1] The cocoons of the larvæ of *Antheræa mylitta* are firm and hard, of a silvery drab colour, egg-shaped, and much larger than those of the true silk-worm. The product is used largely for the manufacture of silk plush and buff-coloured Indian silks.

Other wild silks are the *Eria* silk of India, the *Muga* silk of Japan, the *Fagara* or *Atlas* silk of China, etc.

Woodcuts illustrating the magnified appearance of a number of varieties of silk have been published by Wardle (*J. S. D. & C.*, 1885, 1, 196); and details of the tension, strength, and diameter of the fibres have been recorded by the same author (*J. Soc. Arts*, 33, 671).

The microscopic appearance of Tussah silk has been described and illustrated by Bowman (*J. S. D. & C.*, 1888, 4, 90).

Interesting particulars respecting wild silk have been given by C. Grosseteste (*J. S. D. & C.*, 1888, 4, 155).

Silbermann gives the diameter of silk filaments as follows:

Nature of silk	Average size	Limits of size
Mulberry silk	24 μ.	18–26 μ.
Indian Tussah	45 μ.	40–60 μ.
China Tussah	70 μ.	62–80 μ.

A long series of papers on the chemical composition and structure of different varieties of silks has been given by Strauch (*Zeit. physiol. Chem.*, 1910 and 1911). These may be referred to where details are required as to decomposition products, etc.; similar work has been done by Abderhalden (*Zeit. physiol. Chem.*, 1911, **72**, 1). (Also see Abderhalden and Schmid, and Abderhalden and Walder, *Zeit. physiol. Chem.*, 1910, **64**, 460, and Abderhalden, 1910, **65**, 417 and 1910, **66**, 13.)

Particulars concerning the African wild silks are given by Barwick (*Bull. Imp. Inst.*, 1910, **8**, 150).

The gum of Indian tussah silk gave the following yields of mono-amino-acids calculated on the ash-free material dried at 120°: glycine 11.5, alanine 9.8, leucine 4.8, serine 5.4, aspartic acid 2.8, glutamic acid 1.8, phenylalanine 0.3, tyrosine 1.0, proline 3.0%.

Tussah silk is bleached after degumming in "Bensoap" (1% solution) to which 2–5 kilos of sodium perborate to the 1,000 litres has been added, treatment is carried out at 60–70°. It is then decolourised in a slightly alkaline hydrogen peroxide solution (6 volumes) at 30–40°. After treating with oxalic acid the material is bleached with sodium hydrosulphite. (Beltzer, *Rev. Gen. Mat. Col.*, 1911, **15**, 36.)

Physical Examination of Silk in Mixed Fibres.—The foregoing observations refer to the microscopic appearances of different varieties of silk, but the microscope also serves to readily distinguish silk from cotton, wool, and other textile fibres. The following illustrations indicate the relative appearance of silk and other fibres under a magnifying power of 80 diameters:

Under the microscope, *silk* appears as a slender, homogeneous, solid cylindrical and continuous filament, free from either scales or medullary substance. Sheep's *wool* appears thicker than silk, and has a perfectly circular stalk with tile-shaped scales. *Linen* (flax) is cylindrical and never flat; it is not stiff or twisted, and is characterised by the narrowness of its medullary tube. *Hemp* resembles linen-fibre, being easily broken; its ends branch out stiffly, and its tube is open. *Cotton* fibres are long, flat, and resemble a twisted band. Mercerized cotton is rounder.

Detection of Animal Fibres by Chemical Means.—Animal

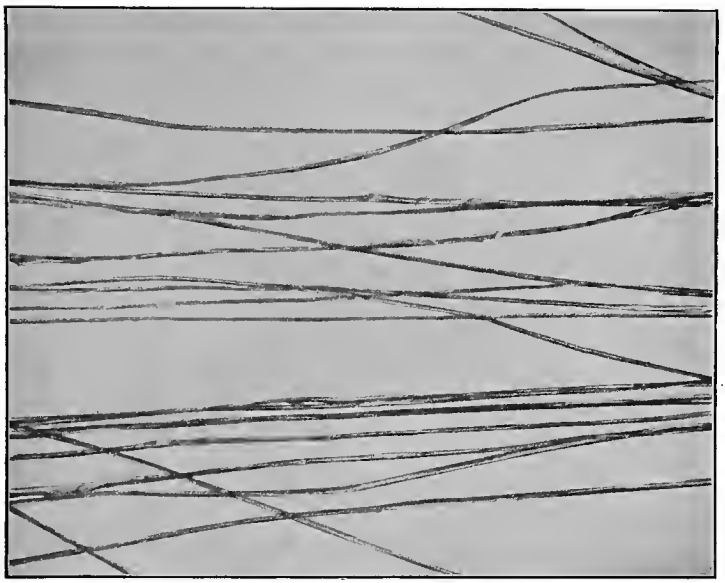


FIG. 53.—Ordinary Silk. × 80. (*Bombyx mori*.)

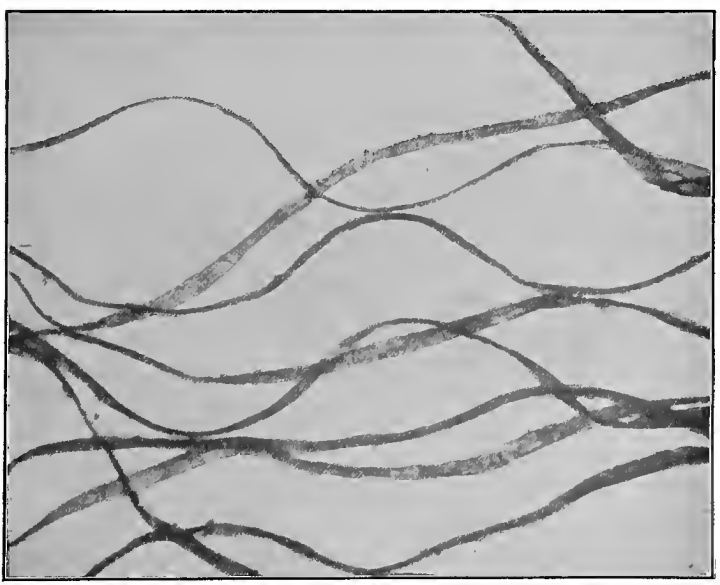


FIG. 54.—Tussah Silk. × 80. (*Saturnidæ*.)

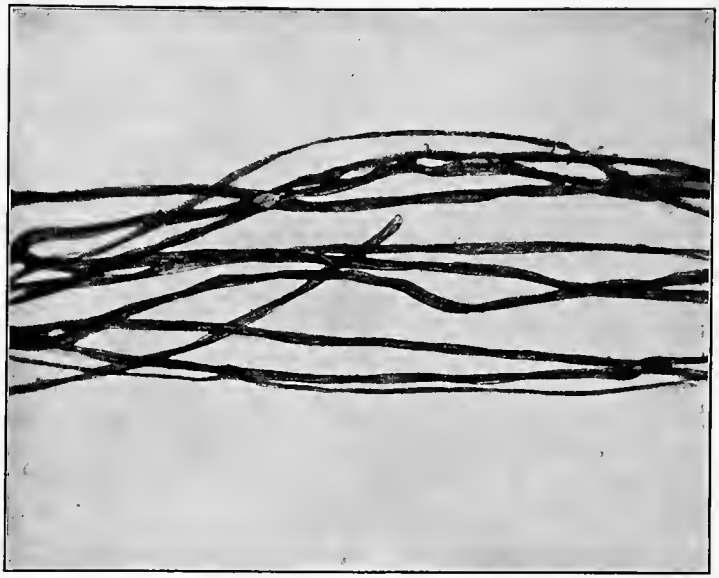


FIG. 55.—Cotton. × 80.

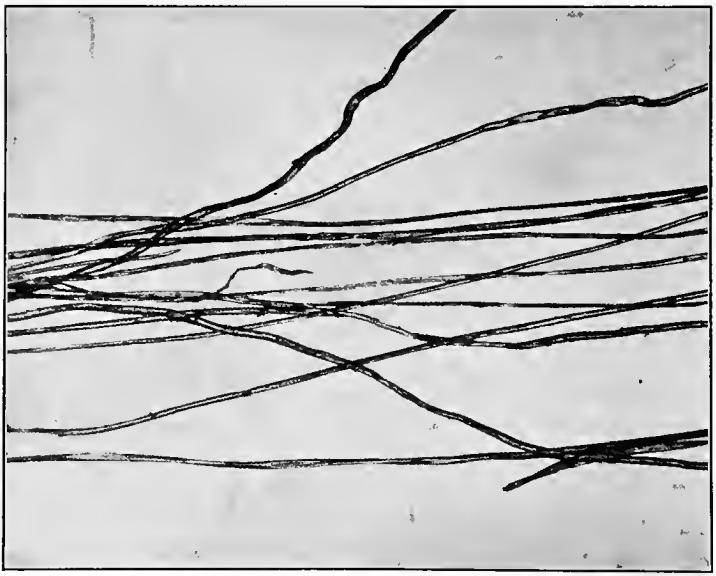


FIG. 56.—Mercerised Cotton. × 80. (*Egyptian.*)

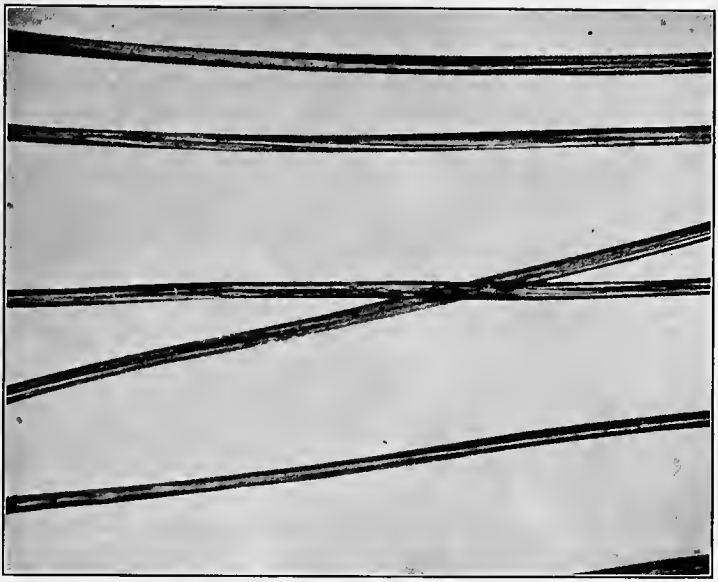


FIG. 57.—Artificial Silk. × 80. (*Glanzstoff.*)

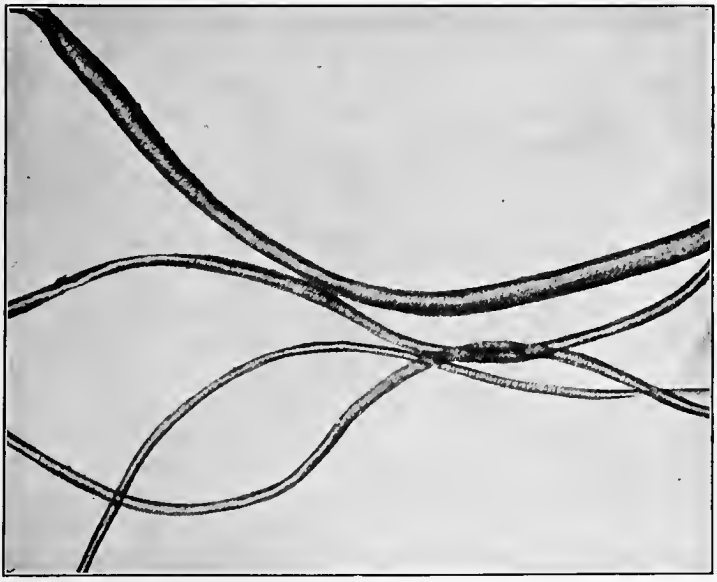


FIG. 58.—Wool. × 80.

fibres, such as silk, wool, and hair, contain nitrogen, and evolve an odour of burnt feathers when ignited. They are dyed by magenta and picric acid without a mordant, and are coloured yellow by nitric acid and red by Millon's reagent. Tussah silk (unbleached) gives a chocolate brown.

The following reactions distinguish silk qualitatively, and to some extent quantitatively, from other fibres.

	Silk, wool, fur, or hair		Cotton or linen
Heated in small test-tube.	Brittle, carbonaceous residue, with smell of burnt feathers. Condensed moisture is alkaline to litmus.		Charring and smell of burning wood. Condensed moisture is acid to litmus.
Boiled in an aqueous solution of picric acid, and rinsed in water.	Dyed yellow.		Unchanged.
Treated with cold nitric acid of 1.2 sp. gr.	Coloured yellow.		No change of colour.
Boiled with Millon's reagent.	Red colouration.		No change of colour.
Moistened with dilute hydrochloric acid and dried at 100°.	Unchanged.		Powders on friction.
	Silk (mulberry)	Wool, fur, or hair	
Heated to boiling with conc. hydrochloric acid.	Dissolved.	Swells, without at once dissolving.	Mostly undissolved.
Boiled with a concentrated solution of basic zinc chloride.	Dissolved.	Unchanged.	Unchanged.
Treated with a cold concentrated solution of ammoniocupric oxide (Schweitzer's reagent).	Dissolved. Not precipitated from the solution by salts.	Undissolved. Dissolves on heating.	Dissolved slowly. Solution precipitated by salts.
Treated in the cold with 10% caustic soda.	Undissolved.	Dissolved.	Undissolved.
Boiled with 2% sodium hydroxide solution.	Dissolved. Solution not darkened by lead acetate; negative reaction with sodium nitroprusside.	Dissolved. Solution gives black or brown precipitate with lead acetate, and violet colour with sodium nitroprusside.	Unchanged.
Behaviour with Molisch's test.	Dissolved; with little colouration.	Undissolved; yellow or brown colouration.	Dissolved; deep violet colour.

Reactions for distinguishing mulberry silk from Tussah silk are given on page 652.

Lecompte's Test Modified.—The fibre or fabric is treated with an acid solution of nitrous acid, prepared by adding excess of hydrochloric acid to a 1% solution of sodium nitrite. The operation is conducted in the dark. After washing it is introduced into an alkaline solution of β-naphthol, prepared by dissolving that substance in a weak solution of sodium hydroxide to which lead acetate has been added to produce lead plumbite. Wool is coloured black by the interaction of the sulphur

present with the lead. Silk is dyed a deep red and cotton or artificial silk remains colourless. The writer finds that the time taken to "diazotise" the fibre must not be less than 30 minutes, and the sodium nitrite solution will act more readily if it be increased to 5%. The free acid must be washed out, before the sample is immersed in the β naphthol-lead acetate solution. A temperature of 100° is necessary to develop the red shade on the silk to its maximum when it is of a dull purple colour.[1]

Dreapers Test.—In order to obviate the difficulties connected with Lecompte's test, the following solution is proposed: A solution containing 2 grm. of acetate of lead, 2 grm. of sodium hydroxide (fused), and 2 grm. of picric acid or 0.3 grm. magenta per 100 c.c. is boiled with the fibre to be examined for two minutes, which is then washed. A silk fibre will be dyed yellow or red respectively; wool will be dark brown or black; cotton will remain colourless. When a thread is thought to contain a mixture of these fibres, it is best examined under the microscope, when the colour test will at once confirm the ordinary microscopical examination. This test may of course be made in daylight and only entails the use of one solution. Artificial silks remain white. (*J. S. D. and C.*, 1913, **29**, 78.)

Grothe (*Zeit. Analyt. Chem.*, 1864, **3**, 153) distinguished silk from wool by the following reactions. The fibre is heated to 130°. Under these conditions wool turns to a golden yellow shade, gives an odour of carbon disulphide and ammonia, and curls up. Silk does not colour under 140° and does not curl. When the fibre is moistened with potassium hydroxide, and then dipped into a solution of copper sulphate, and exposed to the air, wool rapidly turns brown through the formation of cupric sulphide, while silk or cotton remains colourless. Wool dissolved in sodium hydroxide and neutralised with tartaric acid gives with copper sulphate a dark brown colour, while silk gives a fine violet colouration.

Liebermann's Test—Sodium hydroxide is added to a concentrated solution of magenta until the solution is colourless. After filtering, the fibre is dipped into the liquid, withdrawn and well washed with water. Wool or silk dye a red shade, while cotton remains colourless. The colour test is rendered more delicate by immersing the fibre in dilute acetic acid, after washing it in pure water.

Liebermann's reaction and that with picric acid may be employed to render visible the animal fibres in a mixed yarn or fabric. Wool (and hair) fibres may be rendered visble by immersing the material

[1] The writer has varied this test by boiling the sample in the nitrous acid solution for two minutes in ordinary light instead of the longer process in the dark (*J. S. D. and C.*, 1913, 29, 78).

in a dilute boiling solution of sodium hydroxide, to which a little lead acetate has been added. The wool will be turned brown or black from the formation of lead sulphide, while silk and vegetable fibres are unaffected.

In mixtures of silk and wool the writer finds that treatment with concentrated sulphuric acid under the microscope may give valuable information. A thread is placed on a micro slide and covered with an ordinary circular cover. While the sample is being examined a drop of concentrated sulphuric acid is dropped onto the outer edge of the cover. By capillary action it follows the line of the fibre and any silk present is dissolved within 2 minutes leaving the wool intact. The actual process of solution may be observed.

The foregoing tests are of value chiefly for the preliminary examination of undyed fibres or after dyes have been removed.

Quantitative Estimation of Silk.

Various methods of *determining the proportion of silk* in mixed fabrics have been proposed, but most of them are imperfect, and the best are not free from errors, owing to the absence of any reagent which will dissolve either cotton, silk, or wool, without at the same time more or less attacking the other two fibres.

Silk has been estimated in plush by boiling the fabric with a 2% solution of sodium hydroxide, but this reagent acts to a serious extent on cellulose, especially in presence of air. A reagent sometimes recommended for the solution of silk is prepared by dissolving 16 grm. of copper sulphate in 140 to 160 c.c. of water, and adding from 8 to 10 grm. of pure glycerol. A solution of sodium hydroxide is then gradually poured in until the precipitate first formed redissolves, excess of alkali being avoided. It was originally stated that this solution has no action on wool and cotton, while it readily dissolves silk, but Richardson observed that when heated with the copper solution for 20 minutes (the time necessary to dissolve silk from plush), purified cotton lost from 1 to 1.5% of its weight, and became friable and tended to dust out; while woollen fabrics, treated in a similar manner, lost from 9 to 16% of their weight. Hence the reagent is useless for the analysis of fabrics containing wool.

From the solution of cotton or silk in ammonical copper oxide solution, the addition of salts will cause a precipitate in the case of cotton, but not in that of silk which is only thrown out by dilute acids.

Persoz observed that silk dissolved in a boiling solution of oxychloride of zinc, and on this reaction von Remont based the following method of analysing mixed fabrics containing silk, wool, and cotton. Four

quantities (A, B, C, and D) of two grm. each of the air-dried material are weighed out. One of these (A) is kept, and each of the other three boiled for 15 minutes in 200 c.c. of water containing 3% of HCl. The liquid is decanted, and the boiling repeated with more dilute acid. This treatment removes the size, and in some cases the colouring matter. Cotton may be decolourised very quickly, wool less readily, and silk but imperfectly. Light shades on silk can be neglected, but the black dye on silk often forms a considerable part of the weight of the fabric. Its incomplete removal is indicated by the ash left on igniting the fabric containing iron, a test which is more reliable than the incomplete decolourisation of the stuff, since this may occur when only an insignificant amount of colouring matter remains. The acid is removed from the material by washing and pressing, and portion B is then laid aside. To remove the silk, portions C and D are then next placed for 2 minutes in a boiling solution of basic zinc chloride of 1.72 sp. gr.,[1] then thrown into water, and washed first with water acidified with 1% of hydrochloric acid and then with distilled water until the washings are free from zinc. Portion C is pressed and laid aside. For separation of the wool and cotton D is boiled gently for 15 minutes with 60 to 80 c.c. of sodium hydroxide solution of 1.02 sp. gr., and then washed very carefully in water, taking care not to destroy the vegetable fibre. The 4 portions, A, B, C, and D, are then dried for an hour at 100°, and left till the following day fully exposed to the air, so that they may absorb the normal amount of hygroscopic moisture. If a, b, c, and d are the weights of the portions represented by the corresponding capital letters, then:

$a-b$=dye and finish; $b-c$=silk; $c-d$=wool; and d=vegetable fibre. Some analysts make an allowance of 5% on the weight of d, to compensate for loss by the action of the alkali. In the case of a black silk resisting the action of dilute acid, its composition can be arrived at by the method described on page 656.

The foregoing process has been critically examined by Richardson (*J. Soc. Chem. Ind.*, 1893, **12**, 430); working on the air-dry material, and subsequently exposing the treated portions overnight to the air, is apt to lead to misleading results; therefore the material should be thoroughly dried at 100° before being weighed, and the treated portions weighed after being dried at the same temperature.[2] Boiling with water containing 3% of hydrochloric acid acts too generally on the wool and

[1] The reagent is prepared by dissolving 1,000 grm. of zinc chloride in 850 c.c. of hot water, adding 40 grm. of zinc oxide, and heating the mixture until solution is complete.

[2] As textile fibres are hygroscopic, the usual precautions must be adopted to prevent the material from reabsorbing water during weighing.

cotton to render such treatment desirable. Boiling for 10 minutes with 1% hydrochloric acid is more satisfactory. Richardson dipped the material two or three times into the boiling zinc chloride solution, taking care that the total time of immersion did not exceed 1 minute. It is necessary that the zinc solution should be sufficiently basic and concentrated to obtain satisfactory results. Even then, purified cotton loses about 0.5%, and purified wool from 1.5 to 2.0% of its weight.

An ammoniacal solution of nickel oxide has been recommended by Richardson (*J. Soc. Chem. Ind.*, 1893, **12**, 430) as a reagent for separating silk from wool and cotton in mixed fabrics.[1] Silk dissolves rapidly even in the *cold*, and after treatment for 2 minutes, which is the time necessary to dissolve the silk from fabrics other than plush, purified cotton lost 0.45, and purified wool 0.33%. In order to dissolve silk from plush, it is necessary to boil the material with the nickel solution for 10 minutes under a reflux condenser. Cotton loses 0.8% of its weight when thus treated. In the writer's hands this method has given satisfaction.

For the analysis of *plush*, Richardson recommends a brief exposure of the fabric to the action of a boiling solution of basic zinc chloride, as already described, but for the determination of silk in light fabrics, especially when wool is also present, exposure from 1 to 3 minutes (according to the nature of the material) to a cold solution of ammoniacal nickel oxide is to be preferred.

Richardson mentions the case of a plush which contained rather more silk than cotton, and gave the following results:

	By ammoniacal nickel solution	By basic zinc chloride solution	By copper glycerin reagent
Moisture, dye, and finish	11.34	11.00	10.04
Silk	45.60	45.00	47.06
Cotton	43.06	44.00	42.90
	100.00	100.00	100.00

Plush backs are much harder than most cotton fabrics, and successive treatment with acid and with the copper-glycerol reagent gives good results; but the copper reagent is not suited for use with other cotton goods, and wool is dissolved by it to a very serious extent. Richardson found a mixed woollen and silken fabric, after being thoroughly

[1] The reagent is best prepared by dissolving 25 grm. of crystallised nickel sulphate in about 80 c.c. of water, 36 c.c. of a 20% solution of sodium hydroxide is then added, and any excess of alkali carefully neutralised by dilute sulphuric acid. The precipitated nickel hydroxide is redissolved in 125 c.c. of strong ammonia, and the solution made up with water to 250 c.c.

cleansed from extraneous matter and dried, gave with the nickel reagent 25.4% of silk, and with basic zinc chloride 27.0%. He considered the former result the more accurate, as the zinc solution acts to a greater extent on the wool.

Fibre	Fibres actually present; %	Percentage obtained by		
		Ammoniacal nickel solution	Basic zinc chloride solution	Copper-glycerol reagent
Silk	5.84	5.92	5.52	18.80
Wool	76.31	76.58	80.08	64.05
Cotton	17.85	17.50	14.40	17.15
	100.00	100.00	100.00	100.00

In fabrics containing silk, wool, and cotton, the silk may first be dissolved by treatment with the nickel reagent. The portion left insoluble is first treated with very dilute hydrochloric acid (1%), and the wool then dissolved by boiling for 7 minutes with a 2% solution of sodium hydroxide. The foregoing results were obtained from a material which had been very imperfectly dyed, and the constituent fibres of which were easily separated and weighed. It will be observed that fairly accurate results were obtained by the nickel reagent, but not by the copper nor the zinc solution.[1]

The ammoniacal nickel solution has been employed by Merritt Matthews with satisfactory results. The following are the details of procedure, which differ in certain respects from those given above: the yarn or fabric is cut up very fine with a pair of scissors, and thoroughly dried at 100°. 1 grm. of the material thus prepared is treated with 40 c.c. of the cold ammoniacal nickel solution for 2 minutes. The liquid is then filtered, and the residue, consisting of the wool and cotton of the sample, is digested for 2 or 3 minutes in 1% boiling hydrochloric acid, washed free from acid, dried at 100°, and weighed. To dissolve the wool from the cotton, the residue is boiled with about 50 c.c. of a 1% solution of potassium hydroxide for 10 minutes, and the solution filtered. The residue, which consists of *cotton*, is washed free from alkali, dried at 100°, and weighed.

The foregoing method assumes that silk-gum, dyes, and weighting materials have been previously removed. To remove the silk-gum and possibly the dye, the material may be boiled in a 1% solution of soap for one hour, but Richardson has considered that its complete

[1] Richardson considered the solvent action of basic metallic solutions on silk to be due to dissociation of the amino-anhydride of which the fibre is composed, with formation of soluble metallic amino-compounds.

removal is best effected by treatment in the cold with a 2% solution of potassium hydroxide. This has the advantage of decomposing Prussian blue, and facilitating the subsequent removal of iron by 1% hydrochloric acid. After this last treatment the material should be thoroughly washed and dried. Metallic mordants are apt to be imperfectly removed, and their amount must be deduced from the ash left on ignition and subtracted from that of the other constituents. It is sometimes advisable to boil dyed fabrics with methylated spirit and then with ether, to remove certain dyes and oily matters.

No general method for the removal of colouring and weighting materials from fabrics can be suggested. General information on the subject will be found in the following pages.

Recognition of Tussah Silk.—The published tests which distinguish ordinary silk (*Bombyx mori*) from Tussah, cannot be considered as altogether satisfactory, but the writer would place reliance on the results obtained in the following general examination.

Microscopical appearance of the fibre as compared with ordinary silk.

Millon's reaction which gives a chocolate brown colour with degummed Tussah, instead of red with ordinary silk.

Amino Acid Test which gives a deep brown with degummed Tussah, in place of bright red with ordinary silk.

Solubility in Nitric Acid (conc).—Tussah silk as present in a fabric (degummed) dissolves at the boil in 30 seconds and gives a darker colour to the solution than ordinary silk.

Solubility in Hydrochloric Acid (conc) or 5% Sodium Hydroxide Solution.—Ordinary silk completely dissolves in each case at the boil within 5 minutes. Tussah silk does not dissolve, but the filaments become very transparent as compared with cotton, which latter fibre may actually powder down in the acid solution (see page 639).

Dyeing and Weighting Silk.

Silk has a great affinity for the coal-tar colours, which dye it without the use of any mordant, though it is customary to use a soap-bath (boiled-off liquor), with or without the addition of acetic or other weak acid. Indigo-sulphonic acid is used for indigo tints. For black, the silk is treated with ferric acetate, washed and immersed in a bath of potassium ferrocyanide, followed by another iron bath, washing, and immersion in a catechu or other tannin bath, followed by one of logwood and soap.

A systematic method devised by Martinon, for recognising the nature

of dyes on silk, is reproduced in Vol. 5, page 513 *et seq.*, where other methods are also given which apply to animal fibres.

Determination of Weighting in Silk.

In addition to legitimate processes of dyeing, silk is subjected to operations having for their object the introduction of weighting materials. This practice of adulteration is carried out systematically and to an extraordinary extent, the adulterants sometimes amounting to more than the weight of the true silk.[1]

Carter Bell (*J. Soc. Chem. Ind.*, 1897, **16**, 304) gives the following analyses of unweighted and weighted silk.[2] The standard taken for silk was pure boiled-off silk, dried at the ordinary temperature, and assumed to contain 18% of nitrogen.

	Moisture	Ash	Silk
1. White silk	5.1	0.349	100
2. Salmon-coloured Shanghai	5.5	0.246	100
3. Black	7.9	18.7	25
4. White	8.2	50.0	25
5. Shot silk	6.2	34.7	39
6. —	10.2	33.7	32
7. Pink silk	8.5	43.2	54
8. Pink silk	9.4	52.4	43
9. Pink silk	8.1	49.5	46
10. Blue	6.5	35.4	60
11. Gold colour	5.8	41.7	52
12. Cream colour	9.1	45.7	46
13. Pink and green	8.6	34.2	50
14. Pink	8.6	28.2	60
15. Cream	9.3	8.9	87
16. Yellow	8.6	9.1	86
17. Black	8.5	42.9	12
18. Sky-blue	9.1	48.9	37
19. —	8.1	45.9	36

[1] The history, objects, and effects of silk-weighting have been fully discussed by Wardle and Carter Bell (*J. Soc. Chem. Ind.*, 1897, 16, 297).

M. J. Langdon (*J. Soc. Chem. Ind.*, 1897, 16, 405) suggested that the adulteration of silk by weighting should be limited by arrangement between the dyers to the comparatively moderate amounts of 16 to 84 ounces for 16 ounces of silk, according to the nature of the material.

J. Carter Bell (*J. Soc. Chem. Ind.*, 1897, 16, 303) has described a case in which 100 pounds of silk were sent to the dyer with the request that he would weight it to 1,000 pounds. This being done, the weighted silk contained 9% of moisture, under 2% of nitrogen (corresponding to less than 10% of silk), and yielded 43% of ash

In 1897, the silk-weavers of Zurich entered into an agreement for one year with the Swiss silk-dyers, forbidding, on pain of heavy fines, the weavers of import or use, and the dyers to dye, any silk, whether of home or foreign origin, that had been weighted to such an extent as seriously to impair the strength of the fibre. Following this example, the silk-dyers of Crefeld (Germany) agreed that the maximum degree of weighting in the future should be 30 to 40% for taffetas, and 50 to 60% for other kinds of silk (*J. Soc. Chem. Ind.*, 1897, 16, 532).

Highly-weighted silk burns without flame when heated. It smoulders slowly away, leaving the original form intact. Instances of the spontaneous combustion (smouldering) of black silk are on record. (*J. Soc. Chem. Ind.*, 1897, 16, 304).

[2] The practice of weighting silk probably had its origin in the desire to make up the loss of weight (amounting to about 25%) which occurs in the boiling-off process, and competition has increased the amount of weighting materials introduced till they have reached the present proportions.

The sp. gr. of silk has been used by Vignon (*Bull. Soc. Chem.*, 1892, **7**, 247) as a means of ascertaining the proportion of weighting materials present, but the practical utility of the method is lessened by the necessity of knowing the nature of such weighting materials. Vignon ascertains the sp. gr. of the fibre, in its ordinary air-dry condition, in benzene, after removing occluded air by means of the air-pump. The sp. gr. of some textile fibres were thus determined by Vignon and by Silbermann (*Chem. Zeit.*, 1904, **18**, 744) with the following results: Wool, 1.28 to 1.33; cotton, 1.50 to 1.55; mohair (combed), 1.30; hemp (carded), 1.48; ramie, 1.51 to 1.52; linen (spun), 1.50; jute (spun), 1.48; silk (raw), 1.30 to 1.37; boiled-out silk, 1.25. The sp. gr. of silk varies in course of treatment by dyeing, bleaching, and weighting, and in course of mechanical treatment by stretching, soupling, etc.

In order that data as to the approximate amount of weighting present in any given silk may be deduced from its sp. gr., the nature of such weighting and the process of dyeing used must be known, at least approximately.

The theory of silk weighting has been fully discussed by Heermann (*Chem. Zeit.*, 1911, **34**, 829).

The materials used for weighting silk are of varied character. The silk may be immersed in some solution of a metallic salt and then transferred to a bath of salt which will react to form an insoluble precipitate on or in the silk-fibre. Thus iron is precipitated as ferrocyanide, gallotannate, and catechu-tannate; tin as catechu-tannate, tungstate, phosphate, silicate, oxide, etc. The use of tin in the form of diammonium stannichloride, $(NH_4)_2SnCl_6$, commonly called "pink salt," and its precipitation in the fibre as phosphate and silicate, has increased enormously of late years. Chromium compounds are also used, besides sulphates of sodium, magnesium, and barium, and various organic matters (*e.g.*, gelatin, dextrose, logwood, and tannins).[1]

Silk may be weighted with tin in the following manner and this may give the analyst some idea as to the nature of the weighting material present on the yarn. The silk is soaked for 1 hour in stannic chloride solution of 30–35° Bé. After washing it is introduced into 4° Bé. phosphate of soda in which it is raised to the boil. It is then heated in a 5° Bé. sulphate of aluminium bath for 15 minutes, and this is followed by a warm bath of 5° Bé. silicate of soda. This sequence of operations is repeated as often as desired, great care being taken to thoroughly wash the yarn in water between each operation. The

[1] Salts of nearly all the commoner metals have been proposed for use as weighting materials. Detailed information on the methods of weighting silk will be found in articles by Silbermann (*Farb. Zeit.*, ,1896 **8**, 34, 51, 68).

final bath is one of a very fat soap at 40°. In the case of tannin weighting in light colours the silk is steeped in a 4° Bé. pure gall extract (bleached).

In some cases it is necessary to identify and separately determine the various weighting materials present in a sample of silk, but in other cases it is sufficient to know their total weight. For the estimation of such additions as oil, sugar, soap, etc., 5 grm. of the sample of silk should be dried at 100°, and then extracted in succession with ether, alcohol, and boiling water; drying and noting the loss of weight after each treatment.[1]

The percentage of weighting materials is usually expressed on the raw silk, and not on the boiled-off silk. As the raw silk loses 25% of silk-gum in the process of degumming, it follows that 75 parts of conditioned silk represent 100 of raw, and this amount is allowed for in stating the proportion of weighting materials found.

The increase of the volume of silk by dyeing is in practice more important than the increase of weight, and thus the desired effect is produced better by the use of tannin than by the introduction of excessive proportions of metallic compounds.

In examining *white silk*, the total *soluble weighting materials* should be determined by treating a known weight of the sample 4 or 5 times with hot water, washing it thoroughly, and drying it. In consequence of the great and variable hygroscopic character of silk, to obtain

[1] Silbermann (*Chem. Zeit.*, 1894, 27, 744) recommends the following method for the recognition of the nature of the weighting and dyeing materials present in silk: Readily-soluble materials, such as cane-sugar, dextrose, glycerin, magnesium salts, etc., are estimated directly by boiling the silk with water, and testing the extract with Fehling's solution, etc. From 2 to 3 grm. of silk are ignited, and the ash tested for tin (present in the fibre as basic chloride and stannic acid), chromium, iron, etc. Fatty matters, wax, and paraffin are detected by extraction with ether or benzene. The silk is soaked in warm, dilute hydrochloric acid (1:2). If the fibre be almost decolourised by this treatment, only a slight yellow tint remaining, while the solution assumes a deep brownish colour *not* changed to violet by lime-water, it is safe to conclude that the silk has been dyed by alternate passage through baths of iron and tannin. The yellow colour of the fibre is due to a residuum of tannin, and the precise shade (from greenish to brownish-yellow) enables a rough idea to be formed as to the nature of the tanning material used (sumach, divi-divi, catechu, etc.). Decolourisation of the fibre, the acid extract being pink, changed to violet by lime-water, indicates a logwood-black (so-called "English black"). If the fibre retain a deep greenish tint and the solution be yellow and unaffected by lime-water, the black is dyed on a Prussian blue ground. If the latter, as is often the case, has been produced during the final stage of dyeing, this will be shown by its solubility in the acid. A green fibre and pink solution, altered to violet by lime-water, point to a logwood-black on a Berlin-blue ground. In the hydrochloric acid solution, metals, such as lead, tin, iron, chromium, and aluminium, may be determined. Blacks produced by artificial dyes on an iron-tannin or iron-blue catechu ground, are recognised by the colouration imparted to acid and caustic soda solutions. As regards blacks produced solely by the agency of aniline-dyes, etc., treatment with a hydrochloric acid solution of stannous chloride does not affect aniline and alizarin blacks; naphthol-black is altered to a reddish-brown colour, while wool-black becomes yellowish-brown. Aniline and alizarin blacks may be distinguished by means of sulphurous acid, which attacks only the former, turning it greenish. Tannin substances in general may be extracted by alkaline solutions and subsequently precipitated and distinguished by ferric acetate. To remove the whole of the weighting material and dye, it is recommended to boil the silk with acid potassium oxalate, wash with dilute hydrochloric acid, and finally treat with sodium carbonate solution. When iron and tin are both present in the fibre, it is well to extract the tin previously by means of an alkali-metal sulphide. In conclusion, Silbermann gives a number of experimental results, which exhibit the relation existing between the density of silk and the percentage and nature of the loading material. See also Vignon, *Bull. Soc. Chim.*, 1892, 7, 249).

reliable results it is desirable to determine this. After treatment with water and redrying, the samples are placed together under a clock-glass, and when the standard silk has regained its original weight and washed sample is reweighed, the loss represents the matters soluble in water. An aliquot part of the solution may be evaporated to dryness and the residue weighed, to obtain a direct estimation of the matters dissolved. Unweighted silk loses on the average 25% of its weight on boiling off, so that only the loss in excess of this must be regarded as due to weighting, unless the additions be actually identified and determined by other means. *Dextrose* may be directly determined in the solution by Fehling's solution, and *cane-sugar* after inversion. *Sulphates* and *chlorides*, *magnesium*, etc., may be detected and determined as usual. *Stannic oxide* will be left on igniting the silk in porcelain after the above treatment. In its presence, the material burns with difficulty, and the residue retains the shape of the original silk. The weight of the ash (assuming it to be wholly SnO_2) may be calculated to the form in which the tin exists in the weighted silk, namely SnO_2,H_2O, by multiplying it by the factor 1.12.[1] It may be identified as stannic oxide. The possibility of the tin being present as insoluble compounds must also be considered.

The general estimation of weighting in silks is dealt with by Persoz, (*Rev. Gen. d. Mat. Col.*, 1906, **9**, 322).

For the further examination of white silk, Silbermann (*Chem. Zeit.*, 1896, **20**, 472) recommends the following procedure:

A weighed portion of the silk is boiled with dilute hydrochloric acid to decompose any tannin-lakes of tin or other metals, and in the solution tannin is tested for by the addition of an excess of sodium acetate and a ferric salt. If tannin-lakes be present the determination of the weighting materials consists in: (1) Removing tannin from the aqueous solution with gelatin; (2) estimation of tannin in this precipitate, and of sugar, etc., in the filtrate; (3) successive treatment of the silk with dilute hydrochloric acid and sodium carbonate, and precipitation of tannin from both solutions by means of "gum" solution (gelatin?); (4) ignition of the silk and determination of metallic weighting. If the ash be not completely soluble in hot, moderately concentrated hydrochloric acid, it may contain barium sulphate or silica.[2]

To calculate the percentage of weighting material, W, in the silk

[1] A simpler method for the detection of tin is to heat the silk with just sufficient hydrochloric acid to effect its complete solution, dilute the liquid with water, filter, and pass hydrogen sulphide, when any tin will be precipitated as yellow stannic sulphide.

[2] The ash may also contain stannic phosphate and tungstic oxide. It is doubtful whether treatment with hydrochloric acid can be relied on to remove stannic oxide and actual fusion in the ordinary manner with suitable salts may have to be resorted to.

examined, the following formula is employed in which a is the weight of the sample before treatment, b the weight after extraction with water, p the SnO_2 left on ignition, and d the loss of weight during the boiling of the fibre itself. This is taken at 20 to 25 for boiled-out silk ("cuit"), 5 to 9 for souple silk, and 0 to 2 for écru:

$$W = \frac{a(100-d)}{b-1.13p} - 100$$

Dark-coloured and black silk may contain hydroxides of tin, iron, and chromium, fatty matters, tannin, Prussian blue, and various other colouring matters. Fatty matters may be removed and determined by treatment with ether. Treatment of the silk with hydrochloric acid (1.07 sp. gr.) at 50 to 60° dissolves the logwood and leaves the silk a maroon colour in the absence of Prussian blue, or blue black if it be present. In the latter case, on then treating the silk with dilute caustic soda, a solution containing ferrocyanide will be obtained, which will yield a precipitate of Prussian blue when acidified with hydrochloric acid and treated with ferric chloride. The metallic oxides will be contained in the ash left on igniting the silk, and are best examined by fusing the residue in platinum or silver with nitre and sodium carbonate and treating the product with water, when the tin and chromium will be dissolved as stannate and chromate respectively, and the iron will remain insoluble. From the acid filtrate the tin may be precipitated by hydrogen sulphide, and the chromium thrown down from the filtrate by ammonia, or determined by other known means. For the detection of free *tannin*, a portion of the sample should be boiled with water, and a small quantity of ferric acetate solution added, when a blue-black liquid is produced in presence of tannin. Or the sample may be boiled with very dilute hydrochloric acid, and the liquid tested with gelatin solution or ferrous sulphate. Free tannin may be determined by dissolving it from the silk by passing it through an alkaline soap bath at 95°, and finding the loss of weight on redrying.[1]

To rapidly determine the *total proportion of weighting materials*, a known quantity of the silk, previously dried at 110°, should be boiled for 10 minutes with a 2% solution of sodium carbonate and then with dilute hydrochloric acid (250 grm. of the commercial acid per litre).

[1] The following is an outline of the method of analysing weighted silk recommended by E. Kōnigs, Director of the Silk-Conditioning Establishment at Crefeld: (1) Estimate moisture by drying. (2) Fatty matters by extraction with ether. (3) Boil out silk-gum with water. (4) Dissolve out Prussian blue with alkali, reprecipitate with acid and ferric chloride, and ignite precipitate with addition of HNO_3; 1 part of $Fe_2O_3 = 1.5$ parts of Prussian blue. (5) Estimate SnO_2 present in ash of silk and calculate as catechu-tannate of tin; one part of $SnO_2 = 3.33$ parts of catechu-tannate. (6) Estimate total Fe_2O_3, subtract that present as Prussian blue, and the amount naturally in the silk (0.4 to 0.7 %), and calculate the remainder to tannate; 1 part of $Fe_2O_3 = 7.2$ parts of ferric tannate (or 5.1 %, if present as a ferrous compound).

This treatment is repeated up to four times, washing the material between each treatment. Experience will indicate the treatment that silks will stand in this direction. The silk, which will now have become very brittle and must be carefully handled, is dried at 110°, and weighed.

If souple or écru silk be under examination it should be subjected to a final boiling with soap before drying.

By the foregoing treatment all foreign substances are removed, except mere traces of tannin and colouring matters, and in some cases small quantities of metallic compounds. The weight of the ash left on igniting the treated silk, if multiplied by 1.25, will represent pretty nearly the hydrated metallic oxides retained in the washed silk.

A certain loss of silk-substance occurs in the treatment, and hence the proportion of weighting materials found in the sample is somewhat in excess of the truth. But the chief source of error lies in the uncertainty of the allowance to be made for loss in the weight of the silk by "boiling off." For boiled-off silk this (d) is taken at 25%; for souple silk at 8%; for écru at 0. If p be the original weight, and D the weight after boiling, the percentage of weighting, W, may be found by the following equation:

$$W=\frac{(100-d)\times(p-D)}{D}$$

In cases where the treated silk leaves a sensible quantity of ash, a, on ignition the following equation must be substituted:

$$W=\frac{(p-D+1.25a)\times(100-d)}{D-1.25a}$$

The foregoing process is tedious and not very accurate. A preferable plan is to determine the total nitrogen by Kjeldahl's process, after removing any gelatin, Prussian blue, or other extraneous substances containing nitrogen. This is effected by boiling a weighed quantity of the silk (1 or, preferably, 2 grm.) with a 0.2% solution of sodium hydroxide for 1/2 hour.[1] The silk is then removed, washed, and heated to 60° for 1/2 hour in water containing 1% hydrochloric acid, and well washed with hot water. The treatment should be repeated until the silk no longer retains a blue colour. In the case of souple and écru silks, ammonia or ammonium carbonate should be substituted for sodium hydroxide and the discharged silk should be subjected to a final boiling for an hour with a 1% soap solution to remove any silk gum present.

[1] Prussian Blue may be removed by boiling the silk in a solution of acid oxalate of potassium.

Air-dried silk with 11% of water contains 17.6% of nitrogen, and hence the silk in a sample free from Prussian Blue, etc., can be found by multiplying the percentage of nitrogen by 5.68.

Estimation of Prussian Blue.—The actual estimation of Prussian Blue may be carried out by the following means (Williams and Dreaper, *J. Soc. D. and C.*, 1912, **28**, 336) whereby the whole of the nitrogen in the Prussian Blue is converted into hydrocyanic acid and distilled off into $N/10$ sodium hydroxide.

The actual process is as follows: A weighed quantity of the silk is distilled with a convenient quantity of 10% solution of sulphuric acid to which is added 0.1 grm. of pure cuprous chloride dissolved in a few drops of concentrated hydrochloric acid. The hydrocyanic acid is distilled into $N/10$ sodium hydroxide (or even $N/100$) and the solution afterwards treated with $N/10$ silver nitrate. The number of c.c. of $N/10$ silver nitrate required plus 0.01355 represents the actual weight of hydrated Prussian Blue present.

The accurate estimation of the Prussian Blue has the further advantage of yielding under certain conditions a correspondingly exact figure for the pure silk fibre present. By deducting the calculated amount of nitrogen present in the Prussian Blue, from the total nitrogen, as usually determined by Kjeldahl's method, this figure represents in the known absence of any nitrogenous dressing or "silk gum" the nitrogen due to the silk (fibroin) itself.

Any insoluble, or other dressing material, and "silk gum" present must be removed by boiling for say half an hour in 2% sodium carbonate solution (or as we prefer it, 1% sodium carbonate solution and 1% soap solution) which will also remove the Prussian Blue.

In the case of a sample of raw silk dyed black, this last treatment must be repeated. In this way nitrogen figures for the silk, the Prussian blue, and also possibly any nitrogenous dressing or "silk gum" present, can be obtained.

The following results have been obtained in practice:

Experimental Results.

(1) Black weighted silk distilled direct with dilute sulphuric acid and cuprous chloride=

$$\underset{3.68\%}{Fe_4^{III}(Fe(CN)_6)_3} = \underset{5.22\%}{Fe_4^{III}(Fe(CN).2H_2O)}$$

(2) The same extracted repeatedly with a 2% solution of sodium

carbonate, a portion of the extract distilled with dilute sulphuric acid and cuprous chloride=

$$Fe_4^{III}(Fe(CN)_6)_3 \quad Fe_4^{III}(Fe(CN).2H_2O)$$
$$3.67\% \quad = \quad 5.21\%$$

(3) The above sodium carbonate extract acidified and precipitated with an iron salt in excess, filtered, washed, and ignited=2.77%-Fe_2O_3=

$$Fe_4^{III}(Fe(CN)_6)_3 \quad Fe_4^{III}(Fe(CN).2H_2O)$$
$$4.25\% \quad = \quad 6.03\%$$

(4) Above (3) repeated $Fe_4^{III}(Fe(CN).2H_2O)$ = 4.25%

In the known absence of "silk gum" or soluble or insoluble dressing material, the Prussian Blue may be estimated by the difference in the percentage of nitrogen present, before and after extraction of the Prussian Blue from the fibre by a suitable alkali treatment (2% sodium carbonate solution) at the boil, which may be repeated if necessary. This process is recommended by (Farrell and Goldsmith, *J. Soc. D. and C.*, 1912, **28**, 236). It must be remembered, however, in calculating results that anhydrous Prussian Blue is unknown and it is best to average the water of hydration at 20 molecules under the usual conditions of silk dyeing.

The determination of nitrogen must be conducted with great care.[1]

Silk fabrics should be separated into warp and weft, and these analysed separately, since the weft is usually more heavily weighted than the warp.

It is a frequent practice to express the proportion of weighting materials on 100 parts of the silk treated, and not on 100 parts of the product. This plan is adopted in the following statement of results obtained by Gnehm and Blumer (*Rev. Gen. d. Mat. Col.*, 1898, **2**, 133) by a modification of the foregoing process.

	Weighting above pari	
	Actual	Found
A. Japanese trame,[2]	96%	95.71%
B. Japanese trame,[2]	50%	49.66%
C. Yellow Italian organzine,[2]	53%	52.81%

[1] In the analysis of weighted silks Müller recommends removing the weighting material with a .4 to 1% solution of hydrofluoric acid or a saturated solution of oxalic acid (*Zeit. f. Farb. u. Text. Ind.*, 1903, 160).

Gnehm and Blumer treat the silk first with hydrochloric acid at 60° C., and subsequently with sodium carbonate at 80°, repeating both the treatments several (up to seven) times. (See also Gnehm and Schwartz, *Revue Gén. des Mat. Col.*, 1898, **2**, 131.)

[2] *Trame* or *tram*, usually employed for wefts, is the product of the union of two or more

In the case of sample A the loss of weight in discharging (d) was taken at 18%, and in that of C at 22%. Notwithstanding the above results, the varying amounts of loss assumed to occur detract much from the accuracy of this process. Indeed, Gnehm and Blumer state that the results obtained differ from the actual in some cases by 5 to 10%.

The process of silk weighting especially in relation to the increase in volume of the silk fibre, which is the important factor, has been discussed by Heermann in detail. (*Färber. Zeit.*, 1911, **22**, 29, and *Chem. Zeit.*, 1911, **34**, 829.)

Artificial or Imitation Silk or Lustrocellulose.

Of recent years artificial filaments have been prepared from solutions of organic colloids by forcing them under pressure through fine jets into suitable media in which by some process of coagulation fine filaments may be drawn off and wound on to suitable bobbins and combined into composite threads.

These may be of such a diameter that they resemble horsehair or be as fine as silk filaments and reproduce many of its essential characteristics. Thus they have become known in commerce under the name of artificial silk and artificial horsehair respectively. These manufactures from a cellulose basis have been made in increasing quantities until an output of 10,000–12,000 tons has been recorded, and to the fact that in many characteristics these products closely resemble the real article to an extent which may deceive the general public, it is necessary to consider the question of their identification. This is specially the case under modern conditions where composite threads of artificial silk and real silk are in some cases employed.

It must be remembered that many of the tests which have been proposed are not of a very definite order, and are influenced by certain modifications in actual manufacture which will be unknown to the analyst. Care should therefore be taken to make all colour tests, and the like, by direct comparison with recently manufactured samples of these artificial fibres of known origin and manufacture. Recognition of special makes of artificial silk by colour reaction is also rendered difficult by the fact that the modern method of treatment of the filaments by formaldehyde may materially modify or alter such reactions. A microscopical examination must always accompany the chemical

single untwisted threads, which are then doubled and slightly twisted. *Organzine*, generally used for warp-silk, is produced by the union of two or more single threads separately twisted in the same direction, and then doubled and re-twisted in the opposite direction.

one, and the results obtained from a series of tests must generally agree.

Of the many products proposed only those prepared from cellulose need be considered. Gelatin and casein have been suggested as possible raw materials, but the products obtained can hardly compete with those already mentioned, as they lack certain more or less essential qualities already possessed to a reasonable degree by the others.

In the past it has been possible to generally recognise the presence of artificial silk yarns by the comparatively large diameter of their individual filaments, but this is not a certain distinction under modern conditions of manufacture, nor will a mere burning test suffice as a general distinction, as weighted silk may not burn in the characteristic way of ordinary silk (or wool). A more detailed examination of a chemical nature is therefore necessary.

The production from nitrocellulose of an artificial textile fibre resembling silk[2] has been the subject of various patents, of which those of Chardonnet, Du Vivier, and Lehner are the chief. These processes differ in certain details of manufacture. Some of the processes first patented have been superseded. Broadly speaking, artificial silk is now manufactured by: (*a*) nitrating cellulose by treatment with a mixture of sulphuric and nitric acids, taking care not to carry the process of nitration too far; (*b*) solution of the resultant, thoroughly washed, nitrated cellulose in a mixture of alcohol and ether; (*c*) forcing or drawing the viscous collodion (after careful filtration) through minute orifices of glass; (*d*) solidification of the fine thread by immersion in water (Lehner) or evaporation of the solvent (Chardonnet); (*e*) denitration of the fibre by an ammonium sulphide or other reducing agent.

The other processes in general vogue are the cupro-ammonium and viscose ones in which the cellulose is dissolved directly in these respective solvents and subsequently coagulated in filament form.

Of the other possible processes little need be said as they have not reached the commercial stage, although it is possible that the cellulose acetate products may find their way onto the market.

For general convenience the artificial silks may be divided into the following classes:

[1] The products described by these names are quite distinct from that resulting from the deposition on cotton-fibre of silk from its solution in alkali or in ammoniacal copper or nickel oxide (see *J. Soc. Dyers, Col.*, 9, 180).

[2] These products are chiefly used to replace real silk in the weft, and especially in decorative and mixed fabrics, it is capable of being used with lustre and effect equal to silk itself, and costs considerably less in spite of its want of covering power.

Nitrocellulose product.
Cupro-ammonium product (glanzstoff).
Viscose product.
Zinc chloride product.
Cellulose acetate products. } Not on the market
Gelatin and casein products. }

Recognition of Artificial Silk Products.—From their origin, it is generally easy to distinguish the artificial silk from the natural product. To distinguish the different products from one another is more difficult.

In doing this certain tests are applied which may be of the following order:

Microscopical Examination.—Genuine silk exhibits a uniform thickness and roundness as compared with the artificial filaments but this difference is diminishing as time goes on. Irregularity in diameter if present may therefore indicate the artificial product, but it will be seen that this defect is not always present as in the accompanying photomicrograph (Fig. 57). Examined in cross-section the artificial silks generally show an irregular section of varying shape caused by unequal drying in the process of manufacture. This defect is least shown in the nitrocellulose filaments which are squirted into the air instead of into a solution in their manufacture. The presence of drawn-out air bubbles in the filament itself when present is characteristic of the artificial product; these bubbles may vary between 3–10μ in length and 1–3μ in diameter.

Size of Individual Filaments.—This has been discussed under different headings. It forms a ready means of distinguishing most of the artificial silk from the real fibre. The artificial filaments of which the yarns are composed vary in diameter between 35μ and 120μ, while real silk filaments vary from 9–20μ (Hassock, *Oest. Chem. Zeit.*, 1900).

The actual size of the filaments generally differentiates the artificial products from the real silk being greater in the former case in the ordinary 100 denier and above sizes. In the finer counts as in the Thiele silk this may not be so, however.

The microscopical and physical characteristics are dealt with generally by Massot (*Färb. Zeit.*, 1909, **18**, 146, 166 and 182). Minajeff (*Färb. Zeit.*, 1908, **7**, 63) compares under the microscope cotton with the Glanzstoff product.

According to Beltzer (*Monit. Scient.*, 1911, **74**, 633) the following characteristics may be observed in recently manufactured products:

Nitrocellulose Silks (magnification of 250 diams.).—Filaments are straight regular cylinders without longitudinal striæ, but with a fairly regular central canal having a diameter of 1/8–1/10 of the fibre. The diameters of the filaments varies from 0.08–0.25 mm.

Cupro-ammonium Silks.—Fibres have very regular appearance at 250 diams. There is no central canal or excoriations on sides of fibres. Diameter only varies between 0.4–0.5 mm. (Glanzstoff and Givet makes).

Viscose Silks.—According to the method of manufacture these may show at 250 diams.

(a) Fibres with regular appearance, similar to the cupro-ammonium product and without central canals. Straight excoriations on surface. Diameters vary from 0.025–0.06 mm. These are prepared by slow coagulation.

(b) Fibres have a well-marked curved canal and distinct and regular longitudinal striæ. These have been prepared by rapid coagulation.

Cellulose Acetate Silks.—Generally regular and cylindrical without longitudinal striæ or excoriations. Surface smooth. Diameters vary between 0.03–0.08 mm.

Polarised Light.—The phenomena exhibited by artificial filaments is characteristic. With the exception of filaments manufactured from gelatin (which are not on the market) the artificial fibres show double refraction. The experienced observer can distinguish these from natural silk by this means. Cross sections of these artificial silks show in the dark field bright colours, while in the case of artificial gelatin products the colours are absent. Genuine silk has the power of refracting light, but in a way which is easily distinguished from that of the artificial filaments.

Breaking Strain and Elongation.—The relative strength of these fibres in the dry and wet state is often a certain test of their origin. Ordinary silk retains its full strength on wetting, but the cellulose products lose 50–70% of their strength on wetting in cold water for 5 minutes. Care must be taken when making this test as their full strength is regained on drying.

The actual strength of the fibres in relation to their size is a valuable indication as to their origin, but different investigators seem to obtain varying results probably owing to the different methods used for testing. The ordinary silk serimeter should be used with automatic attachment. (See Dreaper, *J. Soc. Chem. Ind.*, 1909, **28**, 1300, and Dreaper and Davis, *ibid.*, 1912, **31**, 100.)

Maschner Test.—This can only be safely applied to artificial silk

which has not been dyed. Equal weights of the fibres to be tested are placed in small flasks standing on white paper. Equal weights of pure, concentrated sulphuric acid are poured over the fibre when the following reactions are noticed.

Nitrocellulose product: { Colourless; after 40–60 minutes a weak, yellowish tone.

Cupro-ammonium product: Yellowish-brown, or yellow at once.

Viscose product: { Reddish-brown at once. After 40 minutes liquor rusty brown.

Certain precautions have to be taken in carrying out this test. The per cent. of moisture should be approximately the same in all cases.

Fehling Solution.—The details of the Fehling test as given by Schwalbe (*Färb. Zeit.*, 1907, **18**, 237) are as follows:

0.2 grm. of each silk treated with 2 c.c. Fehling solution. The Chardonnet silk (owing to reducing action) gives a green colour to solution. Pauly and Viscose silk show no change.

On treatment with ammonio-cupric oxide (Schweitzer's reagent) both true silk and Collodion silk swell considerably before dissolving. On adding hydrochloric acid to the resultant solution of Collodion silk, or even on merely diluting it with water, a white precipitate of cellulose is thrown down.

Concentrated Nitric Acid.—This reagent disintegrates China silk in the cold, also Tussah; artificial silks are unchanged under these conditions, but on boiling they may ultimately dissolve. Silk takes in the first place a deep yellow colour.

Action of Potassium Hydroxide on Artificial Silks.—In a solution of maximum concentration Cellulose silks become discoloured, gelatinised, and transparent but only dissolve after prolonged boiling. In a 40% solution which completely dissolves China silk at 70–85° and Tussah at 75–100° only, cellulose filaments swell at 15° but resist action at the boil for a considerable time.

Sodium Hydroxide (Mercerising Strength).—Fibre swells up. *Cupro-ammonium product.*—Fibres swell and ultimately dissolve. *Viscose product.*—Fibres swell up and dissolve in 12 hours. *Cellulose acetate.*—Fibre swells up.

Action of Zinc Chloride Solution 60%.—Collodion silk dissolves at 145°, China silk at 120°.

Zinc Chloride Iodine Test.—(Schwalbe, *Färb. Zeit.*, 1907, **18**, 237.) 20 grm. $ZnCl_2$, .2 grm. KI and 15 grm. water add 0.1 grm.

iodine. After immersion the samples are washed in water. **Pauly** silk quickly loses its brown shade, while Viscose silk keeps its colour for a long time (bluish-green). The results obtained cannot be relied on.

Iodine in Potassium Iodide.—The sample is moistened with water, and a drop of reagent added. China silk takes a deep brown, Tussah a heavy brown.

Nitrocellulose gives pale yellow. *Cupro-ammonium* products strong violet brown colouration. *Viscose* similar to cupro-ammonium filaments. *Cellulose acetate* irregular result even in same fibre, yellow to brown. (Beltzer.)

Ruthenium Red.—0.1 or 0.01% solution of ammoniacal oxychloride of ruthenium $[Ru_2(OH)_2Cl_4(NH_3)_7 + 3H_2O]$ has been proposed by Beltzer. No colouration is given with nitrocellulose products. The cupro-ammonium products are hardly coloured. Viscose products give a distinct pink. Cellulose acetate produces irregular results, some of the fibres remaining white, others showing pink colouration.

Percentage of Moisture.—The figures which have been given for the relative per cent. of moisture in artificial silks as compared with real silk, are misleading. For instance, China silk has been given at 7.77% against 10.37% for Chardonnet (Collodion) silk and 10.04% for Pauly (cuproammonium) silk. When it is remembered that the natural moisture in China silk is really 9.5–11% these figures have little significance.

Effect of High Temperature.—The result of heating the different products to 200° for 2 hours is interesting (Süvern and Hassock). China silk browns considerably. Tussah silk no change (?). Nitro silks are carbonised to blue-black coal. Pauly silk takes a light yellow shade. The samples lose on the dry weight (at 100°) the following amounts: China 3.18%, Tussah 2.95%, Chardonnet 33.34%, Lehne 26%, Pauly 1.61%, and gave an ash of China 0.95%, Tussah 1.65%, Chardonnet 1.6%, Lehner 1.43%, Pauly 0.96%.

Identification of Nitrocellulose Products.—The residual nitrogen remaining in the fibre substance also enables this class of artificial silks (Chardonnet, Lehner, etc.) to be identified. The fibre is treated with concentrated sulphuric acid until actual solution takes place in the cold. If the solution is coloured yellow the product may be a viscose one or prepared by the cuproammonium process. The solution is divided into 2 portions. To one is added a fragment of meta-phenylenediamine. A blue or brownish-blue colouration (dark) will indicate the presence of a nitrocellulose product. To the other portion

is added brucine hydrochloride. A blood-red colouration indicates a nitrocellulose product. The nitrocellulose products dissolve in cold concentrated sulphuric acid, and 40% chromic acid. They swell in glacial acetic acid, but do not dissolve.

Dimethylaniline is claimed by Walter (*Z. angew. Chem.*, 1911, **24**, 62) as a reagent for detecting any unaltered nitrocellulose in artificial silk prepared from that product. The material is immersed in the dimethylaniline and exposed in porcelain basins to the action of the air in the dark at 13–15°. The colour change to a greenish tint after 24 hours, changing to intense bluish violet at 60 hours, and violet at 156 hours was noticed with nitrocellulose itself or celluloid.

Dyed Samples.—Coppetti (*Ann. Chem. Analyt.*, 1909, **14**, 47) treats dyed samples, (silk and artificial silk,) with hydrofluoric acid (commercial strength) for 5–10 minutes, washes with water and boils up in soap solution, washes, treats with 5% hydrochloric acid and bleaches in hypochlorite before testing. The writer, however, prefers, whenever possible, to discharge the colour with one of the many reducing substances now used in commerce such as "reducite." rongalite, etc.

Dyeing Test. *Nitrocellulose Products.*—Methylene blue and solid green dye the fibres. Acid violet 4BN gives no colouration. Congo red in slightly alkaline solution dyes red. Formaldehyde-treated product gives no reaction with methylene blue, but potassium permanganate gives a colouration with this.

Cupro-ammonium Products.—Permanganate gives bronze colouration; more intense with formaldehyde-treated product. Methylene blue gives hardly any colour on ordinary fibre, and none with formaldehyde-treated yarn. Solid green a paler shade than with nitrocellulose product. Methylene blue very weak colouration. Congo red, pale irregular pink, from weak alkaline solution.

Cellulose Acetate Products.—Permanganate gives strong bronze colouration. Methylene blue, very irregular, some fibres intense blue, others colourless. Solid green gives green colouration. Congo red irregular result.

Viscose Products.—Permanganate gives light bronze increasing on standing. Methylene blue gives blue colouration less intense than with nitrocellulose, but more intense than with cupro-ammonium product. Solid green gives green colouration. Congo red strong colouration in weak alkaline solution. Formaldehyde-treated product gives no colour reactions as above, except with permanganate which is reduced more rapidly than with untreated yarn.

Burning Test.—Silk burns slowly with a smell resembling burnt horn. Weighted silk burns and leaves a skeleton thread of mineral matter. Nitrocellulose, viscose, and cupro-ammonium products burn quickly like cotton. Cellulose acetate filaments burn very much like real silk, with smell of acetic acid. Casein and gelatin products burn like silk. Composite threads of silk and artificial silk are on the market.

Cellulose acetate filaments may come into the market in the future so that the following particulars given by Herzog (*Chem. Zeit.*, 1910, **34**, 347) are of interest. The average diameter of the individual filament is said to be about 42μ. This silk is doubly refractive, having a refractivity equal to that of lemon oil in which it becomes almost invisible. Under the ultra-microscope a sort of cellular structure is observed. The newer product of the Donnersmark factory does not show polarisation between cross nicols. Its sp. gr. is 1.25 (against silk 1.36 and cuproammonium artificial silk 1.50). Its strength is given 226 grm. for an 18-filament thread (as above) in the dry state and 128 grm. in the wet. Its strength is given by this authority as compared with other fibres, per K. per square centimetre, as follows: Silk (dry) 37 K.; the same wet 37 K.; Chardonnet product 12.0 and 2.2 wet; Fismes 7.8 and 1.6 (wet); Lehner 16.9 and 1.5 (wet); Cellulose silk 19.1 and 3.2 (wet); Gelatin silk 6.6 and 0.0; Acetate silk 10.22 and 5.8 (wet). These figures differ materially from those given by other authorities. They are said to have been taken on a Schopper testing machine. It is soluble in cold acetic acid (glacial), not soluble in ammoniacal copper hydroxide, colour yellow by iodine and sulphuric acid. Burns rapidly leaving a puffed up charcoal and gives off a disagreeable odour. An artificial silk prepared from gelatin is the only other one which gives any dichröism when dyed with Congo Red. Its density is very low (1.251); it does not swell in water. The claim for additional strength in the wet state of the cellulose acetate product is not suggested by the above figures when they are compared with known results obtained with other artificial salts of recent manufacture.

Süvern (*Farb. Zeit.*, 1900, **2**, 283) gives the reactions of nitrocellulose artificial silk as compared with the natural China and Tussah silks. It must, however, be remembered that unbleached Tussah gives a chocolate brown with Millon's reagent and not a violet.

According to Silbermann (*J. Soc. Dyers & C.*, 1893, **9**, 163) a concentrated solution (40%) of sodium hydroxide dissolves mulberry silk and Chardonnet's artificial silk in a few minutes, while Tussah silk and Vivier's artificial silk are comparatively unaffected. Boiling with ammonia or soap for 15 minutes has no appreciable effect on Collodion

Reagent	China silk	Tussah	Nitrocellulose artificial silk
KOH, conc. solution	Dissolves on gently warming.	Dissolves on boiling.	Unaltered.
$ZnCl_2$, 60% solution	Dissolves at 120°.	Dissolves at 125°.	Dissolves at 140–145°.
Fehling solution	Dissolves readily on boiling.	Dissolves on boiling.	No change.
Millon's reagent	Violet coloura-tion on boiling.	Same.	No change.
Ash	0.95%	1.15%	1.6 %
Nitrogen	16.6 %	16.79%	0.15%

silk, and boiling dilute acids have no immediate action. This test is not however considered a distinctive one.

A specimen of lustrocellulose examined by Cross and Bevan (*J. Soc. Chem. Ind.*, 1896, **15**, 318) was found to contain: carbon, 43.77%; hydrogen, 6.40; and nitrogen, 0.19%. On boiling with dilute hydrochloric acid (sp. gr., 1.06) the lustrocellulose yielded only traces of furfuraldehyde, and on boiling with dilute sodium hydroxide and Fehling's solution no cuprous oxide separated. By prolonged boiling with a 1% solution of sodium hydroxide it lost 9.14%, which shows a much higher resistance to alkaline hydrolysis than might have been expected.

From the foregoing results, Cross and Bevan conclude that no oxycellulose results from the treatment of the cellulose, and that the permanent hydration-changes which take place are of minor importance.

According to Cross and Bevan, the proportion of moisture in lustrocellulose is much higher than that present in unaltered cellulose, which usually contains from 10 to 12 %; the tensile strength is about two-thirds of that of true silk; and the elasticity somewhat less. Its dyeing capabilities are satisfactory. Lustrocellulose from wood-pulp gives no reaction with iodine, whereas that prepared from cotton-waste is stated to take up a considerable proportion.

P. Truchot (*Analyst*, 1897, **22**, 248) states that artificial silk, or lustrocellulose, has a sp. gr. of 1.490, whereas real silk ranges in sp. gr. from 1.357 to 1.367.

The following figures representing the sp. gr. have been quoted by Süvern.

Italian Silk	1.36
Chardonnett product	1.52—1.53
Lehner product	1.51
Glanzstoff	1.50
Gelatin silk	1.37

There can be little doubt but that these results may be modified by certain details in manufacture.

General reference may be made to the text books by Süvern, Foltzer, and a section of *Nitrocellulose* by Worden, 1911, London, Vol. 1. The following articles may also be consulted: Coux, *Rev. Gen. Mat. Col.*, 1910, **21**, 203., Colin, *ibid.*, 1909, **12**, 40. Dreaper, *J. Soc. Chem. Ind.*, 1909, **28**, 1300, Dreaper, *J. Soc. D. & C.*, 1907, **23**, 7, and Beltzer, *Monit. Scient.*, 1907, **68**, 181, where further particulars of the manufacture may be obtained. Many references will be found in Worden's work.

CHITINOIDS.

The substance known as chitin occurs throughout the invertebrata in the form of an investment to the outermost cellular layer or ectoderm. In many cases, a chitinous composition has been ascribed to structures solely on account of their insolubility in alkali hydroxides and in dilute acids, or even in only one of these reagents. At least two distinct compounds, chitin and conchiolin, have been confounded in this manner. They exhibit essential differences in characters and composition, and, together with spongin, may be conveniently classed together as "chitinoïds." The following table shows the ultimate composition attributed to these three substances:

	Chitin	Conchiolin	Spongin
Carbon	46.32	50.7	47.44
Hydrogen	6.40	6.5	6.30
Nitrogen	6.14	16.7	16.15
Oxygen	41.14	26.1	30.11
	100.00	100.00	100.00

Chitin, $C_{18}H_{30}O_{12}N_2$ or $C_{30}H_{50}O_{19}N_4$.

Chitin, as it occurs in nature, is frequently impregnated with calcareous matter, as in the shells of the crustacea, or with silica, as in the radula of the higher mollusca.

Chitin was prepared by Hoppe-Seyler by boiling the wing-cases of the cockchafer with dilute solution of sodium hydroxide until they became colourless. The product was then boiled in succession with water and dilute hydrochloric acid, and finally exhausted with boiling alcohol and with ether.

Chitin may be obtained by a similar process from the shell of the crab or lobster, but in this case the substance should be previously digested with hydrochloric acid, to dissolve the earthy matters deposited in the chitinous tissue.

Chitin is a colourless, amorphous substance, which, when prepared in the foregoing manner, retains the form of the parts composed of it. It is insoluble in water, alcohol, ether, acetic acid, and in dilute mineral acids. Chitin resists in a remarkable manner the action of alkalies, and can be subjected to a prolonged treatment with their boiling concentrated solutions without undergoing decomposition.

Chitin is dissolved by strong mineral acids. If the chitin prepared as above described be treated with cold, concentrated hydrochloric acid, and the solution diluted with a large excess of water, the chitin is precipitated in a colourless, gelatinous form.[1]

The composition of chitin has been investigated by Ledderhose, under the direction of Hoppe-Seyler and Baumann (*Zeit. physiol. Chem.*, 1878, **2**, 213), who, as the mean result of twelve analyses, found it to contain: Carbon, 45.69; hydrogen, 6.42; nitrogen, 7.00; and oxygen, 40.89%. Analytical results by many investigators will be found in the *Biochemische Lexikon*. Vol. **2**, 191, 627.

Frankel & Kelly (*Monatsh.*, **23**, 132) find that chitin undergoes hydrolysis when heated with acids, with formation of glucosamine and acetic acid, according to the following equation:

$$C_{18}H_{30}O_{12}N_2 + 4H_2O = 2C_6H_{13}O_5N + 3C_2H_4O_2.$$

When chitin is boiled with hydrochloric acid the hydrochloride of glucosamine is formed. This is soluble in water, crystalline, and dextrorotatory, $[\alpha]_D$ 70.6°. The sulphate crystallises in needles. Enoch Zander (*Pflüger's Archiv*, **66**, 545) gives a microchemical test for chitin. The substance is treated with a drop of strong potassium iodide solution and this is followed by conc. zinc chloride. The cell structure takes a violet colour and the chitin a brown one.

[1] C. P. W. Krukenberg (*Zeit. Biol.*, 1886, 22, 480) finds that the action of cold hydrochloric acid on chitin for 1 hour is not one of simple solution. A chlorinated compound is first formed, which swells up in the acid, and on filtering, a cloudy filtrate is obtained, from which about 2% of chitin is precipitated by water or baryta. The filtrate from this precipitate does not contain glycosamine or other decomposition products. After longer action of hydrochloric acid, the chlorinated substance suffers partial or complete dissociation, and the filtered liquid contains a dextrinoid substance of feeble reducing power, together with very small quantities of chitin (precipitable by water) and glucosamine hydrochloride. By the action of 5 and 10% solutions of potassium or sodium carbonate saturated with chlorine, chitin was converted into a substance corresponding with amidulin. After 12 days a small quantity of chitin had dissolved, and after filtering and removing the salts by dialysis a substance was obtained which dissolved readily in cold water, reduced Fehling's solution on heating, and gave, both with neutral and with basic lead acetate, copious precipitates insoluble in excess of the reagent. These characters and its indiffusibility distinguish the substance from glucosamine hydrochloride.

Conchiolin ($C_{30}H_{48}O_{11}N_9$).

This substance is obtained by macerating the shells of mussels or snails in dilute hydrochloric acid, and then boiling with sodium hydroxide solution.

Conchiolin closely resembles chitin, with which substance it was formerly supposed to be identical. It is distinguished from chitin by its larger content of nitrogen, and by yielding leucine when boiled with dilute sulphuric acid, without any sugar-like substance being simultaneously formed.

Conchiolin gives the biuret, Millon, and xanthoproteic reactions, but does not react with lead sulphide.

Spongin.

Spongin, or spongiin, is the characteristic protein substance of sponges.[1] It has been compared to the collagenes and to the fibroïn of silk, but on the whole appears to be best classed with the chitinoïds.

Spongin is obtained when sponge is boiled in succession with dilute hydrochloric acid, dilute sodium hydroxide, water, alcohol, and ether. The product contains 16.15% of nitrogen.

Spongin is unaffected by the reagents employed for its preparation, and is also insoluble in ammonia. It is dissolved slowly by strong alkalies, and is reprecipitated on neutralising the solution. Dilute acids have no action on spongin in the cold, but it is dissolved by concentrated acids.

When boiled with water under pressure, spongin yields no gelatin. Boiling dilute sulphuric acid is stated by Städeler (*Annal. Chem. Pharm.*, **111**, 12) to decompose it with formation of leucine and glycine, but not tyrosine.[2] Kossel & Kutschen (*Zeit. physiol. Chem.*, **31**, 165) obtained arginine, lysine and glutamic acid on hydrolysis with sulphuric acid.

[1] An interesting description of the natural history of sponges has been published by Holmes (*Pharm. Jour.*, [iii], **17**, 991). Information on the methods of *bleaching* sponges will be found in the *Pharmaceutical Journal*, [iii], **14**, 88, and in the *Chemist & Druggist*, **30**, 643. Potassium permanganate is commonly employed. According to a receipe in *Le Moniteur de la Teintnre*. (abst. *J*, *Soc. Dyers & C.*, **4**, 63), sponges may be conveniently bleached by immersing them in saturated bromine-water for several hours. This treatment is repeated till the desired tint is obtained. The sponges are then passed through a bath of dilute sulphuric acid, and finally washed with cold water. It is stated that the treatment in no way injures the quality of the sponge.

[2] The constitution of spongin was studied by Zalocostas (*Compt. rend.*, 1888, **104**, 252. Sponge, after being washed with dilute hydrochloric acid and benzene, was submitted to the action of aqueous barium hydroxide under pressure. The ammoniacal nitrogen formed in the decomposition was equal to about 1/4 of the total nitrogen, as in the case of albumin. Also, for each molecule of carbon dioxide and oxalic acid were found two atoms of ammoniacal nitrogen, as in all protein matters. Zalocostas represents the reaction by the following equation:

$$2C_{40}H_{64}O_{17}N_{12} + 24H_2O = 6NH_3 + 2CO_2 + C_2H_2O_4 + C_2H_4O_2 + 2C_{37}H_{76}O_{24}N_9$$

Spongin. — Oxalic acid. — Acetic acid. — Fixed residue.

This residue consisted of fixed nitrogenous principles. Zalocostas points out that the molecules of water fixed in the reaction are equal to the number of nitrogen atoms, and that the relation of carbon to hydrogen in the fixed residue is as 1 : 2.66, whereas the relation in the case of prot ins and collagenous substances is as 1 : 2. The analysis of this mixed fixed residue showed the presence of leucine, butalanine, tyrosine, glycalanine, etc.

Spongin yields on digestion peptone-like substances which, however, do not give the usual colour reactions (xanthoproteic, Millon's or Adomkiewicz tests).

Spongosterol, ($C_{19}H_{32}O$) has been isolated from ethereal solution of *suberites domuncula*, with a m. p. of 119–120° and giving $[\alpha]_D = 19.59°$. Its crystals give no colouration with 20% sulphuric acid (Henze, *Zeit. Physiol. Chem.*, 1904, **41**, 108).

Cornin forms the framework of corals, and yields indole.

KERATIN SUBSTANCES. KERATOIDS.

When cuticular and allied tissues—including nails, horns, hoofs, feathers, whalebone, scales, etc.—are treated in succession with boiling ether, alcohol, water, and dilute acids, the insoluble residue retains the shape of the original tissue and is known as keratin. The product varies somewhat in its characters and composition with its origin, so that a number of allied substances are comprehended under the general name of keratin. The following table illustrates the composition of the "keratin" obtained from different sources:

Source of keratin	Carbon	Hydrogen	Nitrogen	Sulphur	Oxygen (by difference)	Authority
Feathers	52.46	6.94	17.74	?	22.86	.....
Quills	51.7	7.2	17.9	.....	.....	Scherer.
Wool,[1]	50.65	7.03	17.71	4.61	20.00	Scherer.
Hair (man's)[1]	50.65	6.36	17.14	5.00	20.85	Van Laer.
Fur (white rabbit's)	49.45	6.52	16.81	4.02	23.20	Kühne and Chittenden.
Nails	51.00	6.94	17.51	2.80	21.75	Mülder.
	51.09	6.82	16.90	2.80	22.39	Scherer.
Horn (cow's)	51.03	6.80	16.24	3.42	22.51	Tilanus.
Hoof (horse's)	51.41	6.96	17.46	4.23	19.94	Mülder.
Epithelium	51.53	7.03	16.64	2.18	22.32	Hoppe-Seyler.
Epidermis	50.28	6.76	17.21	0.74	25.01	Hoppe-Seyler.
Whalebone[2]	51.86	6.87	15.70	3.60	21.97	Van Kerckhoff.
Tortoise-shell	54.89	6.56	16.77	2.22	19.56	Mülder.
Neuro-keratin (brain)	56.99	7.53	13.15	1.87	20.46	Kühne and Chittenden

Bourquelot (*Pharm. Jour.*, [iii], **19**, 1035) gives the following limits of composition of keratoids, so far as has been at present recorded: Carbon, 50.3 to 52.5; hydrogen, 6.4 to 7.0; nitrogen, 16.2 to 17.7; oxygen, 20.7 to 25.0; and sulphur, 0.7 to 5.0%.

The following results have been given by Abderhalden and Fuchs (*Zeit. physiol. Chem.*, 1908, **57**, 339) for keratin from 4-year old animals.

[1] Other analyses of the keratin from wool are given below.

[2] Substitutes for whalebone have been protected by various inventors including the following English patents: J. Baier, 1895, No. 10193; W. Hunkemöller, 1896, No. 1214; and Martin & Levy, 1895, No. 1820; 1896, No. 4718.

	Hoof of ox	Horn of ox	Hoof of horse
Dry residue	91.5 %	96.5 %	75.4 %
Ash	0.16 %	0.36 %	0.45 %
Melanin substance	0.12 %	0.96 %	0.9 %
Glutamic acid hydrochloride in ash dry residue.	16.8 %	12.9 %	18.2 %

It appears that while the keratin from different sources is tolerably constant in the proportions of carbon and hydrogen contained in it, the percentages of sulphur and of oxygen vary within limits. This fact is further illustrated by the following results recorded by Mohr (*Zeit. Physiol. Chem.*, 1895, **20**, 403).

Source of keratin	Sulphur, %	Source of keratin	Sulphur, %
Woman's hair; dark blonde	4.95	Pig's hair	3.59
Girl's hair; dark brown	5.34	Sheep's wool	3.68
Boy's hair; red blonde	4.98	Goose feathers	4.59–3.16
Boy's hair; red	5.32	Pig's hoof	2.69
Rabbit's hair	4.01	Calf's hoof	3.57
Calf's hair	4.35	Ox hoof; white	3.49
Horse's hair	3.56	Ox hoof; black	3.45

All the keratoids yield more or less ash on ignition, the proportion in some cases being considerable. The nature of the ash varies with the source of the keratin. Thus hoofs and horns give an ash consisting chiefly of calcium phosphate, while the ash of wool is largely composed of potassium and sodium sulphates. The ash of hair consists chiefly of sulphates of alkali-metals, ferric oxide, and silica. The silica in the ash of feathers is stated by von Bibra to range from 27 to 40%.

The greater part of the sulphur of keratoids is only loosely combined, so that on boiling hair, wool, or feathers with lead acetate and excess of sodium hydroxide, the liquid blackens from formation of lead sulphide. Hoppe-Seyler found that by heating horny substances with bartya-water in sealed glass-tubes, nearly the whole of the sulphur is obtained as barium hydrosulphide.

When heated in the dry state, keratoids swell up, char, and evolve a characteristic odour of burnt feathers.

Many of the keratoids are very hygroscopic, taking up a large proportion of water without affording any indication of its presence beyond the increase in the weight of the substance.

The keratoids are quite insoluble in alcohol or ether, and are not much affected by boiling with water at the ordinary pressure; but, when heated under pressure for a long time to 150 to 200°, they dissolve with

evolution of hydrogen sulphide to a turbid solution which does not gelatinise on cooling, and gives on evaporation a residue insoluble in water.

Keratin yields a turbid solution on prolonged boiling with water at 150–200°. It yields aspartic acid, leucine, and tyrosine on boiling with dilute sulphuric acid. Gümbel gives the following values for its nitrogen content. 1.17 as amide-N., 0.42 as melanin-N., 2.95 as diamino-N., and 11.51 as monamino-N. It yields no plastin or coagulose on digestion, and is the least soluble of the albuminoids, (Smith, *Zeit. f. wissensch. Zool.*, 1883, **19**, 469). Successive treatment with acid, alkali, pepsin, and trypsin will remove all other albuminoids, (Kühne and Chittenden, *Zeit. f. wissensch. Zool.*, 1890, **26**, 291). It is remarkable for its percentage of tyrosine and cystine (14%), gives intense Millon's lead sulphide, and xanthoproteic reactions.

When treated with alkalies keratin substances swell up, and are entirely dissolved by boiling alkaline solutions.[1] Ammonia acts similarly but less strongly. On adding excess of acid to the solution of keratin in an alkali, a white flocculent precipitate is formed and hydrogen sulphide is evolved.

When treated with cold glacial acetic acid, horny substances swell up, and on boiling are largely dissolved. Whalebone is converted into a gelatinous substance by boiling with concentrated acetic acid, but tortoise-shell is little changed by such treatment.

Nitric acid turns keratoid bodies yellow, and on application of heat dissolves them with formation of oxalic acid and other products.

On treatment with cold concentrated sulphuric acid, the keratoids swell up, and on heating dissolve more or less completely. The solution appears to contain syntonin, for on dilution with water and addition of potassium ferrocyanide it yields a flocculent precipitate, and also gives a white flocculent precipitate when exactly neutralised.

When chlorine is passed into water containing a finely divided keratoid, or when a keratoid is treated with bromine water, the substance undergoes no change in external appearance, but after drying it is harsh to the touch, and then dissolves in ammonia with evolution of nitrogen.

The keratoids give the Millon and Adamkiewicz reactions for proteins.

When treated with fuming hydrochloric acid, most keratoid bodies

[1] Smith (abst. *J. Chem. Soc.*, 1884, 46, 1398) states that 0.5–1 % solutions of potassium or sodium hydroxide have no action on keratin; that solutions of 20% dissolve it, while 40 % solutions have a weaker action.

swell up to a jelly and subsequently dissolve; with the exception of hair, which is unaffected by such treatment.

According to Smith (*loc. cit.*) keratin is unacted on either by pepsin or trypsin. The first of these statements is correct, but the second is probably erroneous, since keratoids undergo digestion in the small intestine.[1]

When keratoid substances are boiled with dilute sulphuric acid, they undergo decomposition with formation of aspartic acid, volatile fatty acids (including propionic acid), ammonia, leucine, and from 3.5 to 4.0% tyrosine. The formation of the last substance distinguishes keratoids from the collagenes. Keratoids also differ from the collagenes in not yielding gelatin by the action of superheated water or dilute acids, and in containing a notable proportion of sulphur in a loose form of combination.

"Keratin" is official in the German Pharmacopœia (3rd edition).[1] It is directed to be prepared by digesting shavings of quills with a mixture of equal parts of ether and spirit, and subsequently with an acidified solution of pepsin at 40°. The residue is dissolved in acetic acid by prolonged boiling (30 hours), the solution strained, evaporated to a syrup, and dried on plates. The product forms a brownish-yellow, tasteless, and odourless powder or scales. It should not yield anything to water, spirit, ether, dilute acids, or pepsin-hydrochloric acid; and should not yield more than 1% of ash, nor contain more than 3% of matter insoluble in acetic acid or ammonia.

Small quantities of azelic acid (0.046 grm. from 250 grm.) are formed when this substance is oxidised with potassium permanganate. Similar results have been obtained from horn after extraction with ether to remove grease. (Lissizin, *Zeit. Physiol. Chem.*, 1909, **62**, 226.)

Hair.

Hair is one of the most stable of the keratoid substances, resisting the action of reagents to a marked extent. It is remarkable for the high proportion of sulphur contained in it, and for the presence of a large proportion of silica in the ash left on ignition. The *ash* of hair ranges from 0.5 to 2.0%, and consists chiefly of sulphates of alkali-metals, ferric oxide, and silica, the last constituent sometimes forming 40% of the total ash.[2] Vauquelin states that the iron and

[1] Keratin has been employed for coating pills intended to act on the small intestine. Its resistance to the gastric juice and solubility in the pancreatic juice render it very suitable for this purpose (see Bourqueolt, *Pharm. J.*, [3], 19, 1035).

[2] Gorup-Besanez (*Ann. Chem. Pharm.*, 66, 321) found the hair of the lower animals to contain from 0.12 to 0.57% of silica, whereas human hair contained only from 0.11 to 0.22%.

manganese present in dark hair are replaced in fair hair by magnesia, but this result requires confirmation.

Hair consists of a cylindrical keratinous tube covered with minute scales, the points of which are directed toward the free extremity. Hence three morphological elements are distinguishable: the cuticle, the cortex, and the medullary substance.

Hair is closely allied in structure and chemical composition to fur and wool, and no clearly defined distinction can be drawn between them. The fine hair on some animals closely resembles wool, while the coarse wool on others simulates hair. Wool is peculiar in the wavy or curling nature of the fibre, and is distinguished by the comparatively loose attachment of the outer scales. In hair, these scales usually lie so close to the stem that the serrations are scarcely perceptible, whereas in wool they are strongly in evidence (see Figs. 60 and 63). The structure of wool and hair has been fully described by Bowman (*J. Soc. D. & Col.*, 1885, 1, 109 *et seq.*).[1]

The following illustrations (taken from Swaine Taylor's *Medical Jurisprudence*) show the microscopic appearance of hair and fur from different sources. The magnifying power is 300 diameters in each case, except in the case of No. 4, which is shown only 70 times the natural size.

The cells and the linear markings on the cortical portion afford the chief distinctions between hairs from different sources.

The hair of the human head appears under the microscope as transparent cylinders of various colours, and exhibits markings resembling (but less distinct than) those of wool. As a rule, the hair of women is finer and longer than that of men, and the hair of children finer and more silky than that of an adult. No. *2a*, representing a transverse section of human hair, shows the cortical and medullary portions, and the air-cells in the interior of the cylinder. No. 3 represents the pointed extremity of a hair from the eye-brow. These hairs (and those from the eye-lashes) are thicker and coarser than hairs from the head, and are opaque, except toward the point. No. 4 represents the sheath of the hair (magnified 70 diameters) with the hair issuing from it, a condition which occurs when the hair has been pulled out violently from the skin. When a hair has been indented, cut, or bruised, the

[1] "The difference between wool and hair is rather one of degree than kind, and all wool-bearing animals have the tendency, when their cultivation is neglected, to produce hair rather than wool. Wool and hair, fur being intermediate, are simply modifications of the same root-substance, and the scales of the wool-fibre have a much larger free margin than is the case with hair, being only attached to the stem by about one-third of their length, and in many cases the ends are more or less turned outward, so as to present a much more serrated edge than is the case with hair. The interior of the fibre portion, however, does not differ in the least from that of hair, and can neither be distinguished from it chemically or microscopically."—Bowman.

microscopic appearance of the medullary structure often shows marks of such treatment.

The hair of the lower animals is coarser, shorter, thicker, and less transparent than human hair; but the hairs of the spaniel and skye-terrier are long, silky, and very similar to human hair. The diameter of the hair from a particular animal varies greatly, and cannot be regarded as more than roughly characteristic. The furs or hairs of

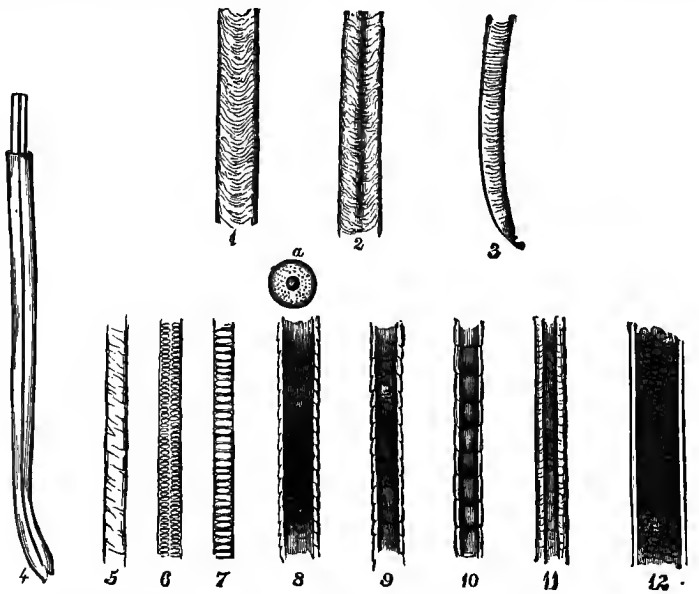


FIG. 59.—Microscopic characters of hairs.

1, Hair of child; 2, hair of adult (*a*, transverse section); 3, conical hair from eye-brow; 4, human hair (×70) with tubular sheath, as torn out by force; 5, hair of spaniel; 6, fur of rabbit; 7, fur of hare; 8, horse-hair; 9, goat's hair; 10, hair of fox; 11, cow's hair; 12, hair of fallow deer.

the rabbit, hare, squirrel, mouse, rat, and other rodents are characterised by dark, transverse cells.

The products of hydrolysis have been given as follows:

Acids	Horse hair	Goose feathers
Glycine	4.7%	2.6%
Alanine	1.5%	1.8%
Aminovaleric acid	0.9%	0.5%
Leucine	7.1%	8.0%
Pyrolidine-2-carboxylic acid	3.4%	3.5%
Aspartic acid	0.3%	1.1%
Tyrosine	3.2%	3.6%
Serine	0.6%	0.4%

When colourless (white) hairs or feathers are boiled with diluted sulphuric acid, they dissolve to a colourless solution; but when black or brown, they yield a similarly coloured liquid, and a black or brown amorphous pigment remains undissolved. This pigment is insoluble in dilute acids or alkalies, and is with difficulty acted on by strong acids, except nitric acid. It is slowly acted on by bromine, forming a substance soluble in water, and is said to yield only traces of ammonia when ignited with soda-lime. The black pigment is present to the extent of about 1% in the feathers of the rook. A mean of 10 analyses of the black pigment from the feathers of several species of *Corvus* showed: Carbon, 55.4; hydrogen, 4.25; and nitrogen, 8.5% (Hodgkinson and Sorby, *J. Chem. Soc.*, 1877, 1, 427).

The pigment of black hair and feathers is changed to yellow by treatment with hydrogen peroxide, an oxidising agent which is extensively employed for bleaching hair and feathers.[1]

Hair-dyes.

These may be divided into two classes, the first contains some compound of a heavy metal which forms a black sulphide. This, by reaction with the loosely combined sulphur present in the hair, occasions the desired darkening in colour. Solutions of lead, bismuth, copper, and silver are those most generally used for the purpose.

Many of these so-called hair-dyes are objectionable, and some are actually dangerous. Erysipelas and inflammatory swellings are liable to result, and well-authenticated cases of lead-poisoning and paralysis have occurred from the use of preparations containing lead.

A widely used article is composed of lead acetate, suspended sulphur, rose-water, and glycerin. In another, said to be capable of dyeing any shade from black to light brown, the free sulphur is replaced by sodium thiosulphate ("hyposulphite"). A chestnut-brown dye contains cupric nitrate and pyrogallic acid, while a black dye is composed of silver and copper nitrates, with ammonia. A preparation which dyes hair almost instantaneously consists of a solution of ammonio-nitrate of silver. This is applied to the hair first, and is followed by a solution of pyrogallic acid.[2] A hair-dye consisting

[1] To produce "golden hair," the grease is first thoroughly removed by washing the hair in soft soap and water, with the addition of a little ammonia. Hydrogen peroxide (of 20 volumes strength = 3 % of available oxygen) is then applied to the hair.

[2] The *Lancet* for January 13, 1877, stated that of 18 samples (three American and the rest English) of so-called hair-restorers, including all the best known, fourteen were found to contain suspended sulphur and lead in varying but always in very considerable quantity. Many were described as "perfectly harmless," "free from injurious substances," etc., and only one was plainly stated on the label to be poisonous if taken internally. Two other samples contained sodium thiosulphate instead of free sulphur. The handbill

essentially of a solution of potassium bismuth citrate has been recommended by Hager, and one containing ammonio-citrate of bismuth, with the addition of sodium thiosulphate, by Naquet (*Year-book Pharm.*, 1873, page 360; 1883, page 326).

If the statement that such preparations as the above are "restorers" and not dyes were correct, they should restore hair which was formerly red to its original colour.

In the second class amino-phenols or similar substances are increasingly employed as hair-dyes. These bodies, especially the salts of *m*-phenylene-diamine and diamino-phenol, resemble pyrogallol in undergoing rapid oxidation when their solutions are exposed to the atmosphere, with the formation of coloured bodies which will dye or stain the hair. According to a patent by Lumière, the dye is prepared by dissolving the hydrochloride or other salt of diamino-phenol in dilute alcohol, and adding sodium sulphite to prevent too rapid oxidation. By varying the strength, various shades of dye are obtained. Unsubstituted 2.6 diaminophenol may also be used.

m-Aminodimethyl-*o*-toluidine or monomethyl-2:4-diaminoanisole have been recommended for dyeing hair, etc., (*Ger. Pat.* 230630). Also *m*-diaminoanisol or *m*-diaminophenetol (*Eng. Pat.*, 5134, 1910). *p*-Phenylenediamine is said to have an irritant effect on the human skin which may be counteracted by the addition of sodium sulphite in equal weight. The colour is subsequently developed with hydrogen peroxide, or these substances may be present in the same solution.

Colman (*Chem. Zeit.*, 1911, **35**, 899) recommends the use of 2.5 parts of tolylenediamine and 5 parts crystalline sodium sulphate in 100 parts of water. Hydrogen peroxide is added and the mixture applied to the hair.

Erlenbach (*U. S. A. Pat.* 992947) obtains blue black tints by using instead of certain mixtures of *p*-phenylenediamine and *m*-diamines, *m*-phenylenediamino, *m*-diaminoanisole, or *m*-diaminophenetole.

Wool.

Wool is closely allied in composition and general characters to fur and hair (page 678), but is usually more elastic, flexible, and curly; and

which accompanied one of these warned purchasers against dangerous hair-restorers containing lead, as likely to lead to paralysis and insanity, and recommended that all such preparations should be tested for lead by potassium iodide. This reagent gives no precipitate of lead iodide in presence of excess of sodium thiosulphate, so that the lead which existed in considerable proportion in the preparation in question would escape detection by the test recommended.

Another sample contained a considerable quantity of lead in solution, but no sulphur or thiosulphate. The remaining preparation was that referred to in the text as containing silver ammonio-nitrate and pyrogallic acid in separate bottles.

the flattened scales, which are observed under the microscope to cover its surface (Figs. 59, 60), give it the property of matting with greater readiness than is the case with either fur or hair. This difference is less apparent in raw wool than in that which has been scoured and treated with a dilute acid.

The microscopic characters of wool have been described and illustrated at length by Bowman (*J. Soc. D. & Col.*, 1885, **1**, 86) and Watson Smith (*ibid.*, 1889, **5**, 12).[1]

FIG. 60.—Sheep's wool (highly magnified), showing the cortical scales and the medullary tube.

The length and diameter of the wool fibre varies between 1.5 and 7 in. and 0.004–0.0018 in. Bowman gives the following data:

Nature of wool	Breaking strain in grm.	Elasticity in % elongation	Diameter of fibre in inches
Leicester	502	0.284	.00081
Southdown	86	0.268	.00099
Amstration Merino	50	0.335	.000517
Saxony	39	0.272	.000338
Mohair	586	0.299	.000170
Alpaca	149	0.242	.000526

[1] Sheep's wool varies enormously in the diameter of the fibres and in other characters The chief varieties of wool from animals other than the sheep are: *Cashmere wool*, from the fine downy hair of the Cashmere goats. *Vicuna wool*, now little used, from the very slightly curly hair of the llama or vicuna goat. *Alpaca wool*, similar to but not quite so fine as vicuna wool, from the alpaco or pako goat of Peru. *Mohair*, or so-called "camel's wool," from the long, slightly curly, silky hair of the angora goat.

A mixture of ordinary sheep's wool with the hair of hares or rabbits is sometimes substituted for vicuna wool. The term "viguna" or vicuna wool is now applied in the wool trade to a mixed fabric of silk and cotton.

Raw wool is far from being an approximately pure keratoid substance. Besides moisture and accidental dirt it contains a considerable proportion of *suint* or wool-soap (page 683), sometimes called "yolk."

The estimation of moisture in wool is an important point. Bowman returns the natural moisture (at 38°) as 8.28 % on the average with the limits 7–10% and as 14% (at 100°). At the English Testing Houses the following percentage of moisture are allowed: Worsted yarn, 18.25%; carded woollen yarn, 17%; noils, 14%; scoured wool, 16% (the silk standard is 11%).

Hummel (*The Dyeing of Textile Fabrics*) has suggested the following method for the analysis of raw wool:

Moisture is determined by drying the wool at 100° in a current of hydrogen or other inert gas.

Wool-fat is determined by extracting the sample with ether. The solution also contains more or less of the oleates of the wool, which are separated from the wool-fat by agitating the ethereal solution with water.

The residual wool is washed repeatedly with cold distilled water, by which more *oleates* are extracted. The solution is added to the water separated from the ether. The wool is next washed with alcohol, and the oleates dissolved added to those recovered from the aqueous solution. The wool is then treated with dilute hydrochloric acid, which removes earthy oleates not soluble in water.[1] The wool is washed with cold water to remove acid, dried, and treated in succession with ether and alcohol. On evaporating these solvents to dryness, a residue of oleic acid is obtained, from which the amount of pre-existing *earthy oleates* can be calculated.

The wool, freed in the foregoing manner from fatty and soapy matters, is dried and teased out over paper to remove dirt, sand, etc. The purified *wool-fibre* is then carefully washed on a fine sieve, dried at 100° and weighed, the amount of *sand* and *dirt* being taken by difference.

The following analyses, by Märcker and Schulze, were made by the foregoing method, and illustrate the results yielded by typical raw wool.

[1] In the case of very dirty wool a considerable quantity of lime is dissolved which has its origin in calcareous dust and not in lime-soap.

	Wool of lowland sheep	Wool of full-bred rambouillet sheep	"Pitchy" wool
Moisture	23.48	12.28	13.28
Wool-fat extracted by ether	7.17	14.66	34.19
Wool-soap extracted by subsequent treatment:			
By water (wool-sweat)	21.13	21.83	9.76
By subsequent treatment with alcohol	0.35	0.55	0.89
By subsequent treatment with dilute hydrochloric acid.	1.45	5.64	1.39
By subsequent treatment with ether and alcohol.	0.29	0.57	.....
Pure wool-fibre	43.20	20.83	32.11
Dirt (by difference)	2.93	23.64	8.38
	100.00	100.00	100.00

The following analyses by Faist further illustrate the general composition of sheep's wool (air-dried).

Description	Mineral matter	Suint and fatty matter	Pure wool	Moisture	Pure air-dry wool
Raw Wools:					
Hohenheim, with little suint	6.3	44.3	38.0	11.4	49.4
Hohenheim, with much glutinous suint.	16.8	44.7	28.5	7.0	35.5
Washed Wools:					
Hohenheim	0.94	21.00	72.00	6.06	78.06
Hohenheim, with difficultly soluble suint.	1.3	40.0	56.0	2.7	58.7
Hungarian, very soft	1.0	27.0	64.8	7.2	72.0
Würtemburg, less soft	1.2	16.6	77.7	3.5	82.2

Wool-fat (Vol. 2, page 495) is remarkable for containing a large proportion of *cholesterol* and *isocholesterol.* It takes up a considerable proportion of water. Purified wool-fat forms the lanolin of pharmacy.

Suint, Wool-sweat or Wool-soap, is the portion of the wool-soap soluble in water. It has a very complex composition, and has been the subject of numerous researches. Suint consists chiefly of the potassium salts of the higher fatty acids, including cerotic acid; with smaller quantities of valerate, butyrate, propionate and acetate; and phosphates, sulphates, and chlorides. Ammonium compounds are present in small proportion.

The *mineral matter* of purified sheep's wool ranges from 1 to 2%, and consists largely of sulphates of the alkali-metals. It is remarkable for containing a notable proportion of silica.[1]

[1] A sample of Lincoln wool which had previously been scoured by soap, thoroughly washed with pure water, and dried, was found by Wood (*J. Soc. Dyers and Col.*, 1885, 139) to yield 1 % of *ash* of the following composition:

	Percentage composition		
	Total ash	Ash soluble in water	Ash insoluble in water
K_2O	31.1	42.3	trace
Na_2O	8.2	17.3	trace
CaO	16.9	4.5	51.2
Al_2O_3 and Fe_2O_3	12.3	3.6	37.7
SiO_2	5.8	4.1	11.1
SO_3	20.5	24.8	trace
CO_2	4.2	3.4	.....
P_2O_5	trace	trace	trace
Cl	trace	trace	.....

Bowman found 2 % of magnesia in the wool-ash from sheep which had pastured on a magnesian-limestone district.

Cholesterol is contained in the wool suint (or yoke) which is removed in the process of wool washing. It melts at 145° (isocholesterol melts at 132°). It has a neutral action, is insoluble in water, soluble in boiling alcohol, ether, chloroform, sublimes at 199°.

Wool-gelatin, according to Gardner and Carter (*J. Soc. Dyers, & Col.*, 1898, **14,** 167), is present in small proportion (1.65%).

Wool-keratin, according to Bowman (*J. Soc. Dyers & Col.*, 1885, **1,** 136), has the following composition:

	Carbon	Hydrogen	Nitrogen	Oxygen	Sulphur	Loss
Lincoln wool	52.0	6.9	18.1	20.3	2.5	0.2[1]
Irish wool	49.8	7.2	19.1	19.9	3.0	1.0[1]
Northumberland wool	50.8[1]	7.2	18.5	21.2	2.3	.....
Southdown wool	51.3[1]	6.9	17.8	20.2	3.8	.....
Mean	50.27	7.05	18.37	30.40	2.90	.....

The mean of Bowman's analyses of wool-keratin correspond to the empirical formula: $C_{43}H_{71}O_{13}N_{13}S$.[2]

When wool is heated to about 140° it begins to decompose, and on heating more strongly it yields an oily substance of intolerable odour, together with large quantities of pyrrole, much hydrogen sulphide, a small quantity of carbon disulphide, and mere traces of oily bases (*Ann. Chem. Pharm.*, **109,** 127).

Wool is affected to an appreciable extent by prolonged boiling with

[1] The "loss" in the first two analyses represents the mineral matter. In the last two this is included in the carbon.

[2] Mills and Takamine (*Trans.*, 1883, **43,** 142) regard wool-keratin as a substance of definite composition, having the empirical formula $C_{42}H_{157}O_{15}N_5S$. This is based on the mean of the analyses of wool-keratin recorded in Gmelin's *Handbook of Chemistry* (**18,** 351); but Whiteley (*Proc. Chem. Soc.*, 1886, **49,** 142) has pointed out that by a misprint the respective percentages of nitrogen and hydrogen were transposed. The corrected average composition of keratin, according to the average of the analyses in question, is C, 49.96; H, 7.11, N, 16.65; S, 3.39; and O (by difference), 22.89 %. These figures correspond to the formula: $C_{41}H_{71}O_{14}N_{12}S$.

water, sensible traces of hydrogen sulphide being evolved. Bowman states that wool which looked quite bright when well washed with tepid water, became quite dull and lustreless under treatment with boiling water, and the appearance under the microscope was materially altered. In presencee of only a very small quantity of alkali, the prolonged action of boiling water gelatinises wool more or less completely.

Wool is not much affected by solutions of soap, borax, ammonium carbonate, or carbonate of alkali-metal, but is acted on by alkali hydroxides, even when very dilute. Knecht found that when wool was boiled for 3 hours with water containing sodium hydroxide in amount equal to 0.3% of the weight of the wool, the fibre was not disintegrated, but when the alkali was increased to 0.6% complete disintegration and almost complete solution of the wool took place.

Schützenberger obtained the following products by decomposing purified wool with an aqueous solution of baryta, under pressure, at 170°:

	%
Nitrogen evolved as ammonia	5.25
Carbon dioxide (separated as $BaCO_3$)	4.27
Oxalic acid (separated as BaC_2O_4)	5.72
Acetic acid (by distillation and titration)	3.20
Pyrrole and other volatile products	1 to 1.5
Percentage composition of fixed residue; containing leucine, tyrosine, and other volatile products: C	47.85
H	7.69
N	12.63
O	31.83

By distilling wool (flannel) with concentrated sodium hydroxide, Williams obtained a distillate containing a large quantity of ammonia, besides butylamine and amylamine.

A fairly accurate separation of wool from cotton and linen can be effected by boiling the mixed fibres for some time with a solution of sodium hydroxide of 1.05 sp. gr. This dissolves the wool, leaving the vegetable fibres unaffected.

On the contrary, the vegetable fibres are affected by acids far more readily than wool, which fact is extensively employed for the recovery of the wool from waste fabrics of mixed nature.[1]

By dissolving carefully washed wool in concentrated baryta water, passing carbon dioxide, and treating the filtered liquid with lead acetate, a precipitate is obtained which, when washed, suspended in water, and decomposed by hydrogen sulphide, gives a solution which, after filtration and evaporation to dryness, yields a dirty yellow, non-deliquescent, acid substance. This body Knecht (*J. Soc. Dyers & Col.*, 1889, **5**, 72) called lanuginic acid. It dissolves slowly in cold,

[1] The goods are exposed in a closed chamber to the action of hydrochloric acid gas for a number of hours. When taken out and passed through suitable machinery the cotton falls to a fine dust, while the wool-fibre or shoddy remains practically unchanged. A similar method (in which sulphuric acid is commonly preferred) is employed for freeing raw wool from the "burrs" frequently observed in it.

but readily in hot water, is sparingly soluble in alcohol, and insoluble in ether. The aqueous solution of lanuginic acid is not coagulated by boiling, but it precipitates both acid and basic colouring matters, forming lakes, and it also yields precipitates with tannin and with potassium bichromate. The compounds of lanuginic acid with colouring matters appear to be definite in composition, and precipitation by night-blue or picric acid may be conveniently employed for their determination. With phosphotungstic acid and with Millon's reagent lanuginic acid behaves like the proteins.

When heated to 100°, this substance becomes soft and plastic, and this property is shared by its lakes, most of which actually melt at the temperature of boiling water. This so-called acid becomes anhydrous at 110°, and on ignition leaves a considerable quantity of ash, consisting chiefly of barium carbonate. By substituting sodium carbonate for baryta in the preparation of lanuginic acid, Knecht obtained a product identical in external properties and all other characters with that previously prepared, but leaving a much smaller quantity of ash on ignition. Knecht finds this substance to have the following ultimate composition: C, 41.61; H, 7.31; N, 16.26; S, 3.35 and O, 31.44%.

By heating wool with five times its weight of water to a temperature of 200–230° for 4 hours, the substance almost wholly dissolved, with liberation of sulphuretted hydrogen, etc., and the solution contained the lanuginic acid. Very similar products were obtained by heating horn and human epidermis with water to 200° under pressure.

When wool is boiled with a solution of sodium hydroxide to which a little lead acetate has been added, it dissolves with brown or black colouration, due to the formation of lead sulphide. It appears, however, that the whole of the sulphur of wool does not enter into this reaction, a portion being in a more stable state of combination. Thus Chevreul found that by treating wool with alkalies the greater part of the sulphur is removed, but he was unable to extract the whole of it in this manner. By steeping wool 28 times in lime-water for 24 hours each time, and washing with hydrochloric acid between each treatment, he succeeded in reducing the proportion of sulphur to 0.46%. The wool treated in this manner was no longer blackened by boiling with lead acetate and excess of alkali hydroxide. Knecht found that lanuginic acid also gives a negative reaction with the alkaline lead reagent, from which fact he considers it probable that the residual sulphur of the wool exists as lanuginic acid, or as some substance which readily yields lanuginic acid as a first product of its decomposition by reagents.

The blackening of wool by boiling with an alkaline solution of lead affords a ready means of distinguishing it from silk, cotton, and linen, neither of which fibres contains sulphur. The recognition of wool in mixed fibres may also be readily effected by the microscope[1] (see further, page 592).

Knecht states that the plumbate reaction is also given with "stoved" silk (sulphur bleached) but this seems doubtful. *p*-Benzoquinone (Scharwin, *Zeit. f. angew. Chem.*, 1913, 254) gives with wool (and silk?) a rose tint darkening through red to almost black.

Knecht has shown (*J. Soc. Dyers & Col.*, 1888, **4**, 72, 104) that when wool or silk is dyed with coal-tar colouring matters of basic nature, a complete decomposition of the dye takes place, the base uniting with the fibre to form an insoluble coloured lake, while the acid remains in the dye-bath. Knecht has further shown that when wool is dissolved in moderately dilute sulphuric acid, a solution is obtained which possesses the property of precipitating any of the acid colouring matters from their solutions, and that when wool is boiled with very dilute sulphuric acid, and then extracted repeatedly with distilled water until all free acid has been removed, the wool can subsequently be dyed a full shade in neutral solutions of the acid colouring matters. From these observations it appears evident that by the action of the sulphuric acid there is produced in the fibre a substance having the property, not previously possessed by the wool, of forming lakes with acid colouring matters.

The behaviour of wool with coal-tar colouring matters suggests strongly that wool-keratin has the constitution of an amino-acid, in which case it should be possible to obtain the corresponding diazo-compound. This reaction has apparently been effected by P. Richard (*J. Soc. Dyers & Col.*, 1888, **4**, 154), though the characters of the product did not agree in every respect with those of known diazo-compounds, and E. Knecht considers the theory untenable (*J. Soc. Dyers & Col.*, 1889, **5**, 75). A very similar substance is yielded by silk.

If moistened wool be treated with chlorine-gas in excess, the chlorine is absorbed with great evolution of heat, hydrochloric acid is formed in large amount, and the wool is converted into a white pulpy substance. By using a limited quantity of chlorine diluted with air, the wool remains intact but becomes more transparent, acquires a glossy, silk-like appearance, a crackling feel, and an increased capacity for absorbing dyes. When chlorinated in the foregoing manner, wool does not readily acquire a yellow colour by subsequent treatment with soap or alkalies (*J. Soc. Dyers & Col.*, 1898, **14**, 175).

The chlorination of wool has been the subject of an English patent (1897, No. 11917).

APPENDIX A.

Note to Page 4.—Objection may be taken to the method described on page 4 and the tables given therein, on the ground that considerable error arises in such cases by weighing the cuprous oxide direct; a relatively large quantity of organic, colloidal matter is precipitated with the cuprous oxide (often from 5 to 10 mgrm.). In all such cases the cuprous oxide should be oxidized to CuO or reduced to metallic copper, so as to burn away the co-precipitated protein material. (W. A. D.)

Note to Page 5.—For an account of provisional methods that have been suggested for the valuation of commercial malt extracts used in the textile industries see papers by R. T. May and by W. P. Dreaper in the *J. Soc. Dyers and Col.*, 1913, **29**, pp. 156 and 157.

Note to Page 7.—In the opinion of one of the Editors (W. A. D.) the method proposed by Bertrand for estimating sugars can only be regarded as an approximate one; the tables given by Bertrand for invert sugar are unreliable, giving results from 3 to 5% low. This is due to the fact that Bertrand, in preparing his tables, inverted the cane sugar by heating with 2% hydrochloric acid at 100° for 10 to 15 minutes, a procedure which invariably destroys lævulose; this is made evident by the production of a distinct yellow colour. As is well known, in inverting cane sugar a temperature of 70° should not be exceeded.

Note to Pages 18 *to* 20.—Abderhalden and Weil (*Zeitsch. Physiol. Chem.*, 1913, **84**, 39) have recently by the hydrolysis of proteins of nervous tissue, isolated an *amino-acid*, $C_6H_{13}O_2N$, which is apparently α-amino caproic acid.

Note to Page 27.—Dr. R. H. Aders Plimmer has communicated to us a new method for separating cystine and tyrosine, which is as follows: "Cystine and tyrosine can be completely and quantitatively separated from one another by means of absolute alcohol saturated with hydrogen chloride. The tyrosine is rapidly converted into its ester and goes into solution. It can be recovered by boiling the solution, when diluted with water, for about 8 hours and then neutralising with ammonia. Almost the whole of the cystine is

insoluble; the portion which goes into solution (perhaps cystine hydrochloride) is precipitated by adding an equal volume of absolute alcohol before filtering. The cystine is not converted into its ethyl ester, since on dissolving the insoluble portion in dilute hydrochloric acid and neutralising with ammonia the cystine is precipitated in the typical hexagonal plates." (Compare *Biochemical Journal*, 1913, **7**, 311.)

Note to Page 190.—Monier-Williams (*Local Gov. Board Food Reports*, No. 17) has proposed the following routine test for fluorides in butter; it can also be applied to cream. 10 grm. of cream are placed in a test-tube, a few drops of hydrogen-peroxide solution added and then 1 c.c. of a solution containing 2% of titanium sulphate in 10% sulphuric acid; a control test is made with pure cream. If the yellow colour is markedly less in the test than in the control the presence of fluorides may be inferred and should be confirmed by the etching test. If there is no difference in colour it may be taken that fluorides are not present. The test will easily show 0.1% of sodium fluoride and even less. When applied to butter 10 grm. are shaken out with petroleum ether and a little water and the test applied to the aqueous layer.

Methæmoglobin.

Note to Page 541.—Dittrich (*Archiv. f. Exp. Path. v. Pharmak.*, 1892, **29**, 247) states that pure methæmoglobin shows only a single characteristic band, $\lambda = 632$. E. Letsche (*Z. f. phys. Chem.*, 1912, **80**, 412) shows by spectrophotometric measurements that oxyhæmoglobin quantitatively passes into methæmoglobin by the action of hydroxylamine. The spectrophotometric quotient is 1.186, identical with the value found by V. Zeynek (*Arch. f. Phys.*, 1899, p. 460). He considers, as does Küster, that methæmoglobin contains less oxygen than oxyhæmoglobin.

APPENDIX B.

Effect of Hydrogen Peroxide on Milk.

In view of the fact that hydrogen peroxide can now be obtained cheaply, of high purity and good keeping qualities, its effects on the constituents of ordinary milk become of much interest. The data here inserted are derived principally from the investigations of M. J. Rentschler of the Laboratory of the Oakland Chemical Company of New York.

Dr. Henry D. Chapin (*Arch. Ped.*, 1893, **10**, 652) early called attention to its possible value as a preservative, showing that it retarded

the acid fermentation, also observed the absence of liquifying bacteria in milk thus treated and noted the decomposing effect of the proteins on the peroxides. Previous to Dr. Chapin's work, Schrodt (*Milch. Zeit.*, 1883, **12**, 785; *abst. in Chem. Zentr.*, 1884, [3], **15**, 67) had experimented with a mixture of hydrogen peroxide and borax. Babcock (*Bull. Agric. Exp. Sta., Univ. Wisc.*, 1889, No. 9) also showed that milk decomposes peroxide. Several other investigators have confirmed this statement and the opinion was formed that the decomposition was due to enzymic action, commonly defined as the "catalase content" of milk. Many were of the opinion that every enzyme possesses this power but in 1901 Loew (*Chemische Energie d. Lebenden Zellen*) showed that only one enzyme has this power. He had obtained it from tobacco and termed it *catalase* and stated that when any other enzyme has this power it is due to adhering traces of catalase.

As a result of recent investigations it appears that hydrogen peroxide will kill the majority of bacteria commonly occurring in cows' milk, and as the substance in itself can hardly be dangerous in small amounts in which it would be used and as its own decomposing products are water and oxygen, it would appear that we have in this reagent a useful method for improving the quality of unclean milks.

NOTE.—It is highly improbable that the Food Laws of the United States would ever permit the addition of any purifying agent to unclean milk as it has been amply demonstrated that milk can be produced in a cleanly way and if kept cold can be preserved a sufficient time for practical purposes.—S. S. S;

INDEX.