

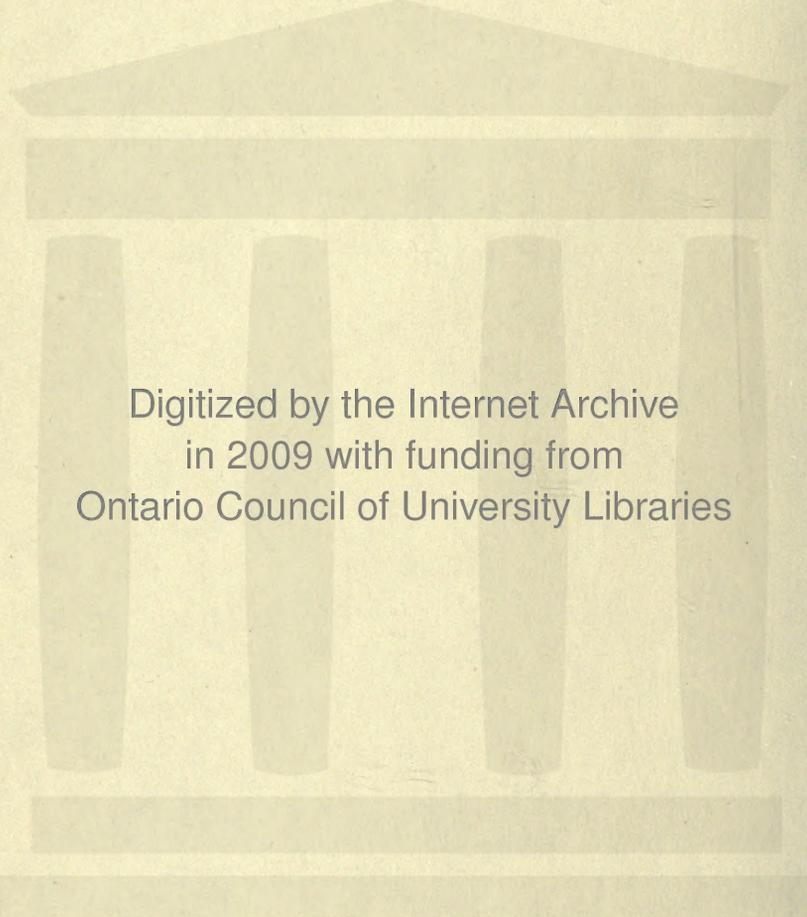


HANDBOUND  
AT THE



UNIVERSITY OF  
TORONTO PRESS





Digitized by the Internet Archive  
in 2009 with funding from  
Ontario Council of University Libraries





THE  
BIOCHEMICAL  
JOURNAL

CAMBRIDGE UNIVERSITY PRESS

C. F. CLAY, MANAGER

LONDON: FETTER LANE, E.C. 4



H. K. LEWIS & CO., LTD., 136, GOWER STREET, LONDON, W.C. 1  
WILLIAM WESLEY & SON, 28, ESSEX STREET, STRAND, LONDON, W.C. 2

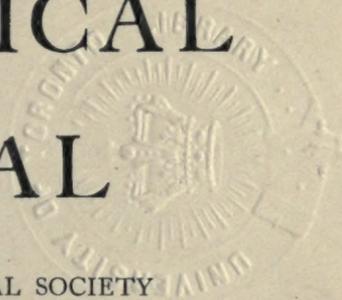
CHICAGO: THE UNIVERSITY OF CHICAGO PRESS  
(Agent for the United States and Canada)

BOMBAY, CALCUTTA, MADRAS: MACMILLAN & CO., LTD.

TOKYO: THE MARUZEN-KABUSHIKI-KAISHA

*All rights reserved*

THE  
BIOCHEMICAL  
JOURNAL



EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

DR G. BARGER

PROF. V. H. BLACKMAN

MR J. A. GARDNER

PROF. F. G. HOPKINS

PROF. F. KEEBLE

PROF. B. MOORE

PROF. W. RAMSDEN

DR E. J. RUSSELL

VOLUME XIV 1920

166814  
5/11/21

CAMBRIDGE  
AT THE UNIVERSITY PRESS

1920



QP  
501  
B47  
v.14  
cop.4

# CONTENTS

## No. 1 (FEBRUARY)

	PAGE
OBITUARY NOTICE: Adrian John Brown . . . . .	1
I. Estimation of Carbon Dioxide, Oxygen and Combustible Gases by Krogh's Method of Micro-Analysis. By H. O. SCHMIT-JENSEN. (With two figures) . . . . .	4
II. On the Deterioration of Cotton on Wet Storage. By N. FLEMING and A. C. THAYSEN. (With Plate I) . . . . .	25
III. Adsorptive Stratification in Gels, III. By S. C. BRADFORD. (With Plate II) . . . . .	29
IV. On the Association of Antitoxins with the Proteins of Immunised Horse Serum. By A. HOMER . . . . .	42
V. The Guanidine Content of Faeces in Idiopathic Tetany. By J. S. SHARPE . . . . .	46
VI. On the Colours of two Sea Anemones, <i>Actinia equina</i> and <i>Anemonia sulcata</i> . Part I. Environmental. By R. ELMHIRST. Part II. Chemical. By J. S. SHARPE. (With Plates III, IV) . . . . .	48
VII. The Penetration of Electrolytes into Gels. I: The Penetration of Sodium Chloride into Gels of Agar-agar containing Silver Nitrate. By W. STILES. (With four figures) . . . . .	58
VIII. Note on the Use of Butyl Alcohol as a Solvent for Anthocyanins. By O. ROSENHEIM . . . . .	73

## No. 2 (APRIL)

IX. An Investigation of the Methods Employed for Cooking Vegetables, with special reference to the losses incurred. Part II. Green Vegetables. By H. MASTERS and P. GARBUTT . . . . .	75
X. On the Theory of Gels. II: The Crystallisation of Gelatin. By S. C. BRADFORD. (With one figure) . . . . .	91
XI. A Note on the Effect of Purgation on the Creatinine Content of Urine. By D. BURNS . . . . .	94
XII. The Preparation of Sørensen's Phosphate Solutions when the Pure Salts are not available. By C. J. MARTIN . . . . .	98
XIII. The Influence of Reaction on Colour Changes in Tyrosine Solutions. By E. C. V. VENN. (With one figure) . . . . .	99
XIV. Barger's Microscopical Method of Determining Molecular Weights. Part I. The Principle of the Method with Reference to the Molecular and Ionic Attraction of Solute for Solvent. By K. YAMAKAMI . . . . .	103
XV. The Formation of Ferrous Sulphide in Eggs during Cooking. By C. K. TINKLER and M. C. SOAR . . . . .	114
XVI. On the Presence of Amylase in Milk and Cheese. By M. SATO . . . . .	120
XVII. The Antiscorbutic Requirements of the Monkey. By A. HARDEN and S. S. ZILVA . . . . .	131

	PAGE
XVIII. The Production in Monkeys of Symptoms closely resembling those of Pellagra, by Prolonged Feeding on a Diet of Low Protein Content. By H. CHICK and E. M. HUME. (With Plate V and one figure)	135
XIX. On the Swelling of Gelatin in Hydrochloric Acid and Caustic Soda. By D. J. LLOYD. (With seven figures)	147
XX. The Anti-Scorbutic Properties of Concentrated Fruit Juices. Part III. By A. HARDEN and R. ROBISON	171
XXI. Observations on Anthocyanins. I: The Anthocyanins of the Young Leaves of the Grape Vine. By O. ROSENHEIM. (With one figure)	178
XXII. The Action of Thrombin upon Fibrinogen. By J. O. W. BARRATT. (With seven figures)	189
XXIII. Effect of Heat on the Anti-Scorbutic Accessory Factor of Vegetable and Fruit Juices. By E. M. DELF. (With four figures)	211
XXIV. The Products of the "Acetone: <i>n</i> -Butyl Alcohol" Fermentation of Carbohydrate Material with Special Reference to some of the Intermediate Substances produced. By J. REILLY, W. J. HICKINBOTTOM, F. R. HENLEY, and A. C. THAYSEN. (With two figures)	229
XXV. A Note on Braunstein's Modification of the Mörner-Sjöqvist Process for the Estimation of Urea. By A. H. TODD	252
XXVI. The Iodometric Estimation of Sugars. By H. M. JUDD	255
XXVII. Dietetic Experiments with Frogs. By A. HARDEN and S. S. ZILVA	263

### Nos. 3 & 4 (JULY)

XXVIII. A Gas Analysis Apparatus Accurate to 0.001 % mainly designed for Respiratory Exchange Work. By A. KROGH. (With four figures)	267
XXIX. The Calibration, Accuracy and Use of Gas Meters. By A. KROGH. (With two figures)	282
XXX. The Relative Value of Fat and Carbohydrate as Sources of Muscular Energy. With Appendices on the Correlation between Standard Metabolism and the Respiratory Quotient during Rest and Work. By A. KROGH and J. LINDHARD. (With twenty-three figures)	290
XXXI. Studies in the Acetone Concentration in Blood, Urine, and Alveolar Air. II: The Passage of Acetone and Aceto-Acetic Acid into the Urine. By E. M. P. WIDMARK. (With four figures)	364
XXXII. Studies in the Acetone Concentration in Blood, Urine, and Alveolar Air. III: The Elimination of Acetone through the Lungs. By E. M. P. WIDMARK. (With three figures)	379
XXXIII. The Metabolism of Carbohydrates. Part I. Stereochemical Changes undergone by Equilibrated Solutions of Reducing Sugars in the Alimentary Canal and in the Peritoneal Cavity. By J. A. HEWITT and J. PRYDE. (With five figures)	395
XXXIV. The Existence in the Bile of an Inhibitor for Hepatic Esterase, and its Nature. By G. M. WISHART	406
XXXV. A Series of Abnormal Liesegang Stratifications. By E. HATSCHKE. (With Plate VI)	418
XXXVI. The Acidity of Ropy Milk. By K. FREEAR and E. C. V. VENN. (With seven figures)	422

# CONTENTS

vii

	PAGE
XXXVII. The Occurrence and Nature of the Plant Growth-Promoting Substances in Various Organic Manurial Composts. By F. A. MOCKERIDGE . . . . .	432
XXXVIII. Rapid Volumetric Methods for the Estimation of Amino-Acids, Organic Acids and Organic Bases. By F. W. FOREMAN. (With one figure) . . . . .	451
XXXIX. Adsorptive Stratification in Gels. IV. By S. C. BRADFORD. (With Plates VII, VIII and one figure) . . . . .	474
XL. The Nature of Yeast Fat. By I. SMEDLEY MACLEAN and E. M. THOMAS . . . . .	483
XLI. The Extraction of the Fat-Soluble Factor of Cabbage and Carrot by Solvents. By S. S. ZILVA. (With seven figures) . . . . .	494
XLII. A Contribution to the Study of Keratomalacia among Rats. By M. STEPHENSON and A. B. CLARK. (With Plates IX, X and eight figures) . . . . .	502
XLIII. Barger's Microscopical Method of determining Molecular Weights. Part II. Its Application to Caseinogen. By K. YAMAKAMI . . . . .	522

## No. 5 (OCTOBER)

XLIV. Oxidising Enzymes. II: The Nature of the Enzymes associated with Certain Direct Oxidising Systems in Plants. By M. W. ONSLOW . . . . .	535
XLV. Oxidising Enzymes. III: The Oxidising Enzymes of some Common Fruits. By M. W. ONSLOW . . . . .	541
XLVI. A Study of some Biochemical Tests. No. 2: The Adamkiewicz Protein Reaction. The Mechanism of the Hopkins-Cole Test for Tryptophan. A New Colour Test for Glyoxylic Acid. By W. R. FEARON . . . . .	548
XLVII. The Heat-Inactivation of Diphtheria Antitoxin. By A. HOMER . . . . .	565
XLVIII. Note on "Scurvy" in Pigs. By R. H. A. PLIMMER . . . . .	570
XLIX. Note on the Oxidation of Quinine with Hydrogen Peroxide. By M. NIERENSTEIN . . . . .	572
L. Glycine and its Neutral Salt Addition Compounds. By H. KING and A. D. PALMER . . . . .	574
LI. Note on the Production of a Contracting Clot in a Gel of Gelatin at the Isoelectric Point. By D. JORDAN LLOYD. (With Plate XI) . . . . .	584
LII. An Experimental Study of the Effect of certain Organic and Inorganic Substances on the Bread-making Properties of Flour and on the Fermentation of Yeast. By H. MASTERS and M. MAUGHAN . . . . .	586
LIII. Ammonia Excretion, Amino-acid Excretion and the Alkaline Tide in Singapore. By J. A. CAMPBELL. (With three charts) . . . . .	603
LIV. Cuorin. By H. MACLEAN and W. J. GRIFFITHS . . . . .	615
LV. Factors influencing Alkaloidal Content and Yield of Latex in the Opium Poppy ( <i>Papaver somniferum</i> ). By H. E. ANNETT . . . . .	618
LVI. Digestibility of Germinated Beans. By D. M. ADKINS . . . . .	637
LVII. The Effect of Pyruvates, Aldehydes and Methylene Blue on the Fermentation of Glucose by Yeast Juice and Zym in Presence of Phosphate. By A. HARDEN and F. R. HENLEY. (With two figures) . . . . .	642

	PAGE
LVIII. The Distribution of Inorganic Iron in Plant and Animal Tissues. By H. W. JONES . . . . .	654
LIX. The Nomenclature of the so-called Accessory Food Factors (Vitamins). By J. C. DRUMMOND . . . . .	660
LX. Researches on the Fat-soluble Accessory Substance. III: Technique for carrying out Feeding Tests for Vitamin A (Fat-soluble A). By J. C. DRUMMOND and K. H. COWARD . . . . .	661
LXI. Researches on the Fat-soluble Accessory Substance. IV: Nuts as a Source of Vitamin A. By K. H. COWARD and J. C. DRUMMOND . . . . .	665
LXII. Researches on the Fat-soluble Accessory Substance. V: The Nutritive Value of Animal and Vegetable Oils and Fats considered in Relation to their Colour. By J. C. DRUMMOND and K. H. COWARD. (With four figures) . . . . .	668

---

### No. 6 (DECEMBER)

LXIII. A Modification of the Barcroft and Winterstein Microrespirometers. By N. K. ADAM. (With one figure) . . . . .	679
LXIV. The Fermentation of Cellulose in the Paunch of the Ox and its Significance in Metabolism Experiments. By A. KROGH and H. O. SCHMIT-JENSEN . . . . .	686
LXV. A Method for Obtaining Uncontaminated Specimens of Urine from the Billy Goat; with some Notes upon the Normal Metabolism of this Animal. By R. A. PETERS. (With six figures) . . . . .	697
LXVI. The Action of Sea Water on Cotton and other Textile Fibres. By C. DORÉE . . . . .	709
LXVII. A Note on the Differentiation of the Yellow Plant Pigments from the Fat-Soluble Vitamine. By M. STEPHENSON. (With one chart) . . . . .	715
LXVIII. Note on the Vitamine Content of Milk. By F. G. HOPKINS. (With four figures) . . . . .	721
LXIX. The Effects of Heat and Aeration upon the Fat-soluble Vitamine. By F. G. HOPKINS. (With five figures) . . . . .	725
LXX. Researches on the Fat-soluble Accessory Factor (Vitamin A). VI: Effect of Heat and Oxygen on the Nutritive Value of Butter. By J. C. DRUMMOND and K. H. COWARD. (With three figures) . . . . .	734
LXXI. The Action of Ozone on the Fat-soluble Factor in Fats. Preliminary Note. By S. S. ZILVA . . . . .	740
LXXII. The Nutritive Value of Lard. By J. C. DRUMMOND, J. GOLDING, S. S. ZILVA and K. H. COWARD. (With seven figures and Plate XII) . . . . .	742
LXXIII. The Iodimetric Estimation of Sugars. By J. L. BAKER and H. F. E. HULTON . . . . .	754
LXXIV. The "Ammonia Coefficient" of Pregnancy. By W. C. CULLIS and E. E. HEWER. (With three charts) . . . . .	757
LXXV. The Penetration of Electrolytes into Gels. II: The Application of Fourier's Linear Diffusion Law. By G. S. ADAIR . . . . .	762
INDEX . . . . .	781

## OBITUARY NOTICE.

### ADRIAN JOHN BROWN.

BIOCHEMISTRY has suffered a severe loss by the sudden death of ADRIAN J. BROWN, which followed closely upon that of his wife last July. Adrian Brown was born in 1852 at Burton and educated at the local Grammar School, whilst he received his chemical training at the Royal School of Mines under Frankland. After acting for a short time as private assistant to Dr Russell at Bartholomew's Hospital he was appointed chemist to the brewing firm of Messrs Thos. Salt & Co. of Burton-on-Trent, where he remained until 1899. In that year he was appointed to the newly-founded Chair of Malting and Brewing in Birmingham, and in 1904 became Professor of the Biology and Chemistry of Fermentation and Director of the School of Brewing, posts which he occupied until his death. His time at Birmingham was largely occupied with his professional duties and he trained a large number of students, many of whom entered the brewing industry. He was a most genial and sympathetic teacher taking great pains with his students, by whom he was greatly respected and beloved. In connection with his teaching he published *Laboratory Studies for Brewing Students* (1904), a practical text-book of much value. He was the first Examiner in Biological Chemistry to the Institute of Chemistry, the recognition of this subject in 1901 as an avenue of approach to the Institute marking an important stage in the development of Biochemistry in this country. He was an early member of the Biochemical Club, served on the committee, presided over the meeting held in Birmingham in 1912 and always took the greatest interest in the proceedings of the Club and of the Society into which it naturally developed. He was elected a Fellow of the Royal Society in 1911 and was President of the Institute of Brewing from 1917-1919.

He had a delightful home at Northfield, outside Birmingham, and was never happier than when discussing the treasures of his garden with a guest or demonstrating to him the abnormal production of marsh gas in the pond.

As an enthusiastic teacher and investigator in the laboratory he commanded the respect and admiration of all who came into intimate contact with him whilst his geniality of character and charming personality endeared him to a large circle of friends.

Adrian Brown was one of the circle of scientific men engaged in the brewing industry at Burton-on-Trent in the seventies of last century, of which such a charming and interesting account has been given by his brother, Horace T. Brown, in his *Reminiscences*. His own chief contributions to the scientific production of the "Bacterium Club" consisted in the study of two oxidising

organisms, *B. acetii* and *B. xylinum*, and a series of researches on the conditions of reproduction and fermentation of yeast.

One of the most important results of his investigation of *B. acetii* and *B. xylinum* (obtained from "mother of vinegar") was the clear demonstration that their oxidising action was strictly selective. Both of these organisms were shown to oxidise dextrose to gluconic acid, whereas they were without action on laevulose. Mannitol, on the other hand, was converted into laevulose, which was not further affected. *B. xylinum* was distinguished by producing a very bulky membranous growth, which appeared to be composed of cellulose, and which was made alike from dextrose and laevulose, and yielded a dextrorotatory reducing substance on hydrolysis. Subsequent investigations by O. Emmerling (1899) showed that the membrane in addition to cellulose contained a nitrogenous substance which yielded glucosamine on hydrolysis.

The investigation on the reproduction and fermentation of yeast also led to highly important results. In the first place he discovered the remarkable fact that when a medium is inoculated with yeast, reproduction ceases when a certain definite maximum number of cells is present and that if a number in excess of this be introduced *ab initio*, no increase whatever occurs, the added cells showing no signs of budding. This phenomenon was subsequently (1905) traced to the curious relations which subsist between yeast and oxygen. A certain concentration of oxygen in the cell appears to be necessary for the occurrence of cell division, and if the number of cells added is so great that this concentration is not reached no growth occurs. When a smaller number of cells is employed, these appear to take up the dissolved oxygen from the medium and if the growth is then allowed to proceed in the ordinary way (*i.e.* practically anaerobically in presence of the carbon dioxide produced by fermentation of the sugar in the medium) a certain definite number of cells is ultimately produced, whatever the seeding has been, "for the amount of available oxygen at the commencement of the experiments is constant, and it is conceivable that its power of stimulation is constant and governs the power of reproduction of the cells as a whole, irrespective of the number present." If the culture is continuously aerated throughout growth, the number of cells produced is much larger. He further demonstrated that Pasteur's conclusion that fermentation was life without air was not correct with respect to yeast, for in presence of abundant oxygen more and not less sugar was fermented than in its absence by equal numbers of yeast cells. A masterly criticism of Pasteur's experiments conclusively showed that these did not lead to a true expression of the fermentative powers of the yeast employed and that there was accordingly no experimental evidence to show that the fermentation of sugar by yeast was not independent of the presence or absence of oxygen in the surrounding medium. Three years later the discovery of zymase by Buchner brilliantly confirmed this conclusion and afforded a simple explanation of the facts. It was no doubt the observation, made in the course of these experiments, that the rate of fermentation of

sugar by a given number of yeast cells was, over a large range (5–20 %), independent of the concentration of the sugar, that led Brown to study the action of invertase, for which the ordinary law of mass action had been found to apply by O'Sullivan and Tompson in 1890. Investigation showed him that in reality the invertase behaved like the yeast cells, the initial rate of fermentation being constant over a considerable range of concentration, but that the products of the inversion inhibited the action of the enzyme and caused the pseudo-monomolecular course of the change. It was to explain this constancy of initial rate that he introduced the idea that combination occurred between the enzyme and substrate before the latter was decomposed, and lasted for a definite interval of time.

Perhaps the most interesting of all his scientific work was that occasioned by his discovery in 1907 that the seed of the barley is provided with a remarkable semi-permeable membrane. He noticed that the seeds of "blue" barley (*Hordeum vulgare* var. *caerulescens*) when steeped in dilute sulphuric acid became soft and swollen but retained their blue colour, although the blue pigment, which is situated in the aleurone cells, is readily turned red by acid. To quote his own words, this showed "that the covering has the property of resisting the passage of the acid, whilst it allows water to diffuse freely into the interior of the grain. So much is this the case that a dilute solution of sulphuric acid may be concentrated by steeping barley in it." Normal sulphuric acid was in fact thus concentrated from 49 to 76 g. of  $\text{H}_2\text{SO}_4$  per litre. The resistance of the membrane to so concentrated an acid is very remarkable and a study of its behaviour to other substances, showed that the property was selective, substances like alcohol and acetic acid passing freely through the membrane, whilst salts in general, cane-sugar and tartaric acid could not penetrate it. The rate and extent of absorption of water from solutions of various substances could readily be measured and in short, in the words of Armstrong, an exquisite and invaluable method of studying the osmotic effect had been devised. The subject was pursued further in conjunction with Worley and Tinker more particularly in its physical aspects.

In addition to the work briefly outlined in the foregoing Brown made a number of investigations on various subjects including the heat of fermentation, the inheritance of colour in barley grains, *Bacillus subtilis*, and in conjunction with Ward on the antiseptic properties of hops.

It is characteristic of the man that although his scientific output was not large in amount, it dealt with problems of a fundamental nature and led in every instance to some discovery of real importance and value.

ARTHUR HARDEN.

# I. ESTIMATION OF CARBON DIOXIDE, OXYGEN AND COMBUSTIBLE GASES BY KROGH'S METHOD OF MICRO-ANALYSIS.

BY HANS OLUF SCHMIT-JENSEN.

*From the Laboratory of Zoophysiology, University of Copenhagen.*

*(Received November 14th, 1919.)*

## INTRODUCTION.

For the study of respiratory processes of small organisms and for many other biological purposes it is essential to have methods for analysing small air-bubbles. In 1907 two such methods were described by August Krogh [1908], viz. a method of microscopical gas-analysis, applicable to air-bubbles of 1.00-0.01 mm.<sup>3</sup>, and a method of micro-analysis for volumes between 1 and 20 mm.<sup>3</sup>.

The principle of the latter method is based upon the fact that small air-bubbles placed in a fluid behave much as semi-fluid fat drops and easily allow transport from one place to another without disunion. For analysis an air-bubble is sucked up into a capillary tube of uniform calibre about 0.25 mm. in diameter and measured. A length of 100 mm. corresponds to a volume of about 5 mm.<sup>3</sup>. Gas-absorptions are next performed one after the other in a funnel at the lower end of the capillary tube. Each absorption process being finished the air-bubble is sucked into the capillary tube again and measured anew. From the difference in length before and after absorption the absorbed quantity may be calculated. As the technique has been described by Krogh [1908, 1915] elsewhere in all details, I shall refer to these publications for further particulars.

The method is applicable to estimations of carbon dioxide and especially oxygen. Krogh has also applied the apparatus for the absorption-analysis of carbon monoxide (unpublished experiments).

In the autumn of 1916 I applied to Professor Krogh in order to obtain analyses of some gas-samples originating from gas cysts of the intestines of pigs, a pathological question I was engaged in studying. It soon turned out however that satisfactory results could not be obtained by the ordinary macro-gas-analysis, each estimation requiring samples from numerous cysts. Consequently Professor Krogh advised me to try micro-analysis of the contents of each single little cyst. As estimations of hydrogen and eventually methane were essential for the solution of the question under consideration

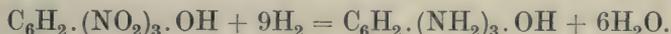
methods for micro-analysis of these gases had to be prepared. The results of these efforts are given below.

My best thanks are due to Professor August Krogh for his steady interest and help but for which I should not have been able to overcome the many difficulties met with during the work.

I also wish to express my sincere thanks to cand. pharm. K. L. Gad-Andresen, assistant at the laboratory, for numerous control-analyses by a Haldane-apparatus of the different gas-mixtures used for the micro-analyses.

#### ABSORPTION-ANALYSIS OF HYDROGEN.

It is well known that palladium, especially in colloidal form, is a good absorbing agent for hydrogen. Paal and Hartmann [1910] recommend a mixture of colloidal palladium and sodium picrate in watery solution for gas volumetric estimations of hydrogen. In this mixture palladium will act as a catalyst and rapidly transmit the hydrogen to the picric acid which is changed into 2.4.6-triaminophenol according to the formula:



1 g. picric acid consumes 834 cc. hydrogen for complete reduction.

There could be little doubt that this reagent would be well adapted to micro-analysis and the results obtained answered our expectations.

The preparation can under ordinary conditions be procured ready for use from Kalle & Co., Biebrich a. Rh. Paal and Hartmann state that Kalle's preparation even exceeds their own in absorbing power. I could not however at that time procure the reagent from Kalle and had consequently to prepare colloidal palladium myself according to the methods of Paal and Amberger [1904].

The preparation was ready for use in February 1917; it should theoretically contain about 45 % palladium, but as the palladium chloride used did not dissolve quantitatively the percentage was supposed to be about 35 % only.

The following solution of this preparation was now made according to the formula marked *A* in Paal and Hartmann's publication [1910, p. 249], the lower palladium percentage of the present preparation being considered:

0.66 g. colloidal palladium,  
0.42 g. sodium picrate,  
20.00 g. distilled water.

The solution was prepared on September 26th, 1917, and in October and November the same year was used for the analyses given below. The solution does not keep its activity for long periods; and half a year later, in May 1918, it absorbed only very slowly and anything but quantitatively. Colloidal palladium in substance also gradually loses its solubility.

Three series of analyses of different hydrogen mixtures were made by means of this palladium solution. The apparatus used had a capillary tube of uniform calibre about 0.25 mm. in diameter. The tube as well as the funnels

were filled with distilled water, slightly acidulated by means of sulphuric acid, a precaution observed once for all for the sake of carbon dioxide but of no importance for other gases. The samples to be analysed were prepared by mixing a certain quantity of hydrogen with an ample amount of atmospheric air in a 100 cc. gas-sampling-tube. Each mixture was analysed in a Haldane-apparatus to establish the exact composition. The gas was led from the sampling tube through a narrow leaden tube to the funnel, which was for safety's sake immersed in water until the air-bubble had been taken in. The bubbles analysed had a volume of 5 mm.<sup>3</sup> on an average. The length of the bubble being measured the funnel was filled with alkaline pyrogallate solution. Oxygen has to be removed before hydrogen absorption owing to the fact that palladium catalyses oxygen and hydrogen with formation of H<sub>2</sub>O. After the estimation of oxygen the funnel was filled with palladium mixture and hydrogen absorbed and determined.

It soon turned out that it was highly essential to maintain definite durations of the two absorption processes in order to obtain uniform results, partly to avoid loss of hydrogen by diffusion during oxygen absorption, partly to secure a complete absorption of hydrogen. A stop watch was advantageously used for this purpose and is on the whole much to be recommended to secure a uniform course of micro-analyses, where diffusion plays a part unknown in ordinary gas-analysis.

It was found in analyses of atmospheric air that 1 *minute* will suffice for a complete absorption of oxygen provided the bubble is moved up and down in the lower parts of the capillary tube twice or thrice during the process.

With regard to hydrogen absorption by the palladium mixture used it was established that 2½ *minutes* are enough to secure a complete absorption, provided the bubble is moved up and down in the lower parts of the tube *incessantly* during absorption.

Before giving the results I shall briefly mention a source of error which at the beginning caused many fallacious results. Accidentally the cause of these errors was detected when on one occasion I happened to keep the funnel under observation through the lens at the moment when pyrogallate solution was added. In the funnel was left as small a quantity of water as possible in order to exclude supersaturation when water and pyrogallate solution were mixed and the lower end of the air-bubble was consequently found very near the point where the bore widens into the funnel. At the moment when water and pyrogallate solution ran into one another I saw the end of the bubble break and the meniscus draw a little backwards into the capillary tube. This observation taken together with the result of the calculation—the oxygen percentage far too high, the hydrogen percentage far too low—cleared up the matter, a minimal part of the sample had been lost in the funnel. The phenomenon was now studied a little further and it was found that bubbles of atmospheric air never broke under conditions such that mixtures of hydrogen broke almost constantly. Furthermore it was established that air-bubbles will

break the easier the higher their percentage of hydrogen. The phenomenon is not seen when palladium mixture is added.

It is therefore essential always to keep the lower end of an air-bubble one to two millimetres within the mouth of the capillary tube when pyrogallate solution is added. As soon as this precaution was observed the phenomenon disappeared totally.

*Series no. 1. 18. x. 17.*

Percentage of

O<sub>2</sub>: 11.2, 11.4, 11.0, 11.0, 10.9, 11.3, 11.5, 11.2, 11.4, 11.2. Mean 11.21.

$\sigma^*$ :  $\pm 0.2$ ,  $\sigma_m \dagger$ :  $\pm 0.06$ . Systematic error:  $-0.06 \pm 0.06$ . Macro-analysis 18. x.: 11.15 % O<sub>2</sub>;

19. x.: 11.15 % O<sub>2</sub>.

H<sub>2</sub>: 46.9, 46.8, 46.9, 46.9, 47.2, 46.7, 46.8, 46.9, 46.6, 46.9. Mean 46.86.

$\sigma$ :  $\pm 0.16$ ,  $\sigma_m$ :  $\pm 0.05$ . Systematic error:  $+0.81 \pm 0.05$ . Macro-analysis 18. x.: 47.67 % H<sub>2</sub>;

19. x.: 47.67 % H<sub>2</sub>.

\*  $\sigma$  = standard deviation. †  $\sigma_m$  = standard deviation of mean.

*Series no. 2. 1. xi. 17.*

Percentage of

O<sub>2</sub>: 13.7, 13.6, 13.5, 13.8, 13.9, 13.6. Mean: 13.68.

$\sigma$ :  $\pm 0.15$ ,  $\sigma_m$ :  $\pm 0.06$ . Systematic error: —. Macro-analysis 1. xi. 17: —.

H<sub>2</sub>: 35.7, 35.9, 35.9, 35.5, 35.6, 35.6. Mean: 35.70.

$\sigma$ :  $\pm 0.17$ ,  $\sigma_m$ :  $\pm 0.07$ . Systematic error:  $+0.49 \pm 0.07$ . Macro-analysis 1. xi. 17: 36.19% H<sub>2</sub>.

*Series no. 3. 2. xi. 17.*

Percentage of

O<sub>2</sub>: 17.8, 17.8, 17.7, 17.8, 17.5, 17.5, 17.9, 17.8, 17.6, 18.0. Mean: 17.74.

$\sigma$ :  $\pm 0.13$ ,  $\sigma_m$ :  $\pm 0.04$ . Systematic error:  $+0.13 \pm 0.04$ . Macro-analysis 3. xi.: 17.87 % O<sub>2</sub>.

H<sub>2</sub>: 15.3, 15.3, 15.3, 15.0, 15.6, 15.4, 15.3, 15.3, 15.2, 14.9. Mean: 15.26.

$\sigma$ :  $\pm 0.2$ ,  $\sigma_m$ :  $\pm 0.06$ . Systematic error:  $+0.13 \pm 0.06$ . Macro-analysis 3. xi.: 15.39 % H<sub>2</sub>.

*A modified Micro-Analysis Apparatus with Combustion Funnel.*

The only technical difference between this apparatus and the ordinary one as used for the hydrogen analyses is, beside the combustion funnel, the increase of the diameter of the capillary tube to about 1 mm. In the apparatus employed the capillary tube was found to be of uniform calibre, a length of 100 mm. corresponding to a volume of about 85 mm.<sup>3</sup>, about 17 times as much as the same length represents in a 0.25 mm. capillary tube.

The objects of this essential innovation were these:

1. To obtain air-bubbles so big that it might be possible to insert a platinum wire into them for combustion analysis. It had proved easy enough to construct platinum instruments small enough for air-bubbles of a volume of only 5 mm.<sup>3</sup> but the insertion of them into such a small bubble was made impossible by the surface tension, the bubble escaping when meeting the wire.

2. To obtain better carbon dioxide analyses, the results by means of the ordinary apparatus being far too irregular, affected as they were by considerable accidental as well as systematic errors.

I soon became aware of several peculiarities which had to be considered when accurate results were to be obtained by this apparatus.

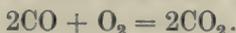


In the calculation of the results the temperature-correction is introduced simply by deducting or adding 0.1 % of a measured length for every 0.2° the temperature has changed.

A *combustion funnel* for this apparatus was constructed to render micro-analysis of combustible gases possible. For technical particulars I refer to the following chapter where the final shape of the entire arrangement for micro-analysis will be described.

#### *Carbon Monoxide.*

Two series of estimations were made, one by combustion and one by absorption. The equation for combustion of this gas is:



As the carbon dioxide formed cannot be measured with sufficient accuracy owing to diffusion, combustion has to be followed by absorption of  $\text{CO}_2$  by potash and the CO calculated as two-thirds of the final contraction. It is consequently unnecessary to read off before the  $\text{CO}_2$  has been absorbed. For absorption analyses acid cuprous chloride solution prepared according to the directions given by Winkler [1910] was used. As the process of absorption takes place rather slowly it is advisable to replace the reagent once during the absorption. Oxygen is also absorbed by this reagent and has therefore to be removed first by pyrogallate solution.

The gas-mixture employed was prepared by adding pure carbon monoxide to atmospheric air. The same mixture was used for both series.

#### *Series no. 4 a. 3. v. 18.*

##### *Combustion analyses.*

Capillary tube and both funnels filled with slightly acidulated distilled water. Volume of samples about 110 mm.<sup>3</sup>. Five seconds' combustion at a heat just enough to make the platinum wire visibly glowing. Absorption of  $\text{CO}_2$  by potash.  $\text{CO} = \text{two-thirds contraction} + \text{CO}_2$ .

##### Percentage of

CO: 20.6, 20.7, 20.7, 20.5, 20.6, 20.5, 20.6, 20.7, 20.6. Mean: 20.62.

$\sigma: \pm 0.08, \sigma_m: \pm 0.03$ .

#### *Series no. 4 b. 3. v. 18.*

##### *Absorption analyses.*

Apparatus filled with water as in series no. 4 a. Volume of samples about 71 mm.<sup>3</sup>. Oxygen absorption by pyrogallate  $\frac{3}{4}$  minute, the sample being moved up and down thrice. Carbon monoxide absorption: first portion of cuprous chloride solution = 1 minute; second portion of cuprous chloride solution =  $2\frac{1}{2}$ -3 minutes; the sample being kept incessantly in motion the whole time.

##### Percentage of

$\text{O}_2$ : 16.7, 16.8, 16.4, 16.6, 16.6, 16.7. Mean: 16.63.

$\sigma: \pm 0.14, \sigma_m: \pm 0.06$ .

CO: 20.4, 20.4, 20.7, 20.5, 20.4. Mean: 20.48.

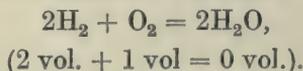
$\sigma: \pm 0.12, \sigma_m: \pm 0.05$ .

Macro-analysis of the gas-mixture was not made, as the Haldane-apparatus was temporarily out of order.

As will be seen the CO values of the absorption estimations are slightly lower than those obtained by combustion. The latter express no doubt the exact percentage of CO, a systematic error of any importance being improbable.

#### *Hydrogen.*

One series of combustion analyses was made. The equation is:



Hydrogen is calculated as two-thirds of the contraction.

#### *Series no. 5. 6. v. 18.*

The gas-mixture was prepared by adding pure hydrogen to atmospheric air. Capillary tube and funnels filled with acidulated water. 10 seconds' combustion as in series no. 4 *a*. Two-thirds  $\times$  contraction =  $\text{H}_2$ .

Percentage of

$\text{H}_2$ : 18.4, 18.4, 18.5, 18.6, 18.5, 18.5. Mean: 18.48.

$\sigma$ :  $\pm 0.09$ ,  $\sigma_m$ :  $\pm 0.04$ . Systematic error:  $+0.29 \pm 0.04$ . Macro-analysis 6. v. 18: 18.77%  $\text{H}_2$ .

Compare further hydrogen estimations in the following chapter.

#### *Carbon Dioxide.*

The difficulty of the micro-estimation of  $\text{CO}_2$  is due to the enormous rapidity with which this gas diffuses in most fluids, and measures which impede loss by diffusion are necessary to secure satisfactory analytical results. To this end Krogh [1908] recommends either to fill the funnel with conc. acidulated glycerol or to collect the sample in the funnel of the analysis apparatus over mercury.

I have myself spent much time seeking a satisfactory solution of this problem. As my experiences may perhaps be of some use to others working on this line and as they in my opinion clearly illustrate the essential factors to be considered in the micro-estimation of  $\text{CO}_2$  I shall sketch the different methods tried before good results were gained.

Loss by diffusion in the absorption funnel was first considered and this part of the apparatus filled either by mercury or by conc. acidulated glycerol. Mercury gave considerably higher percentages than those obtained over water, and somewhat better results than conc. glycerol but there was still a loss in the rest of the apparatus. To prevent this the upper parts of the apparatus were filled with a saturated solution of sodium chloride, acidulated by hydrochloric acid, the absorption funnel remaining filled with mercury. This salt solution was soon replaced by a mixture of equal parts of glycerol and saturated sodium chloride solution both acidulated by hydrochloric acid. This mixture proved to be an important step forward and has ever since been used permanently in the apparatus for all gases. In the sequel it will be named "g.s. mixture."

The mixture in question is thin enough for the movements in a capillary tube 1 mm. in diameter, but requires to a still higher degree than water movements of uniform speed. Augmentation of the glycerol content cannot be recommended, a mixture of 75 %, being too thick, caused accidental errors up to 1 %.

It was now justifiable to omit the filling of the funnel with mercury as being both troublesome and time-wasting. As the whole apparatus for several reasons cannot be filled throughout with mercury a systematic error bigger or smaller will always be present in CO<sub>2</sub> analyses.

This error being constant it will hardly pay to spend much time on diminishing it below a reasonable size which can be attained without particular arrangements.

Three series of CO<sub>2</sub> estimations of different mixtures were now made by this improved technique. In these and all the following series the apparatus was entirely—capillary tube as well as absorption funnel—filled with “g.s. mixt.”

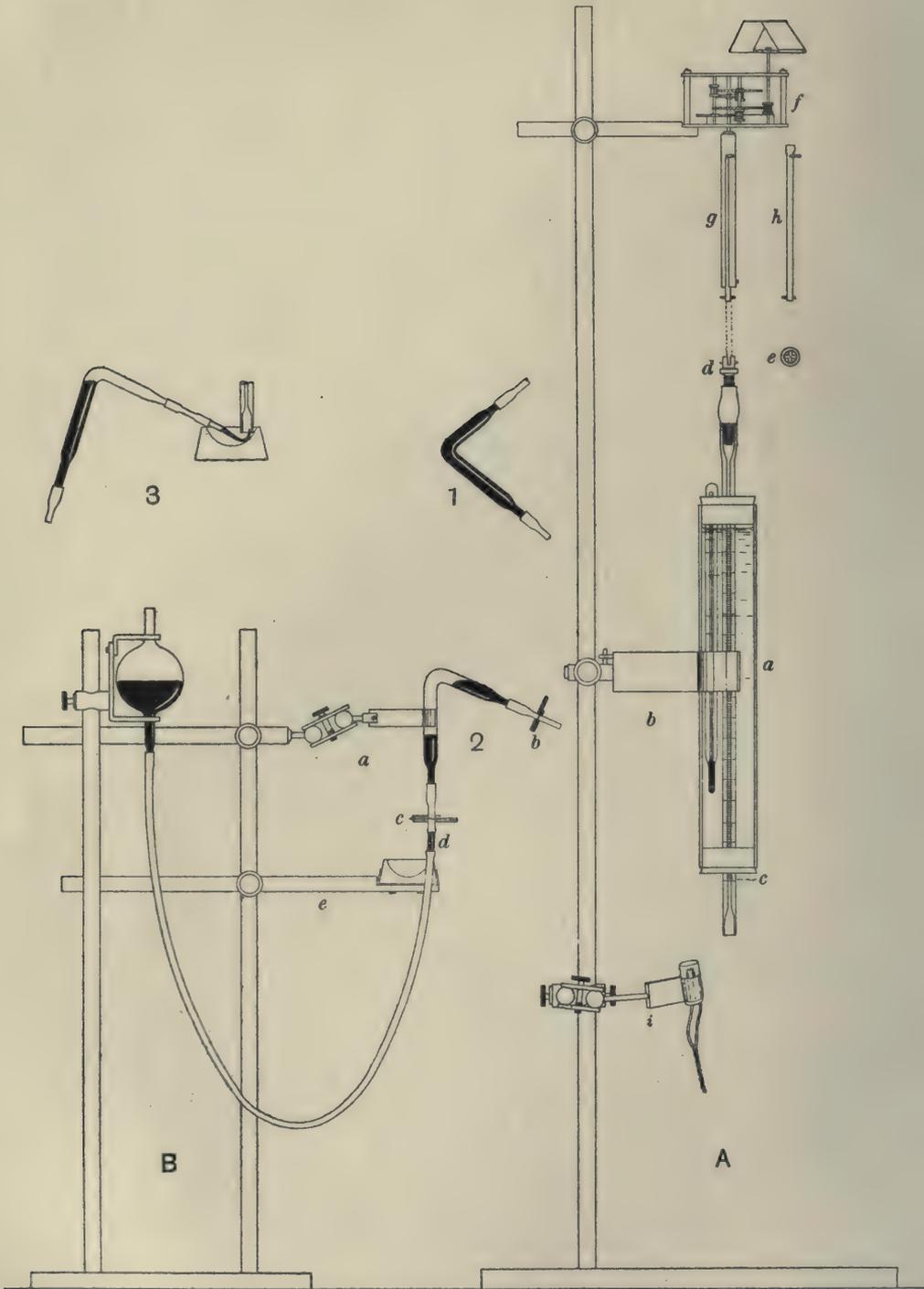
<i>Series no.</i>	6	7	8
	(18. v. 18)	(21. v. 18)	(21. v. 18)
Percentage of	CO <sub>2</sub>	CO <sub>2</sub>	CO <sub>2</sub>
	23·8	51·3	60·8
	23·8	51·8	60·7
	23·8	51·4	60·4
	23·8	51·6	60·6
	23·7	51·5	60·5
	23·8	51·5	60·8
	23·9	51·4	60·4
	23·9	51·3	60·9
	23·8	51·4	60·6
	24·1	51·4	60·5
Mean	23·84	51·46	60·62
$\sigma$	± 0·1	± 0·15	± 0·18
$\sigma_m$	± 0·03	± 0·05	± 0·06
Systematic error	+ 0·59 ± 0·03	+ 0·95 ± 0·05	+ 1·21 ± 0·06
Macro-analysis	24·43	52·41	61·83 % CO <sub>2</sub>
	(18. v. 18)	(23. v. 18)	(18. v. 18)

The accidental errors in these series are evidently of no importance as compared with the systematic ones. The latter are not proportional as might be expected to the concentrations of CO<sub>2</sub>, the lower percentages having a relatively higher systematic error than the higher percentages.

Compare on this point the CO<sub>2</sub> analyses of the next chapter.

#### THE MODIFIED MICRO-ANALYSIS APPARATUS COMBINED WITH CLOCK-WORK AND COMBUSTION FUNNEL.

This construction represents the final shape of the apparatus I have come to during my work. The arrangement will be readily understood from Fig. 1 and annexed explanations.

Fig. 1. About  $\frac{1}{4}$  nat. size.

*Analysis apparatus.* The apparatus is, as to the capillary tube, identical with the one described in the preceding chapter, the diameter being about 1 mm. The clock-work has necessitated a little change. The side-tube has been removed and the screw inserted in the enlarged top funnel. The screw is tightened by a very stiff mixture of pure india-rubber and vaseline melted together. The tube is filled with "g.s. mixture." This fluid undergoing no bacterial decomposition the capillary tube keeps clean for months.

When the apparatus has to be used, the screw is wound as far down as possible, the "g.s. mixture" removed from the absorption funnel by means of a pipette and replaced by a fresh solution, a small quantity of which is sucked up into the capillary tube. By following these directions one may always be sure that the gas is brought into contact with a fluid of constant composition.

Owing to the wide diameter the tube is easily cleaned without the aid of an aspirator.

*Clock-work.* The object of the clock-work is to secure absolutely uniform speed when the gas is sucked up into the capillary tube for measurement. All movements towards the absorption funnel have to be performed by revolving

#### DESCRIPTION OF FIG. 1.

##### A. Entire arrangement for Micro-analysis.

- a*, capillary tube with water jacket and thermometer;
- b*, clamp and bosshead with arrangement for stopping in vertical position;
- c*, division taken as zero in measurements;
- d*, screw of vulcanite with brass top;
- e*, screw seen from the top with cruciform incision of the brass top;
- f*, clock-work with wings for regulating the speed;
- g*, metal tube fixed to the axle of the clock-work, containing a movable iron-pin to fit incisions of *d*;
- h*, iron-pin alone;
- i*, combustion funnel, carried by a clamp (compare *B*, *a*) adjustable to any angle. The conduction wires connect the platinum wire with accumulators and rheostat.

##### B. Stand for Micro-receivers.

- a*, elastic clamp with double ball-and-socket joint for fixation of micro-receiver;
  - b* and *c*, clips on india-rubber tubing of micro-receiver;
  - d*, piece of glass tube for disconnection from mercury reservoir;
  - e*, clamp holding square glass pot with excavation for "g.s. mixture" for immersion of the absorption funnel of *A*, *a*.
- 1, position 1 of receiver, being used when the receiver has to be filled with mercury or when the last gas has to be driven out of the shorter limb for analysis;
  - 2, position 2: gas has just been sucked in, receiver clipped at both ends and levelling-tube raised again;
  - 3, position 3: gas from the longer limb is driven out through a narrow curved glass cannula into the absorption funnel, immersed in "g.s. mixture" (*e*). A drop of mercury regularly left in the drawn out part of the shorter limb is pushed in front of the gas so removing any foreign gas present.

the screw by hand. The most suitable speed for sucking in gas is about 100 mm. in 30 seconds. Twice the speed may very well be applied but the screw is, according to my experience, rather liable to become leaky after some time when revolved so quickly. The speed is regulated by means of a pair of wings adjustable to different angles. In order to secure the most uniform speed obtainable I have furthermore wound the clock-work before each measurement.

Coupling to the screw is effected in a few seconds and disconnecting as quickly. When the iron-pin is fixed in the top of its tube the clock-work never causes any trouble during the other manipulations of the analysis apparatus.

*Combustion funnel* (Fig. 1, *A*, *i*). This small instrument fixed by a clamp and a bosshead may be raised to the absorption funnel. The construction needs some explanation.

Two platinum wires, 4 cm. in length, 0.3 mm. in diameter, are flattened by a hammer at one end to an extent of about 1 mm. Incisions 1 mm. deep following the longitudinal axes of the wires are made in the middle of the flattened parts by means of a pair of scissors. The wires are insulated by platinum imbedding glass drawn out to thin threads and wound round the wires over the blowpipe. (Enamel might be tried for this purpose.) The insulation has to begin just beneath the flattened parts and must cover the wires to a length of about 3 cm. A cork, 8 mm. long, 15 mm. in diameter, is soaked in paraffin (melting point 60–70° C.) till all air is driven out of the pores. Two holes of the diameters of the insulated wires are bored near the centre of the cork 2 mm. apart from one another and the wires are inserted with the uninsulated ends first (no paraffin must touch the upper end). The flattened points have to be 15 mm. above the cork. A platinum wire 0.05 mm. in diameter is next fixed between the flattened points of the insulated wires acting as carriers of the current. Fixation is obtained by winding the wire many turns round the points. The length of the glowing-wire ought to be 5–6 mm., it may be shaped in the form of a single spiral winding for instance. Sharp curves must be avoided the wire being sure to fuse in such places. The advantage of making the wire amply long is that a broken wire may easily be welded together afterwards by simply crossing the ends till contact is obtained and increasing the current to a bright white heat. The whole arrangement allows of the replacement of a fused wire in a few minutes. The uninsulated ends of the wires on the other side of the cork are soldered to ordinary conducting wires connected to a four volt accumulator battery, a rheostat and a key.

The cork is now inserted into a piece of glass tube, 2.5 cm. long and 15 mm. in diameter. The funnel thus being formed is next filled with water (air-bubbles being avoided!) up to the glowing-wire and is now ready for use.

The rheostat may of course be of any construction at disposal, but it is essential to the task in question, that it can regulate from the slight heat of a not glowing-wire through just visible glowing to a bright white heat. A rheostat of 10 ohms fulfilled these requirements.

The heat of the glowing-wire must be regulated according to the different gases. For hydrogen the heat of a "dark" wire is sufficient, the extra heat produced at the combustion making the wire glow for a moment. For carbon monoxide a just visibly glowing-wire will do, while methane requires at least a bright red heat, the extra heat produced by the process causing the wire to glow momentarily at a bright white heat. I have often raised the temperature to a bright white heat during my methane analyses, but it is hardly essential and rather risky for the wire.

As the combustion processes under these conditions take place instantaneously it is neither necessary nor advisable to keep the wire aglow for more than five seconds. When the wire is inserted into an air-bubble and the temperature of the wire raised to a bright white heat during five seconds, the volume of the bubble will not suffer any change at all unless combustible gases are present. When the same procedure is extended to last ten seconds or more the bubble will increase slightly in volume owing to emigration of gas from the heated water in the funnel.

In estimating both hydrogen and methane the measurement after combustion of hydrogen may very well be done without removing the combustion funnel.

*The course of a complete Analysis ( $\text{CO}_2$ ,  $\text{O}_2$ ,  $\text{H}_2$ ,  $\text{CH}_4$ ,  $\text{N}_2$ ).*

1. *Preparations.* The absorption funnel and capillary tube are filled with fresh "g.s. mixture" by means of a pipette and the screw as described before. The lower end of the funnel is inserted into the glycerol basin (Fig. 1, B, e). The clock-work is wound and connected to the screw (Fig. 1, A, d). The gas-sample is taken into the funnel and the clock-work started. When a sufficient amount of gas is sucked into the capillary tube, the clock-work is stopped, disconnected from the screw, the glycerol basin removed and the superfluous gas allowed to escape from the funnel by turning the apparatus. The lower meniscus of the sample being placed at zero by means of the restarted clock-work the first measurement is performed and noted together with the temperature of the water-jacket.

2. *Absorption of carbon dioxide.* The first measurement being finished the lower meniscus of the sample is taken down near the mouth of the funnel, "g.s. mixture" removed by a pipette and without any washing of the funnel replaced by 10% potash. The sample is now taken down into the funnel, the upper meniscus being kept in the capillary tube but 2-3 mm. above the funnel. *It is an essential point that the fluid in the funnel must never come into contact with the "g.s. mixture" above the upper meniscus.* Absorption takes place instantaneously. As soon as the clock-work has been rewound and connected the sample is sucked back into the capillary tube together with a short column of potash below the lower meniscus stopped at zero. Second measurement. Temperature noted.

3. *Absorption of oxygen.* The second measurement being done the sample is taken down near to the funnel and the potash without any washing of the funnel

replaced by pyrogallate solution. Absorption-time about three-quarters of a minute (established beforehand for a given apparatus and pyrogallate solution by means of an oxygen estimation in atmospheric air!). The sample has to be moved up and down thrice to secure a complete absorption. The clock-work is wound anew and connected. The sucking in has to be stopped when the lower meniscus has reached a few millimetres within the capillary tube and any trace of pyrogallate is removed thoroughly from the funnel as well as the capillary tube by means of a pipette filled with distilled water. This being done the lower meniscus of the sample is taken down near the funnel and the water replaced by "g.s. mixt." The sample is next taken up to zero by means of the restarted clock-work. Third measurement. Temperature noted.

4. *Combustion of hydrogen.* The third measurement being done addition of a suitable excess of oxygen for the combustions has to take place. Either pure oxygen or atmospheric air can be applied; in the latter case it will often be necessary to remove part of the sample in order to make room for the necessary excess of oxygen (*vide* calculation below). Application of pure oxygen is advantageous as it generally allows the whole of the sample to be retained. The addition is performed in the following way. The lower meniscus of the sample is placed 1–2 mm. above the funnel, a bubble of oxygen taken into the funnel and sucked up—the screw being turned by hand—into the capillary tube below the sample but *separated* from it by a short column of "g.s. mixture." When a sufficient amount is sucked in, the apparatus is turned so that superfluous oxygen may escape from the funnel. The two air-columns are now quickly taken down into the funnel and united there. The rewound clock-work is started and the enlarged sample taken up. Fourth measurement. The difference between the fourth and third measurements represents consequently the oxygen added. The lower meniscus is next placed near the funnel and a thorough washing of the funnel—inside as well as outside—performed in order to remove any trace of glycerol. The lower 4–5 mm. of the capillary tube are equally washed by moving the screw up and down. The air-bubble may now be taken down into the absorption funnel the upper meniscus being kept 2–3 mm. within the capillary tube as emphasised before, the combustion funnel filled with water is cautiously raised, so that the glowing-wire is found in the centre of the bubble, and a current sent through the wire *without* making it glow visibly. If hydrogen be present the wire will glow up for a fraction of a second. The current is broken off by the key as soon as this has taken place and the sample is sucked up by clock-work without removing the combustion funnel. Fifth measurement. Temperature noted. Two-thirds of the contraction is hydrogen.

5. *Combustion of methane.* The fifth measurement being made the sample is taken down into the funnel again, the glowing-wire eventually centred anew in the bubble and the current *gradually* increased until the wire reaches a bright red heat. The heat should be increased cautiously as otherwise the wire may fuse owing to the extra heat produced at first by the combustion.

If methane be present the wire will for an instant glow up from a bright red to a bright white heat. The combustion is now finished, the combustion funnel removed and a small amount of potash cautiously added by means of a curved pipette to the water in the funnel so as to absorb the carbon dioxide produced by the combustion process. The clock-work is wound and started. Sixth and final measurement. Temperature noted. One-third of the contraction is methane.

6. *Nitrogen.* Calculated as residue.

*Calculation.* The calculation of the percentages, easily performed by a slide rule, does not require to be specially mentioned except in a case where the sample has been diminished during an analysis in order to make room for atmospheric air for combustion.

The new volume of the sample must consequently be corrected for the carbon dioxide and oxygen removed at the beginning of the analysis. The following example will explain the case.

Reading No.	Temp. ° C.	Length	Correct. length	Diff.	%
1. ... ..	13.6	110.6	110.3	8.3	7.5 CO <sub>2</sub>
2. CO <sub>2</sub> absorbed ... ..	13.8	102.4	102.0	0.7	0.65 O <sub>2</sub>
3. O <sub>2</sub> absorbed ... ..	13.8	101.7	101.3		
4. <i>Sample diminished to</i> ... ..	13.9	32.2	31.9		
5. Plus atmosph. air ... ..	13.9	147.3	147.0	2.1	4.0 H <sub>2</sub>
6. Combustion of H <sub>2</sub> ... ..	14.0	145.2	144.9		
7. Combustion of CH <sub>4</sub> plus CO <sub>2</sub> absorbed	14.1	123.7	123.3	21.6	20.7 CH <sub>4</sub>

$$\text{CO}_2 = \frac{100 \times 8.3}{110.3} = 7.5 \%$$

$$\text{O}_2 = \frac{100 \times 0.7}{110.3} = 0.63 \%$$

*Correction of reading no. 4:*

$$31.9 \times \frac{110.3}{101.3} = 34.7 \text{ mm.}$$

$$\text{H}_2 = \frac{100 \times 2.1 \times 2}{34.7 \times 3} = 4.0 \%$$

$$\text{CH}_4 = \frac{100 \times 21.6 \times 1}{34.7 \times 3} = 20.7 \%$$

### *Carbon Dioxide.*

The gas-mixtures for these series were prepared of pure CO<sub>2</sub> and atmospheric air mixed in a 100 cc. receiver. As the clamp (Fig. 1, B, e) for the glycerol basin was not ready when these analyses were made the clamp carrying the analysis apparatus had to be taken out of its bosshead down to a basin with glycerol on the table. The absorption funnel being quite filled with gas, the apparatus and the basin were raised together. The basin was not removed till the sample had been sucked up into the capillary tube. In order to avoid this troublesome and time-wasting removal of the analysis apparatus from the stand the clamp (Fig. 1, B, e) was constructed. The glycerol basin is thereby raised to the absorption funnel and fixed before the sample is taken into the funnel.

Series no.	9	10	11
	(28. v. 19)	(30. v. 19)	(18. vi. 19)
Percentage of	CO <sub>2</sub>	CO <sub>2</sub>	CO <sub>2</sub>
	—	21.8	—
	11.3	21.8	—
	11.0	22.0	—
	11.0	21.9	32.8
	11.4	22.0	32.9
	11.0	21.8	33.0
	11.0	21.8	33.0
	11.0	22.0	33.0
	11.2	21.9	33.0
	10.9	22.0	33.0
	11.3	21.9	32.8
	11.1	21.9	33.0
	11.2	21.9	33.0
Mean	11.12	21.90	32.95
$\sigma$	$\pm 0.16$	$\pm 0.08$	$\pm 0.09$
$\sigma_m$	$\pm 0.05$	$\pm 0.02$	$\pm 0.03$
Systematic error	$+ 0.58 \pm 0.05$	$+ 0.71 \pm 0.02$	?
Macro-analysis	11.70 %	22.61 %	(lost)
	(28. v. 19)	(30. v. 19)	

The *accidental errors* of these analyses have as it may be seen been reduced to such a minimum, that they may wholly be attributed to the unavoidable errors of reading. When the length of an air-bubble is about 100 mm. an error of  $\pm 0.1$  mm. corresponds to  $\pm 0.1$  %.

The *systematic errors* ought theoretically to be strictly proportional to the percentages of CO<sub>2</sub>, found in these analyses. This proportionality is neither absolute in these series nor in the series no. 6, 7 and 8 of the preceding chapter, the tendency being that the lower percentages have a relatively higher systematic error than the higher ones. I have reason to believe that this condition will be still more pronounced at CO<sub>2</sub> percentages higher than the 22.6 % of series no. 10, but as such percentages are not commonly met with during biological work I have not tested the point. The training of the analyst is no doubt of great importance when dealing with a gas diffusing so rapidly and it is to be recommended that every operator should establish his individual systematic error as regards different CO<sub>2</sub> percentages. This is done by means of a series of micro-analyses and a macro-analysis of each CO<sub>2</sub>-mixture. The use of the fixable glycerol-pot, mentioned above, might possibly have given more uniform errors in the present series.

The fact that the errors of these two series are considerably higher than that of the series no. 6 is no doubt wholly due to the slower movement of the clock-work, more time being left for diffusion in the funnel.

### Hydrogen.

The gas-mixture used for the analyses given below was prepared by adding pure hydrogen to atmospheric air. It proved exceedingly explosive, many analyses being spoiled at the beginning. Good results were obtained, when the

current was interrupted instantaneously when the slightest trace of contraction was seen. The wire would nevertheless glow up and the combustion proceed quietly.

The oxygen percentages given are calculated by adding a third of the contraction to the values found by pyrogallate absorptions after the combustions.

A series of absorption analyses was also made, the absorbing agent being the palladium mixture mentioned at the beginning of this paper. The results proved contrary to my expectations very bad. The loss of hydrogen by diffusion during oxygen absorption was both considerable and inconstant. This method was therefore given up as inapplicable, the combustion method being far superior to it.

*Series no. 12. 18. iii. 19.*

Percentage of

H<sub>2</sub>: 21.0, 21.0, 20.9, 20.8, 20.9, 20.7. Mean: 20.88.

$\sigma$ :  $\pm 0.12$ ,  $\sigma_m$ :  $\pm 0.05$ . Systematic error:  $+0.12 \pm 0.05$ . Macro-analysis (18. iii.): 21.0 % H<sub>2</sub>, O<sub>2</sub> (see text above): 16.6, 16.6, 16.8, 16.7, 16.8, 16.7. Mean: 16.70.

$\sigma$ :  $\pm 0.09$ ,  $\sigma_m$ :  $\pm 0.04$ . Systematic error:  $(-0.2 \pm 0.04)$ . Macro-analysis (18. iii.): (16.5 % O<sub>2</sub> by calculation).

The accidental and systematic errors are equal.

In all ordinary circumstances, where the utmost accuracy is not wanted, one may take for granted that there is no systematic error upon analyses of hydrogen, when the percentage does not exceed about 20.

*Methane.*

As it proved difficult to prepare a methane pure enough for gas-analysis by chemical methods, the gas was procured from nature in the following way.

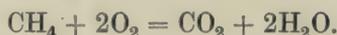
A big glass flask was nearly filled by a mixture of water and the fresh contents from the paunch of a cow. The flask was alternately saturated by CO<sub>2</sub> and evacuated. When the saturation was supposed to be as complete as attainable the flask was placed at 37° C. for 24 hours. A violent fermentation began after some hours, the gas produced being collected in a large receiver. The gas was freed from CO<sub>2</sub> by means of a Pettenkofer's absorption tube with potash. Macro-analysis of the gas so procured showed 92.9 % methane and 7.1 % nitrogen, the CO<sub>2</sub> formed in the combustion process being exactly equal to half of the contraction.

This concentrated methane mixture was used for the series to be given below. The plan of this series is somewhat different from that hitherto followed. It was namely thought desirable to imitate the natural conditions as closely as possible, where oxygen or atmospheric air generally has to be added to the sample. To this end small amounts of the methane mixture were measured in the analysis apparatus, whereupon atmospheric air or pure oxygen was added. Explosions were not seen in any case.

The oxygen was generated by means of a wash bottle containing oxylith on to which water was dropped. The addition of pure oxygen is most advantageous, as it allows of taking in much bigger samples than are possible when

atmospheric air is used. An oxygen-generator ought therefore always to be at hand during these combustion analyses.

The equation of combustion is:



As the contraction and the carbon dioxide formed cannot be measured separately owing to loss of  $\text{CO}_2$  by diffusion into the water of the funnel, combustion is followed immediately by absorption of  $\text{CO}_2$ . One-third  $\times$  (contraction + carbon dioxide) = methane.

*Series no. 13. 10. ii. 19.*

Macro-analysis 10. ii. 19: 92.9 % methane,  
7.1 „ nitrogen.

Micro-analyses:

A. A small sample is measured off and a larger amount of atmospheric air freed from  $\text{CO}_2$ <sup>1</sup> is added afterwards.

1.	92.9 % $\text{CH}_4$	T 13.8	11.3	corrected	11.0	equal to 10.2 mm. pure methane = 6.1 % of the mixture
	+ atmospheric air	T 13.8	167.0	„	166.7	
	Combustion + $\text{CO}_2$	T 13.9	136.3	„	136.0	$30.7 \times \frac{1}{3} = 10.2 \text{ mm.} = 6.1\%$
	Alkaline pyrogallate	T 14.0	125.4	„	125.0	11.0 mm. oxygen left after combustion
2.	Calculated content	12.3 mm.	$\text{CH}_4 = 7.3 \%$			
	Analytical result	12.0 mm.	$\text{CH}_4 = 7.2$			
3.	Calculated content	11.5 mm.	$\text{CH}_4 = 6.8$			
	Analytical result	11.3 mm.	$\text{CH}_4 = 6.7$			

B. A larger amount of pure oxygen is measured off and a smaller sample added afterwards.

4.	Pure oxygen	T 14.0	142.5	corrected	142.3	12.7 = 11.8 mm. $\text{CH}_4 = 7.6 \%$
	+ 92.9 % $\text{CH}_4$	T 14.1	155.2	„	154.9	
	Combustion + $\text{CO}_2$	T 14.2	120.1	„	119.7	$35.2 \times \frac{1}{3} = 11.7 \text{ mm.} \text{ CH}_4 = 7.6 \%$
5.	Calculated content	23.6 mm.	$\text{CH}_4 = 14.8 \%$			
	Analytical result	23.2 mm.	$\text{CH}_4 = 14.6$			
6.	Calculated content	16.2 mm.	$\text{CH}_4 = 9.7$			
	Analytical result	16.0 mm.	$\text{CH}_4 = 9.6$			

As it appears from the methane volumes re-calculated into per cent. the errors lie between 0 and + 0.1 %; in a single case they amount to + 0.2 %. Consequently it may be taken for granted that there is practically no systematic error in analyses of methane mixtures of 1–10 %. Mixtures containing 11–15 % may have a systematic error lying between + 0.1 and + 0.2 %.

#### FRACTIONAL COMBUSTION OF GAS-MIXTURES, CONTAINING BOTH HYDROGEN AND METHANE.

The fact that a heated platinum wire which is not visibly glowing causes combustion of hydrogen while on the contrary a wire of a bright red or white heat is required for combustion of methane led to the question whether it

<sup>1</sup> Carbon dioxide-free atmospheric air is always at hand in the potash flask.

might be possible to estimate both gases by fractional combustion. The results obtained answered our expectations.

The technique is quite the same as in ordinary combustion analyses, the only factor to be considered being, that the combustion of hydrogen takes place at the lowest possible heat by means of a "dark" wire. At the very moment the combustion occurs causing the wire to glow momentarily the current has to be broken off by a key. If the current is not interrupted instantaneously at this moment a trace of methane will also be burned.

Whether higher percentages of hydrogen can be estimated together with methane without causing a smaller or larger systematic error upon the methane percentage is a question open to future experiments. Dilution of the sample by means of an ample amount of atmospheric air will no doubt be the simplest procedure in such cases.

The methane for these analyses was obtained by means of evacuated receivers with connecting tubes and thin steel needles from the intact paunches of freshly killed cows at a slaughter-house. The gas thus collected was freed from carbon dioxide by passage through a Pettenkofer's absorbing tube filled with potash. A micro-analysis of the gas thus obtained showed a content of 52.7 % methane.

The gas-mixture employed for the series below was prepared by adding roughly estimated parts of this methane mixture, pure hydrogen, pure oxygen and pure nitrogen.

*Series no. 14. 11. vi. 19.*

Percentage of

H<sub>2</sub>: 5.3, 5.3, 5.5, 5.3, 5.6, 5.4. Mean: 5.40.

$\sigma$ :  $\pm 0.13$ ,  $\sigma_m$ :  $\pm 0.05$ . Systematic error:  $+ 0.05 \pm 0.05$ . Macro-analysis 11. vi. 19: 5.45 % H<sub>2</sub>.

CH<sub>4</sub>: 9.3, 9.3, 9.3, 9.4, 9.2, 9.3. Mean: 9.30.

$\sigma$ :  $\pm 0.06$ ,  $\sigma_m$ :  $\pm 0.03$ . Systematic error:  $+ 0.23 \pm 0.03$ ; Macro-analysis 11. vi. 19: 9.53% CH<sub>4</sub>, 4.12 % O<sub>2</sub> left after combustion.

It will be seen that the methane is left totally intact by the hydrogen combustion when the directions given are followed.

A MICRO-RECEIVER FOR SMALL GAS SAMPLES.

The quantities of gases met with in nature during biological work are often so scanty that they are lost when taken into the ordinary gas sampling tubes. Krogh has for these reasons mounted the analysis apparatus in a transport-box, serving at the same time as a stand, so that the apparatus is handy for use in nature, on board a ship or elsewhere. The small air-bubbles are then led directly into the absorption funnel of the apparatus and analysed at once. This may be practical in many cases, when only estimations of carbon dioxide and especially oxygen are wanted, but it will hardly be possible to obtain any accuracy when the combustible gases have also to be determined by the technique described in this paper. This condition led to the construction of a suitable micro-receiver for collecting and storing small gas samples during transport until analysis can take place in the laboratory.

The essential point of this simple little apparatus, making it differ from the ordinary receivers in use, is the circumstance that it has no stopcocks, the gas being forced to bubble in through a column of mercury. The sample is thus locked up between two columns of mercury. The construction and use will readily be understood from Fig. 1, *B*. The receivers are easily made by means of a blow-pipe. The dimensions are: diameter of glass tubing 6 mm.; length of longer limb 70 mm., 25 of which are drawn out for connection with



Fig. 2. Transport-box for 24 micro-receivers. The cover contributes to fix the receivers during transport

[About  $\frac{1}{4}$  actual size.]

a narrow rubber tubing; length of shorter limb 50 mm., being equally drawn out. The angle between the two limbs measures 70–80°. Narrow india-rubber tubing, which is not apt to stick together, is used for connection. The rubber tubings are closed by means of small stiff clips, made of spring steel. A micro-receiver of these dimensions may hold about 1.7 cm.<sup>3</sup> gas at ordinary pressure locked up between two mercury columns 3 cm. in height. As all contaminating air is driven out of the connecting tubing and cannula by means of mercury, even smaller air-bubbles may be taken over into the absorption funnel without loss.

A transport-box for 24 micro-receivers will be seen in Fig. 2.

As it could not be excluded beforehand that a minute diffusion might take place along the sides of the tube past the mercury, which is known not to adhere to glass like most fluids, the following control experiments were carried out.

The mercury columns serving for locking up the samples measured on an average 3 cm. in height in all three experiments. A certain pressure was established in all the receivers before they were clipped.

*Experiment no. 1.* Tightness test for carbon dioxide.

Micro-analysis of gas-mixture before filling of micro-receiver (series no. 10):

			21.90 % CO <sub>2</sub>
„	after 15 days	(1)	21.2 „
		(2)	21.3 „

The loss has been about 0.65 % CO<sub>2</sub> in the course of 15 days.

A calculation of the loss per 10 % in 24 hours makes it about 0.02 %.

*Experiment no. 2.* Tightness test for carbon dioxide.

Micro-analysis of gas-mixture before filling of micro-receiver (series no. 11):

			32.9 % CO <sub>2</sub>
„	after 4 × 24 hours	(1)	32.4 „
		(2)	32.2 „
		(3)	32.3 „

The loss has been about 0.65 % carbon dioxide in the course of 96 hours.

A calculation of the loss per 10 % in 24 hours makes it about 0.05 %.

*Experiment no. 3.* Tightness test for hydrogen.

Micro-analysis of gas-mixture before filling of micro-receivers *A* and *B*:

(1) 11.9, (2) 12.0, (3) 12.0 % H<sub>2</sub>.

Micro-analysis of gas-mixture in receivers *A* and *B* after 24 hours:

*A* (1) 12.0, (2) 12.0 %,

*B* (1) 12.1, (2) 12.0 %.

No loss of hydrogen has taken place in the course of 24 hours.

*Discussion of results.* The issue of experiment no. 3 ensures the absolute tightness of the receivers even for hydrogen within 24 hours. The losses of carbon dioxide in 24 hours would as seen by the calculations of the results of the two first experiments be quite insignificant, the high percentages and the exceedingly long time being taken into consideration. It may also be possible that no diffusion has taken place at all, the losses being due to unnoticed impurities of the mercury.

The conclusion drawn from these preliminary experiments is, that the micro-receiver described is absolutely reliable within 24 hours. Gas samples ought consequently when the utmost accuracy attainable is wanted to be analysed before this time has elapsed.

## SUMMARY.

1. The micro-analysis apparatus described by Krogh is also applicable to hydrogen and carbon monoxide estimations, a solution of colloidal palladium plus sodium picrate and an acid cuprous chloride solution serving respectively as absorbing agents.

2. A modification of this apparatus with combustion funnel is described. It is possible by means of this apparatus to estimate  $\text{CO}_2$  and  $\text{O}_2$  by absorption,  $\text{H}_2$  and  $\text{CH}_4$  by combustion in a given air-sample of about 80 mm.<sup>3</sup>—the size of a small pea. CO may be estimated either by absorption or combustion according to circumstances.

$\text{N}_2$  (+ inert gases) is calculated as residue.

3. Fractional combustion of hydrogen and methane appearing together is practicable by means of this technique.

4. The accidental error (standard deviation) in the present micro-analyses corresponds on an average to an error of reading of  $\pm 0.1$  mm.

5. The systematic error differs for the different gases. While being without any practical importance in estimations of  $\text{O}_2$ ,  $\text{H}_2$ ,  $\text{CH}_4$  and probably also CO, it has to be considered in carbon dioxide analyses.

6. A micro-receiver for small gas samples is described.

## REFERENCES.

Krogh (1908). *Skand. Arch. Physiol.* **20**, 279.

— (1915). *Abderhalden's Biochem. Arbeitsmeth.* **8**, 495.

Paal and Hartmann (1910). *Ber.* **43**, 243 (compare *Abderhalden's Biochem. Arbeitsmeth.* 1915, **8**, 406).

Paal and Amberger (1904). *Ber.* **37**, 124.

Winkler (1910). Quoted by Müller in *Abderhalden's Biochem. Arbeitsmeth.* **3**, 646.

## II. ON THE DETERIORATION OF COTTON ON WET STORAGE.

BY NANCY FLEMING AND AAGE CHRISTIAN THAYSEN.

(Received December 4th, 1919.)

(With Plate I.)

It is a well-known fact that exposure to damp renders cotton extremely brittle and short-fibred, and increases its alkali solubility. The extent of this damage is often very considerable, may in exceptional cases cause the complete destruction of the cotton affected, and will often entail the loss of from 10-15 % of the cotton in the form of exceptionally short fibres, the so-called "fly."

In a preliminary report to the Admiralty, dated 19/3/19, an account was given of some experiments carried out in connection with the investigation of this deterioration on wet storage. The conclusion arrived at was, that cotton does not deteriorate on wet storage in the absence of micro-organisms, so that bacteria, in the wider sense of this word, must be considered responsible for the breakdown of the fibres. It was furthermore found that the activity of the bacteria can be brought to a standstill, and the deterioration prevented, if the moisture content of the cotton is reduced to below 9 %.

In view of the commercial importance of this breakdown of the cotton fibres, it was thought desirable to extend the investigation and to examine the types of micro-organisms responsible for the damage; secondly, to establish the source of the infection and the changes taking place in the structure of the fibres by the action of the bacteria, and finally, to determine to what extent this damage to the fibres is noticeable in the various types of cotton.

The present paper deals with some of the results obtained in the investigation of the changes caused by bacteria to the structure of the cotton fibre, and with the method worked out for the quantitative determination of this type of deterioration of cotton.

An investigation of the changes in structure of the attacked fibres was not found practicable by direct microscopical examination, as they were too minute to be examined carefully, even by the oil immersion lens. It was decided, therefore, to make use of Cross and Bevan's viscose process for the treatment of the fibres before the microscopic examination. The viscose process has already been used by Balls [1918] for the demonstration of the daily layers of growth in the cell wall of cotton hairs. When applied as suggested by this author, it causes the cotton fibre to swell to about five times its normal size.

The result of the application of the viscose process to the damaged cotton fibres proved most instructive. Some of the observations made will be outlined in the following pages.

The technique of the method, as applied for our purpose, was substantially the same as that used by Balls, except that, when using 15 % Na(OH), it was found possible to dispense with the application of alkali *in vacuo*.

In our investigation we proceeded as follows. From 0.1 to 0.3 gram of cotton of the sample to be examined was boiled for a short time in a 1 % Na(OH) solution, then washed in running water, dipped in a 1 % acetic acid solution, and again washed in water. After removal of most of the adhering water the cotton was transferred to a small stoppered glass bottle with a wide opening and 1.5 cc. of 15 % Na(OH) solution and 1.5 cc. of carbon disulphide were added. In this mixture the fibres were left for 15-45 minutes, then placed on an ordinary glass slide, covered with a cover slip, and a drop of tap water left to diffuse under the latter. The preparation was now ready for microscopic examination.

Fig. 1, Pl. I, shows the behaviour of normal fibres after the viscose treatment. The daily growth rings, described by Balls, are very marked. Particularly characteristic in most of the fibres is their prevalent beaded appearance. This, no doubt, is brought about by the cuticle giving way to the internal pressure created by the rapidly swelling cellulose. The cuticle can in many cases be seen rolled up between two individual beads as a narrow ring of high refractive power. In other fibres the cuticle seems to have burst longitudinally, and in this case forms a corkscrew band round the swollen cellulose.

If an attacked fibre is treated by the viscose process, quite a different effect is obtained, which varies with the type of micro-organism responsible for the deterioration.

Figs. 2 and 3 are from photographs taken from attacked fibres after treatment. In Fig. 2 a streptothrix was responsible for the action, while a cellulose-decomposing schizomycete caused the breakdown of the fibre in Fig. 3.

It will be noticed that in Fig. 2 the cuticle, and most of the cellulose, has been perforated in numerous places. The cuticle has apparently in this case lost its power of resistance, and the cellulose swells uniformly. No beads are observed.

The same can be said of the fibres in Fig. 3, where the swelling, as already mentioned, is due to a cellulose-decomposing schizomycete. The cuticle of the fibres in this specimen seems to have been eaten away over wide areas. It looks as if the destruction in this case was progressing uniformly from the surface toward the interior.

Fig. 2 explains the appearance of the large number of short fibres found in deteriorated cottons. They are produced by the action of a streptothrix, a form of micro-organism which is always found in large numbers in weathered cottons.

The damage of the true schizomycete is due, less to the formation of fly, than to a general weakening of the fibre.

The fibres, from which Figs. 2 and 3 were taken, had been exposed to the action of micro-organisms for a considerable time—over a month. It appeared of interest, therefore, to determine how soon after exposure to dampness, the action of the micro-organisms would be noticeable on the fibres. Samples of various grades of cotton were, therefore, wetted with tap water and left at ordinary room temperature. It was found, that a cotton waste sample with a bacterial content of about 1 million per gram, showed signs of extensive damage after five days' exposure. On the other hand, a carefully picked sample, taken direct from the bolls, and with only 20,000 micro-organisms per gram, could be left damp for twenty days before the fibres were visibly attacked. If, however, such samples were infected with the characteristic micro-organisms, the damage was considerably accelerated.

Fig. 4 shows a sample of ordinary cotton waste which has been exposed to dampness for five days. It will be noticed that very few of the fibres have any of their cuticle left intact.

Another point of interest is the difference between the ways in which the streptothrix and the true bacteria attack the fibres. The two types of damage are so markedly different, that it is almost possible by the microscopic examination to indicate where the fibres were damaged by a streptothrix, and where by bacteria.

These remarkable changes, found to occur in the structure of the cotton fibre after exposure to dampness, made it natural, on this basis, to attempt the elaboration of a method for the quantitative determination of damaged fibres in good cotton.

Most raw cottons, of American and Indian origin, have at one time or other been exposed to rain or excessive dampness, and others may have been deliberately wetted. In both cases the result is a damage to the fibres, which in its earlier stages, cannot at present, be detected. It is true, that where the moisture content of the cotton remains high—over 9 %—a determination of the amount of water present will be a useful indication of damage; but, in many cases, where the wetting occurred on the farms or after ginning, the cotton is likely to dry up when taken to other districts, for instance for transport to Europe. Moreover the determination of the moisture content can only be an indication, and not a quantitative method by which the cotton merchant is able to determine how far the fibres of a particular cotton have been damaged, and how much of it he is likely to lose during its subsequent treatment at the mills.

The alkali solubility test would, no doubt, in many cases of excessive damage, give good results; but where it is a case of detecting 10, or 5, or perhaps even 2 % of damaged fibres, this method is not sufficiently sensitive. In a later paper it is hoped to substantiate this assumption.

The method for the detection and quantitative determination of damaged fibres in cotton by microscopic examination, as worked out on the basis of the above observations has, so far as it has yet been tried, given very satis-

factory results. It may be of interest to give a few examples of the results obtained.

A sample of damaged cotton, in which all fibres were found to be attacked, was carefully mixed with various quantities of good cotton, picked direct from the boll, and containing normal fibres only. A sample of this mixture containing 30 % of damaged fibres was, on examination, found to have 29.6 %. A second determination of the same sample gave 29.5 %, a third 27.3 %. A mixture made up to contain 10 % was found to have 8.9 %; a 5 % sample 5.4 %, a 4 % sample 4.6 %, and a 2 % sample 2.0 % and 2.5 %.

The broad outlines of the method as now in use<sup>1</sup> are as follows. The sample to be examined is treated with the carbon disulphide and alkali mixture; the fibres are then spread out on a slide, as already described, and counted under the microscope. From each sample three preparations are made. The average of 10 counts from each preparation gives the required figure.

#### CONCLUSIONS.

In cotton which has been exposed to dampness the action of micro-organisms materially alters the structure of the fibres, giving rise to the formation of exceptionally short fibres, the so-called "fly."

The extent of this damage can be quantitatively determined by a method outlined in this paper.

#### REFERENCE.

- Balls (1918). The existence of daily layers of growth in the cell wall of cotton hairs. Author's MS.

<sup>1</sup> The details of the technique will be published later.

28



Fig. 1.  $\times 45$ .



Fig. 2.  $\times 45$ .

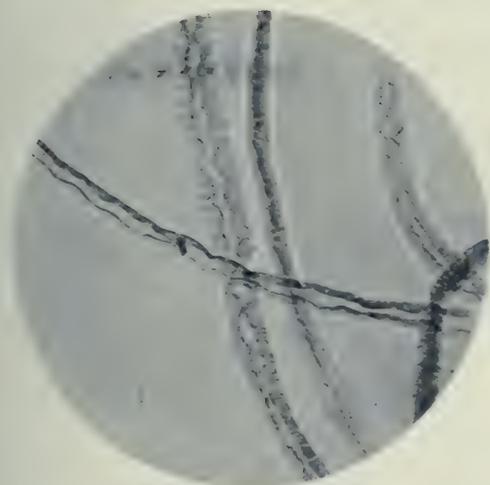


Fig. 3.  $\times 45$ .

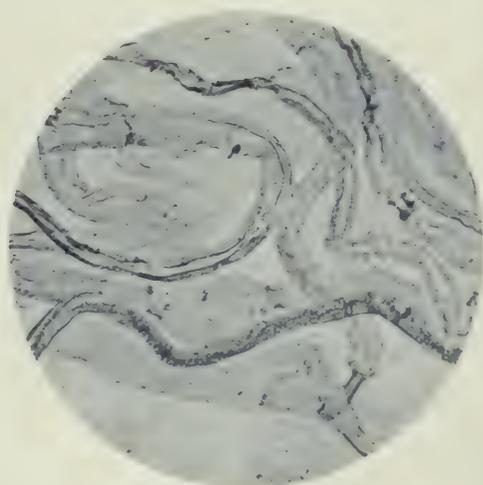


Fig. 4.  $\times 45$ .



### III. ADSORPTIVE STRATIFICATION IN GELS, III.

BY SAMUEL CLEMENT BRADFORD.

(Received December 11th, 1919.)

(With Plate II.)

THE first record of the formation of noticeable structures by the diffusion of precipitating reagents appears to have been made by Runge [1855], who experimented with a number of pairs of inorganic reagents in blotting paper. In 1874 Monnier and Vesque observed the production of large crystals by slow diffusion through strips of filter paper and gels respectively. Three years later Ord allowed ammonium oxalate to diffuse through an isinglass gel in an open tube immersed in calcium chloride solution. The precipitate consisted first of growth forms changing into spherites towards the calcium chloride. The diffusion of solutions into gels was studied extensively by Pringsheim [1895] in 1890 and 1892. The results of his experiments were communicated to the Berlin Academy in 1891 and 1892, but not published until 1895. Pringsheim allowed two solutions, capable of interacting with the formation of a precipitate, to diffuse in opposite directions through gelatin gels contained in the horizontal portion of a graduated U-tube of special form. The rates of diffusion for different concentrations and various reagents were observed, and the important law discovered that, after the meeting of the reagents, diffusion took place only from the hypertonic into the hypotonic reagent. From the number of his experiments it would be surprising if Pringsheim had not observed cases of band formation, especially as U-tubes favour this result. However, such concentrated solutions were employed, from  $N/2$  to  $12N$ , as usually to prevent stratification of the precipitate. Nevertheless examination of his illustrations shows the formation of two bands in several cases. As a botanist he refers to the occurrence: "In many cases not one but two separated precipitates are produced, which may perhaps be due to dissociation phenomena."

The first observation of a series of layers must be ascribed to Lupton [1892]. Unfortunately Lupton did not investigate the phenomenon, and the nature of his bands is left to conjecture. But in 1896 Liesegang [1896], continuing Pringsheim's experiments, obtained the remarkable series of silver dichromate rings now well known. The original experiment was made with a drop of silver nitrate on a glass plate coated with 5 per cent. gelatin containing a small quantity of potassium dichromate. Liesegang rightly perceived the

importance of the phenomenon and commenced an experimental study, the first part of which was published [1898] in a pamphlet which has not been available in London until recently. In these researches he used mostly ammonium dichromate in gelatin gels contained in glass tubes inverted in aqueous silver nitrate. He showed that the spaces between the layers are completely free from unchanged ammonium dichromate, since silver nitrate poured over a plate preparation produced no further effect. Plates examined with X-rays showed dark shadows, corresponding to the A-lines as he called them, with the spaces almost clear. "There is extraordinarily much more silver in the A-lines than in the spaces."

Liesegang also obtained "essentially similar structures" with other insoluble precipitates. Ammonium chromate gave rings almost exactly like those of the dichromate. Using lead nitrate, instead of the silver salt, the bands were wholly like the silver dichromate ones, but not so sharp, and the spaces were filled with a cloudiness due to yellow lead chromate. A-lines were also obtained with lead iodide and thiosulphate, the latter giving sharp white lines separated by glass clear spaces, and with silver ferric oxalate and Prussian blue. Liesegang perceived that wider study of the phenomena might enable biologists artificially to imitate the structures of living organisms. He suggested that banded agates might have arisen in this way and warned microscopists that their methods of staining might lead to the production of fresh structures in the objects under examination.

After having inspected Liesegang's "astonishing" preparations, Ostwald interpreted the A-lines as due to supersaturation, remarking that "Precipitates which appear in the colloidal form give no A-lines, neither do those which are ordinarily (i.e. visibly) crystalline; whatever is the reason in such cases has not yet been discovered" [1897]<sup>1</sup>. The posthumous theory of the late Lord Rayleigh [1919] is essentially similar.

The supersaturation theory was subjected to mathematical analysis by Morse and Pierce [1903]. Working with potassium chromate in the gel, the reaction takes place in accordance with the relation:



For equilibrium in the presence of the solid phase the concentrations must satisfy the relation:

$$\text{Ag} \cdot 2 \times \text{CrO}_4'' = k, \dots\dots\dots(1)$$

where  $k$  is the solubility product. Morse and Pierce inquired whether there is a relation in the case of a supersaturated solution similar to (1), with, however, a different constant, that defines the limit of supersaturation, e.g.

$$\text{Ag} \cdot 2 \times \text{CrO}_4'' = H. \dots\dots\dots(2)$$

Assuming that the formation of a precipitate has no influence on the progress

<sup>1</sup> It has previously been shown, Bradford [1917], that the adsorption theory explains both these facts.

of the diffusion, by the application of Fick's law, they were able to obtain the formula:

$$H = \frac{4U_0^2V_0}{\pi^{\frac{3}{2}}} \left[ \int_0^{\infty} \frac{x}{2a\sqrt{t}} e^{-\beta^2} d\beta \right]^2 \cdot \int_0^{\infty} \frac{x}{2b\sqrt{t}} e^{-\beta^2} d\beta, \dots\dots\dots(3)$$

where  $x$  is the distance of a band from the surface of the gel,  $t$  the time from the commencement of the experiment,  $U_0$  and  $V_0$  are the original concentrations of the reaction components,  $a^2$  and  $b^2$  their diffusion constants, and  $\beta$  is merely a constant of integration.

From this Morse and Pierce deduced that  $\frac{x}{\sqrt{t}}$  must be constant, which they were able to verify, that the change of concentration of the solution in the gel can be neglected and that:

$$H = 1.4 \times 10^{-6} \left( \frac{\text{mol.}}{\text{litre}} \right)^3$$

and

$$a^2 = 1.54 \left( \frac{\text{cm}^2}{\text{day}} \right).$$

This indicates that, with  $N$   $\text{AgNO}_3$  and  $N/75$   $\text{K}_2\text{CrO}_4$ , precipitation occurs at each new band when the concentration of the silver ion is about  $N/200$ , that of the chromate being unaltered at  $N/75$ . Moreover, since the ordinary solubility product of silver chromate is  $5.1 \times 10^{-13}$ , the supersaturated gelatin at the concentration of precipitation held in solution  $\sqrt[3]{\frac{1.4 \times 10^{-6}}{5.1 \times 10^{-13}}}$  or 140 times the amount of silver chromate required to saturate it in the presence of the solid phase. It will also be noted that the figure, 1.54, found for the diffusion constant of silver nitrate in gelatin is much greater than that in pure water, 1.13 to 1.20, determined by Kawalki. These deductions are very remarkable and it will be endeavoured to show that they are contrary to fact. Morse and Pierce also obtained bands in gelatin with lead sulphate; silver oxalate, carbonate, phosphate, thiocyanate and bromide; cobalt hydroxide; barium oxalate; mercurous bromide and carbon dioxide. They also found that bands were produced in capillary tubes in aqueous solution, which broke down after the formation of two or three.

The supersaturation theory has been criticised by a number of writers. Bechhold [1905] considered that, in addition to supersaturation, the production of banded precipitates was influenced by the solubility of the precipitate in one or other of the salts present or formed by the reaction, and by the coagulating or inhibiting effect of ions, as in the case of suspensions and colloid solutions. By mixing 1 cc. of silver nitrate solutions of different strengths with 3 cc. of  $M/100$   $(\text{NH}_4)_2\text{Cr}_2\text{O}_7$  in 2.5 per cent. gelatin sols, Bechhold found that deep turbidity was produced with a concentration as low as  $M/533$  of  $\text{AgNO}_3$ .

The metastable product can be calculated from this value:

$$\text{Ag} \cdot 2 \times \text{Cr}_2\text{O}_7'' = \left[ \frac{1.88 \times 10^{-3}}{4} \right]^2 \cdot \left[ \frac{3 \times 10^{-2}}{4} \right] = 1.64 \times 10^{-9}.$$

It will be seen that although silver dichromate is more than twice as soluble

as the chromate, yet the metastable product calculated from Bechhold's figures is very much less than that deduced from the supersaturation theory for the latter substance by Morse and Pierce.

Since nitrates tend to liquefy the gelatin, Bechhold thought they should favour the formation of a precipitate, and this would be the effect of the ammonium nitrate formed in the reaction. Similar experiments were therefore made both with  $N/160$  and  $N/320$   $\text{AgNO}_3$  mixed with  $M/100$   $(\text{NH}_4)_2\text{Cr}_2\text{O}_7$  in 5 per cent. gelatin containing increasing amounts of  $(\text{NH}_4)\text{NO}_3$ . The turbidity increased to a maximum, diminished and again increased. Parallel experiments in water without gelatin showed similar maxima and minima, though somewhat less definite. These latter results are particularly interesting as they suggest that ordinary supersaturated solutions are affected by ions in the same way as sols. The question of the relation of sols to supersaturated solutions is in urgent need of further study, which would probably throw much light on the nature of solution.

Bechhold realised that, if the ammonium nitrate formed by the reaction plays a part in the ring formation, an increase in the concentration of this salt must cause a change in the ring structure. The bottom of a glass dish was covered half with a layer of 5 per cent. gelatin containing  $M/20$   $(\text{NH}_4)_2\text{Cr}_2\text{O}_7$  and half with chromated gelatin with addition of  $N/4$   $(\text{NH}_4)\text{NO}_3$ . A drop of silver nitrate was placed over the division. The rings in the ammonium nitrate half were appreciably wider than in the other half and very slightly closer, so that separation of the bands was not apparent till a greater distance from the centre. This shows that the formation of the bands is undoubtedly affected by the presence of soluble salts in the gel. However the concentration of the  $(\text{NH}_4)\text{NO}_3$  was six times greater than would be produced in an ordinary experiment. Similar ideas, though without further direct experimental support, were suggested later. In a lecture at the Royal Institution in 1912, Sir J. J. Thomson supposed that the soluble substances produced by the reaction might act in a similar way to certain bodies known as stabilisers which prevent the precipitation of colloidal solutions, and Freundlich and Schucht [1913] thought that the formation of a precipitate might be due to autocatalytic coagulation by electrolytes.

It seemed desirable to add increments of by-product more comparable with the concentration in which they are usually produced. Tubes containing (1) 20 cc. of  $N/20$   $\text{Na}_2\text{CO}_3$  agar gel and (2) the same with  $N/20$   $\text{NaCl}$  were treated with 5 cc. of dilute  $\text{CaCl}_2$  solution. Diffusion proceeded at the same rate in each tube with the formation of bands at distances below the surface of the gel in mm. as follows:

(1)	20.5	22.5	25	28	32.5	35.5
(2)	20.5	22.5	24.5	—	33	35.5

In another experiment 15 cc. of  $N/5$   $\text{K}_2\text{CrO}_4$  solution was poured on (3) 15 cc.  $N/200$   $\text{Pb}(\text{NO}_3)_2$  agar gel, and (4) the same gel with addition of  $\text{KNO}_3$  to  $N/100$ .

The tubes developed beautiful series of clear-cut strata of lead chromate very close together, the average distance of the last twenty layers being respectively 0.70 and 0.67 mm. Comparison with Bechhold's result shows that any influence of the by-product of the reaction is to reduce the distance between the strata, i.e. to inhibit separation of the bands, but such effect is practically negligible.

Hatschek [1911, 1, 2; 1912] used a number of fresh compounds and studied for the first time reactions in various gels, including the inorganic gel of silicic acid. He showed that precipitates tend to form larger crystals in gels than in aqueous solution and that the particles of banded precipitates are usually microscopic spherical aggregates, and was able [Hatschek and Simon, 1912] to explain by his results many features of the occurrence of gold in quartz. He [1914] showed that the supersaturation theory is inadequate to explain the complicated phenomena of stratified precipitation. If, as Ostwald thought, the silver dichromate is deposited on the ring from the neighbouring region which is supersaturated in regard to this salt, it should be impossible to produce a second set of rings in a tube already containing one, or to obtain a stratified precipitate in a gel previously sown with crystals of the insoluble precipitate. Nevertheless he obtained perfect stratifications of lead iodide in agar containing suspended lead iodide [1912] and his pupil, de Whalley, succeeded in producing a second system of lead chromate bands in agar, superposed on a finer system of strata of the same substance [Hatschek, 1914]. These experiments appear to show that bands can form although supersaturation is prevented.

Liesegang [1914] showed that strata of silver dichromate do not form in gelatin which has been purified from mineral matter by soaking in slowly changing water. It is necessary to add gelatose and a small definite amount of (citric) acid for each particular concentration of soluble dichromate in the gel. The bands are however thicker and more widely spaced than in the unwashed gelatin. Further addition of acid yields a continuous mass of colloidal silver dichromate. Moreover it has been found that neither silver chromate nor dichromate forms bands in agar gel. Indeed the nature of the gel appears to have a very remarkable influence on the form of the precipitate in all cases. This was first insisted upon by Hatschek as a necessary consideration for a complete theory of the phenomenon. Hatschek's view of the inadequacy of the supersaturation hypothesis was endorsed by Wo. Ostwald [1917].

Experimental study reveals many minor points for which it is difficult, or impossible, to account on Ostwald's hypothesis. Moreover it seems particularly hard to devise a direct proof of the supersaturation theory. The only experimental evidence so far adduced in its favour appears to be the verification by Morse and Pierce of the constancy of the ratio of the distance of a band from the surface of the gel and the square root of the time of formation. This is by no means a direct proof of the theory. Moreover, as already pointed out, the results deduced at the same time for the metastable product and the diffusion

constant of silver nitrate, as well as the non-diffusion of hypotonic reagent are remarkable. Indeed the latter is at once seen to be incorrect by working with a highly-coloured solute in the gel.

It appeared to be a simple matter to make a direct test of the value of the metastable product which could be compared with that required by the supersaturation theory. 5 per cent. gelatin gels of Nelson's "crystal" leaf gelatin were prepared containing decreasing amounts respectively of potassium dichromate and chromate. These were treated with dilute solutions of silver nitrate of suitable concentration. The silver nitrate was always hypertonic, so that, by Pringsheim's law, the precipitate should always be formed in the gel.

The value found by Morse and Pierce for silver chromate was  $H = 1.4 \times 10^{-6}$ . Since the solubility of silver dichromate is 0.0<sub>3</sub>19 mols per litre, as compared with 0.0<sub>4</sub>8 mols per litre for the chromate, the value of the metastable product for silver dichromate, which would correspond to that found by Morse and Pierce for the chromate, would be  $\frac{1.4 \times 10^{-6}}{5.1 \times 10^{-13}} \times (1.9 \times 10^{-4})^3 = 1.9 \times 10^{-5}$ . Below these values of  $H$  no precipitate should be formed. Yet it has been seen that Bechhold obtained a deep turbidity in 2.5 per cent. gelatin sols with concentrations of dichromate corresponding to  $H = 1.64 \times 10^{-9}$ . Since reactions in gelatin gels must take place in the liquid dispersion medium [Bradford, 1918] which contains only a few tenths of one per cent. of gelatin, the protective effect of the gels could not be greater than in the sol state. The experiments in gels are tabulated below:

			Table I.
$K_2Cr_2O_7$	$AgNO_3$	$H$	
$M/200$	$M/50$	$2.0 \times 10^{-6}$	Red surface precipitate in flakes, faint bands at 1.6, 2.3 mm. Then beautiful black spherites of $Ag_2Cr_2O_7$ , up to 0.17 mm. diam., to depth of 1.5 cm.
$M/250$	$M/50$	$1.6 \times 10^{-6}$	Red surface layer in flakes, faint bands at 1.3, and 3.6 mm. A few spherites below
$M/300$	$M/50$	$1.33 \times 10^{-6}$	Red dusty ppt. above, faint layers at 0.8 and 3 mm.
$M/350$	$M/50$	$1.1 \times 10^{-6}$	Faint dusty layer above, very faint layer at 0.4 mm.
$M/300$	$M/100$	$1.6 \times 10^{-7}$	Fine dusty ppt. on surface. Ochre coloration below.
$M/400$	$M/100$	$1.25 \times 10^{-7}$	Dusty red ppt. on surface. Ochre coloration below.
$M/500$	$M/100$	$1.0 \times 10^{-7}$	No distinct ppt. on surface. Ochre coloration.
$M/1000$	$M/100$	$5.0 \times 10^{-8}$	No distinct ppt. on surface. A few black spherites of ? $Ag_2Cr_2O_7$ , 0.03 mm. in diam. to depth of 4 or 5 cm. below surface.

			Table II.
$K_2CrO_4$	$AgNO_3$	$H$	
$M/200$	$M/70$	$1.0 \times 10^{-6}$	Beautiful series of bands at 0.0, 0.4, 0.8, 1.3, 1.9, 2.3, 3.1*, 4.7*, 6.8*. Clusters of spherites at 7.0 and 9.0 mm., largest 0.02 mm. diam.
$M/400$	$M/70$	$5.0 \times 10^{-7}$	Thin red layers at 0.0, 0.6, 1.6, 2.5, 4.0, broken at 5.2 mm. Spherites down to 12.4 mm., largest 0.01 mm. diam.
$M/800$	$M/70$	$2.5 \times 10^{-7}$	Red surface layer with faint band in ochre opalescence extending to 1.5 cm.
$M/400$	$M/140$	$1.25 \times 10^{-7}$	Fine red ppt. on surface.
$M/800$	$M/140$	$6.25 \times 10^{-8}$	Fine ppt. on surface.
$M/800$	$M/210$	$2.75 \times 10^{-8}$	Faint ppt. on surface.

\* Layers composed of spherites.

It will be seen that with  $H = 1.0 \times 10^{-6}$ , which is already less than Morse and Pierce's figure, silver chromate still forms a beautiful series of bands. The concentration at the bottom of the tube must be very much less than this value. In all the experiments there was some subsidiary white precipitate, as always occurs with silver nitrate in gelatin. Actually, with very low values of  $H$ , the amount of white precipitate was much greater than that of the chromate, so that the effective value of  $H$  for the silver chromate must have been considerably less than that corresponding to the concentrations used. As the ionic product becomes smaller, the density of the precipitate in the surface layer of the gel becomes less until, even under the microscope, it is difficult to determine whether the precipitate is formed in the gel or in the solution. Pringsheim's law indicates that the precipitate is always in the gel. But, without making any assumption in regard to this, it is certain that silver dichromate and chromate respectively form precipitates with values of  $H$  lower than  $1.1 \times 10^{-6}$  and  $2.5 \times 10^{-7}$ , corresponding to the appearance of a layer below the surface. These are respectively 16 and 6 times less than indicated by the supersaturation theory<sup>1</sup>. These experiments are sufficient to invalidate the assumption made by Morse and Pierce, that the formation of a precipitate does not affect the head under which diffusion takes place. That is to say the precipitate does affect the course of the diffusion.

Consideration of the facts (*a*) that the hypotonic reagent is removed from the spaces between the layers and (*b*) that only precipitates which are either colloidal or extremely finely divided separate into strata, leads naturally to the conclusion that it is the adsorbing action of the large surface of the precipitate which removes the solute from the layer of gel in its vicinity, so that the hypertonic reagent, diffusing through, has to traverse an exhausted zone of gel before meeting with sufficient solute to produce a new band.

Microscopic examination of the precipitates which occur in banded form usually shows that they consist of spherites. In the case of silver chromate and dichromate in gelatin, and manganese sulphide in agar, the individual particles are generally too small for their nature to be determined. In the majority of cases the beautiful spherical particles are easily visible, sometimes, as in the case of copper carbonate in agar and calcite in silicic acid, the spherites are large enough to show distinct banding and give a dark cross and colour rings in polarised light. The origin and structure of spherites has been discussed as far as seemed possible at present in a previous paper [Bradford, 1918]. Further work is in progress. For the purpose in view it is sufficient to recall that these apparently globular crystals actually consist of a great many radial needles, so that small spherites have an enormous specific surface and may be regarded as being in the colloidal state. A few precipitates, such as calcite in silicic acid gel, appear first, in the more concentrated regions, as tiny perfect rhombohedra, which become more aggregated, as the velocity of crystallisa-

<sup>1</sup> These figures receive confirmation from some experiments to be published shortly.

tion diminishes with dilution of the reagents, and finally occur as perfect spherulites of macroscopic size.

In all the cases examined, therefore, banded precipitates have very large specific surface and may be expected to behave in the same way as finely divided solids in adsorbing dissolved substances from their vicinity. This effect occurs in the filtering of solutions through fine powders, where the dissolved particles are retained by the solid. It is well illustrated by Dreaper and Davis' night-blue experiment.

The marked adsorption of solutes containing like ions by sparingly soluble solids is generally recognised, and may well be akin to the remarkable attraction of nutrient material from solution by growing crystals, both being probably of electrical origin. Indeed the adsorption of solute in the formation of banded precipitates may be looked upon as comparable with the mechanism of crystal growth. Consequently it can hardly be doubted that precipitates, formed in media which can hold them in place, will tend to remove the soluble reaction components from their vicinity, and this effect will be the more marked the greater the specific surface of the precipitate. Moreover, since the reagent diffusing into the gel is always in excess, any of the solute in the gel, which is adsorbed, will be precipitated with the formation of fresh surface. The adsorbing surface is, therefore, always "clean" with regard to the solute in the gel.

As the adsorption progresses, solute will diffuse from the more concentrated region of the gel into the depleted zone, and a concentration gradient will be set up, the diffusion becoming more rapid as the concentration in the neighbourhood of the precipitate diminishes. The process will continue until the concentration of the adsorbed solute, at the surface of the precipitate, is reduced to the value corresponding to the solubility product of the solid under the particular circumstances. When this happens, the concentration of the solute in the adjoining region of gel must be very much less than this value, but will gradually increase in the direction away from the precipitate. In this manner a considerable zone of gel will be practically exhausted of solute. Any hypertonic reagent diffusing into this region will not cause the formation of a precipitate, since the ionic product will be less than the critical value. The growth of the band under consideration will then have ceased. A new layer will eventually be commenced at a distance from it, where the hypertonic reagent finds sufficient concentration of solute in the gel. In this paper the actual process of precipitation, whether through the formation of sols or supersaturated solutions, is not considered. Much further work appears to be necessary before the mechanism of precipitation in gels can be completely elucidated. It will be seen, however, that although some kind of supersaturation may, and probably usually does, occur to a greater or less degree before precipitation, this is not necessary for the formation of separate bands, and could hardly account for the occurrence of bands of very insoluble substances, such as silver chloride, which the writer has obtained several millimetres apart in agar, this gel having much less protective effect than gelatin.

It will thus be seen that the adsorption theory is almost diametrically opposed to the supersaturation theory. For whereas the latter hypothesis attributes the formation of bands to the influence of supersaturation preceding precipitation and requires that no appreciable diffusion shall occur of the solute dissolved in the gel, the former theory needs no assumption as to the mechanism of precipitation and claims exhaustion of the solute from the region of the gel in the vicinity of the precipitate.

Direct proofs of adsorption are readily forthcoming. By working with deeply coloured solutes it is possible to watch the progress of the adsorption in the neighbourhood of the precipitate. With silver chromate in gelatin the white subsidiary precipitate due to impurities makes it difficult to observe the exhaustion of the gel. But with lead chromate in agar the effect is easily seen. When viewed for the first time the phenomenon is almost astonishing. This is shown in Fig. 1, Pl. II, which is reproduced from a previous paper.

It has been objected that the adsorption theory takes no account of the influence of the gel. However, it has been shown [Bradford, 1917] that the different effects obtained with the same insoluble substance in different gels can be explained as due to the influence of the gel, or reaction medium, on the factors  $K$  and  $P$  in von Weimarn's formula, which determine the specific surface of the precipitate and therefore its adsorbing power. By the application of von Weimarn's theory it is possible to obtain silver chromate and dichromate strata in agar gel which, in absence of subsidiary precipitate, are, if possible, more beautiful than in gelatin. It is hoped to publish these experiments shortly. The same recipes give good rings in filter paper.

Both these and plate preparations in agar show the adsorption effect even more strikingly than with lead chromate. A ring of perfectly colourless gel gradually develops round each band of precipitate, the precipitate ceases to grow, and nothing can be seen except the slowly widening colourless zone. Suddenly a fresh ring commences with a deep reddish-yellow coloration at the edge of the colourless zone where it joins the yellow colour of the soluble chromate.

These experiments with coloured solutes show conclusively that the gel is actually exhausted of solute in the vicinity of the precipitate. Whether the effect be due to adsorption, or to any other cause to be determined, it is sufficient to account for the separation of the bands.

There is however little room for doubt that the colourless zone is really due to adsorption. It can easily be shown that finely divided powders actually have the power of producing a similar result. Naturally it would be inferred that the effect would be less noticeable in this case, since, without excess of hypertonic reagent, the adsorbing surface would quickly become contaminated. This is why a fresh series of bands can form upon an old one. Columns of fuller's earth were embedded in 1 per cent. agar gels above the same gels deeply coloured with various dyestuffs. Some of these showed results more intense than others. With night-blue the effect was most extraordinary.

Within about twenty minutes a perfectly colourless narrow zone could be seen below the adsorbing column. This quickly grew, and in 24 hours had reached a depth of four or five millimetres. All this time the sharpness of the colour transition was remarkable. Practically the whole of the region of gel affected was nearly colourless, the change to deep blue taking place almost suddenly. Gradually the colour gradient became less steep as the exhausted zone increased, but the effect persisted for months. Fig. 2 shows the effect after a few days. With catalpo no. 1 and magenta in 1 per cent. agar the result is equally striking and lends itself better to photography, Fig. 3 (after 17 days). From these experiments there can be no doubt that adsorption is capable of causing the extraction of soluble substances from a considerable zone of gel. Similar experiments were also performed with lead chromate as adsorbent and potassium chromate and dichromate as solutes. The concentration of potassium chromate in Fig. 1 was  $N/20$ , but since  $N/200$  best gives bands of lead chromate, this concentration with  $N/300$  and  $N/100$  was used. The adsorption effect became visible almost instantaneously. It is best seen by standing with the back to the light and holding a piece of white paper sloping backwards at about  $45^\circ$  behind the tube. An exceedingly narrow zone of a much lighter colour immediately becomes visible beneath the column of lead chromate. The effect however increases much more slowly than with the colloidal substances previously examined. Presumably this is due to contamination of the adsorbing surface. Unfortunately also the colour of the dilute gel is not easy to photograph. Fig. 4 shows the effect with  $N/300$   $K_2CrO_4$  after 30 days. The columns of adsorbent were made into a thin cream with water and poured on to the agar gel, care being taken to prevent the lead chromate penetrating between the gel and the tube. By using lead nitrate solution, of the same strength as the potassium chromate in the gel, instead of pure water to moisten the lead chromate, the contamination of the adsorbing surface was reduced and the adsorption progressed almost as rapidly as with dyes. It seemed natural to suppose that this greatly increased effect might be at least partly electric in origin, the chromate anion being attracted by the divalent cation. To test this, equivalent concentrations of sodium hydroxide were used instead of lead nitrate. The adsorption was nearly as great as with the lead nitrate. In confirmation, aluminium sulphate was employed as a trivalent cation. If the adsorption is electrical, the effect should be very much greater in this case. This was fully borne out in the result. A narrow perfectly colourless zone immediately became visible below the lead chromate and the adsorption progressed at least as rapidly as with the dye experiments. Fig. 5 illustrates the condition after two days, with  $N/100$  dichromate and aluminium sulphate.

The suggestion is indicated that the mordanting effect of aluminium salts is electrical in origin. The results are also interesting in connection with Harrison's [1911] electrical theory of dyeing. It may also be pointed out that Lewis' experiments [1908] which showed that the surface tension of

water-oil films decreased with increase of temperature, do not necessarily apply to adsorption by solids. If the surface tension of the solid-liquid is due to unbalanced molecular attractions at the common boundary, and if the thermal expansion of the liquid is greater than that of the solid, the unbalanced forces will increase with rise of temperature thus augmenting the surface energy and consequently the adsorption. In confirmation it will be noted that in the case of two liquids such as ether and water, one of which has a much greater thermal expansion than the other, the interfacial surface tension has been found to increase with rising temperature [Antonov, 1907]. The influence of temperature on adsorption by solids has not been studied. But Freundlich [1907] found that charcoal adsorbs more acetic acid from water at higher temperatures, and Pelet and Grand [1907] found the same for methylene blue and crystal ponceau adsorbed by silica; kieselguhr and charcoal. It may be noted, however, that, with solids such as fibres having very great specific surface, their thermal expansion would be greater than in a less disperse state owing to the comparatively large volume of the solid in the surface layer where the intrinsic pressure is released.

In all these adsorption experiments the exceedingly narrow zone immediately beneath the adsorbent is always distinctly less coloured than the remainder of the exhausted region. This is more noticeable in the actual specimens, but can be made out in the photographs.

Numerous facts observed during experimental study appear to lead directly to the assumption of adsorption. The following are particularly striking:

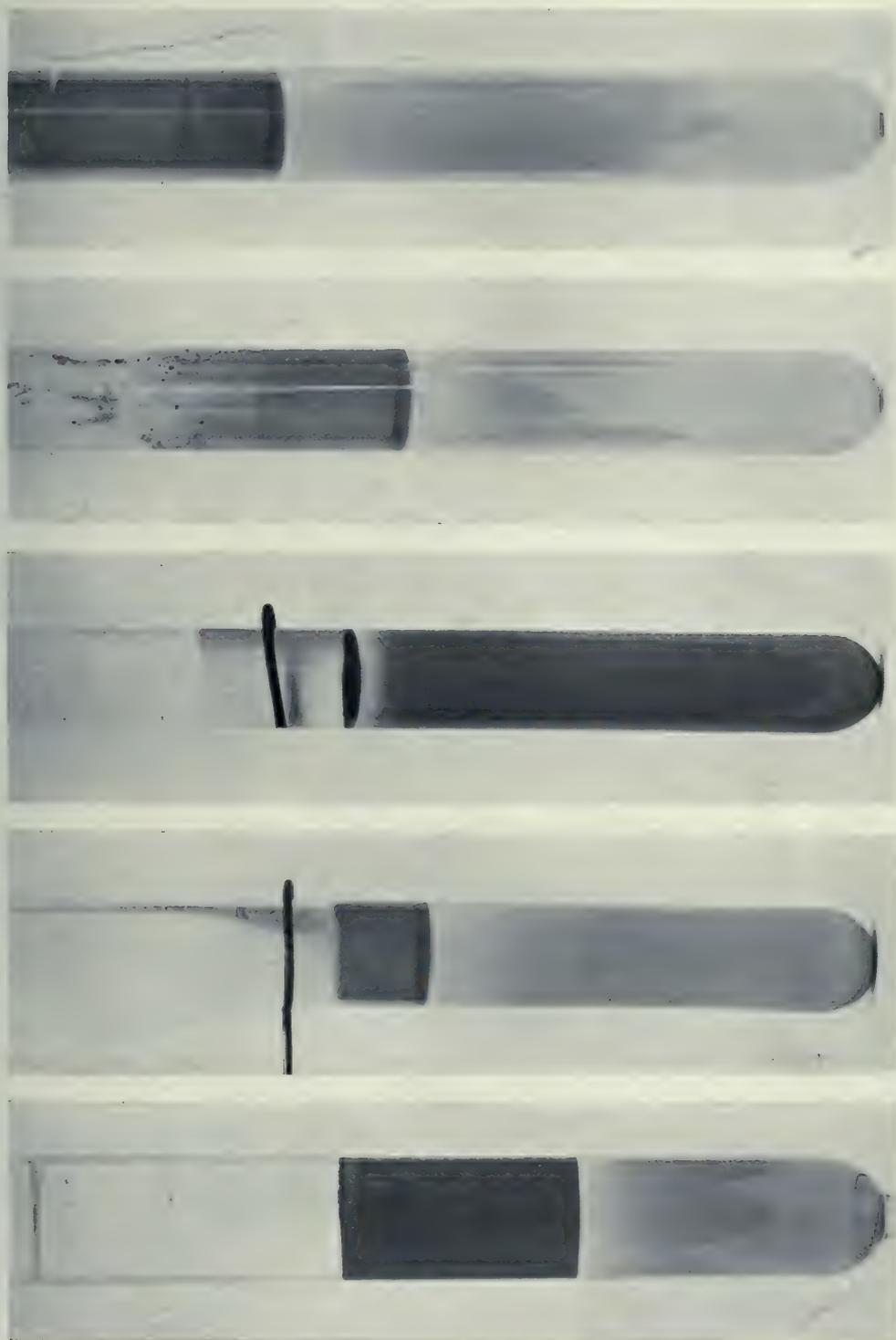
(1) The formation of bands usually ceases long before the bottom of the tube is reached. The hypotonic reagent is invariably so strong that this effect can only be due to the solute in the gel having been exhausted from the lower regions of the tube. This fact also controverts the deduction from the supersaturation theory that the diffusion of the solute in the gel is negligible.

(2) With certain more colloidal precipitates, e.g. manganese sulphide in agar, a tendency is observed to form large spherical aggregates which have much the same structure and density as the bands, except that they frequently show concentric banding. When one of these spherical aggregates begins to form in the zone where a band will shortly appear, the band does not extend to join the sphere, but a spherical cavity, some 2 or 3 mm. wide, is left surrounding the concretion from which the surface of the cavity is everywhere equidistant. This effect must be due to the solute having been exhausted from the neighbourhood of the aggregate. Indeed its spherical form almost certainly implies that nutrient material has accrued equally from all directions.

(3) A similar phenomenon occurs frequently with certain reagents in agar gel. The hypertonic solution accidentally flows down between the gel and the glass, forming a new base from which it diffuses into the gel producing a precipitate with a surface convex inwards, the direction of growth being thus at right angles to that of the precipitate forming down the tube from the upper surface of the gel. It is then observed that this latter precipitate does

not form in the neighbourhood of that growing from the side. It leaves, instead, a clear zone between them which increases in width downwards as the accidental precipitate has had longer time to exhaust the gel in its vicinity.

An interesting feature is sometimes observed in these experiments which appears to afford direct evidence of the energy of the diffusing particles of the solute in the gel. When the agar gel is prepared by straining through muslin, instead of filtering, it has a slightly cloudy, or more opalescent appearance, which is probably due to the presence of larger gel aggregates. During the growth of the first continuous column of e.g. lead chromate in the tube, the region of gel immediately beneath the precipitate loses not only its colour, but also its opalescence, as if the cloudiness had begun to subside, and the clear space gradually extends towards the bottom of the tube. It seems that in such cases the more agglomerated particles of the gel are broken up by the impacts of the rapidly moving solute particles in their diffusion towards the precipitate. In some cases solid particles appear actually to be driven through the gel towards the precipitate. These may be either larger portions of more or less gelatinous matter which have escaped straining, or, apparently they may even consist of particles of the precipitate. It frequently happens with a heavy precipitate that it appears gradually to settle down through the gel. Possibly this may be due to some bacterial or other alteration in the structure or substance of the gel by exposure to the atmosphere. But cases in U-tubes hardly admit of this interpretation. The following is a typical experiment.  $N/30$  NaCl and  $N/10$  AgNO<sub>3</sub> solutions were poured into the respective limbs of a U-tube the lower portion of which was filled with pure 1 per cent. agar gel. With the meeting of the reagents in the gel merely a slight diffuse precipitate was produced at first. After two days this filled the area of the tube for a distance of about 1 cm. and had become somewhat less tenuous. The edge of the precipitate towards the sodium chloride had now developed a distinct, though not very dense, disc of silver chloride. The position of this disc was marked on the tube. The disc then expanded into a column of precipitate which formed continuously from this point, gradually becoming more dense. In the meantime, however, the light diffuse precipitate gradually diminished and in four days had practically disappeared. In thirteen days even the commencement of the denser part of the column had become distinctly less marked and had shifted gradually through about 1.5 mm. At this point the concentration of the silver nitrate would probably have been about  $N/50$ . The disappearance of the precipitate must either have been due to peptisation by this dilute solution, or to the tiny particles having been driven through the gel by the bombardment of the silver nitrate molecules. In this experiment the denser edge of the growing column attained a maximum and appeared as a distinct disc of dense precipitate at about 12 mm. from the commencement of the column. The less dense column continued to grow with the production of a series of such denser bands. The first five were equally spaced, about 1.5 mm. apart, the sixth, at about twice that distance from the





previous band, was the last observed thirteen days from the beginning of the experiment. This production of equally spaced discs is worthy of note, since it has been argued that agates with equidistant bands could not have been due to diffusion into gels. Another similar experiment with 5 cc., respectively, of  $N/10$   $K_2CrO_4$  and  $N/5$   $Pb(NO_3)_2$  gave eight successive discs with clear spaces between, all approximately 1.15 mm. apart, followed by a further series 0.9 mm. apart.

## REFERENCES.

- Antonov (1907). *J. Chim. Phys.*, **5**, 372.  
Bechhold (1905). *Zeitsch. physikal. Chem.*, **52**, 185.  
Bradford (1917). *Biochem. J.*, **11**, 14.  
— (1918). *Biochem. J.*, **12**, 352.  
Freundlich (1907). *Zeitsch. physikal. Chem.*, **57**, 446.  
Freundlich and Schucht (1913). *Zeitsch. physikal. Chem.*, **85**, 660.  
Harrison (1911). *J. Soc. Dyers and Col.*, **27**, 279.  
Hatschek (1911, 1). *J. Soc. Chem. Ind.*, **30**, 255.  
— (1911, 2). *Kolloid Zeitsch.*, **8**, 193.  
— (1912). *Kolloid Zeitsch.*, **9**, 11.  
— (1914). *Kolloid Zeitsch.*, **14**, 115.  
— and Simon (1912). *Trans. Inst. Min. Met.*, **21**, 451.  
Lewis (1908). *Phil. Mag.* (vi), **15**, 499.  
Liesegang (1896). *Phot. Archiv.*, 321.  
— (1898). *Chemische Reaktionen in Gallerten*, Düsseldorf.  
— (1914). *Zeitsch. physikal. Chem.*, **88**, 1.  
Lupton (1892). *Nature*, **47**, 13.  
Morse and Pierce (1903). *Physical Review*, **17**, 129.  
Ostwald (1897). *Zeitsch. physikal. Chem.*, **27**, 365; *Lehrb. all. Chem.*, 2te. Auf. **2**, ii, 778, 780.  
— Wo. (1917). *Introduction to theoretical and applied Colloid Chemistry*, 107.  
Pelet and Grand (1907). *Kolloid Zeitsch.*, **2**, 41.  
Pringsheim (1895). *Jahrb. wiss. Bot.*, **28**, 1.  
Rayleigh (1919). *Phil. Mag.*, **38**, 738.  
Runge (1855). *Der Bildungstrieb der Stoffe*, Oranienburg.

## IV. ON THE ASSOCIATION OF ANTITOXINS WITH THE PROTEINS OF IMMUNISED HORSE SERUM.

BY ANNIE HOMER.

(Received December 12th, 1919.)

THE data compiled from results obtained in the routine concentration of antitoxic horse sera show that the average loss of antitoxin, due to its association with "salt-insoluble" globulin, is greater for antitetanic than for antidiphtheritic plasma or serum thus:

Method of concentration employed	Percentage loss of antitoxin experienced with	
	Antitetanic plasma	Antidiphtheritic plasma
Banzhaf-Gibson	22	17
Banzhaf (1913)	18.5	12.5
Homer (1916, 1918)	7.5	2.5

In each of the above processes the proteins of the plasma or serum, prior to their fractional precipitation by ammonium sulphate, are subjected to a heat-denaturation which not only increases their precipitability by sulphates but also leads to a conversion of "salt-soluble" protein into a "salt-insoluble" condition.

A comparatively low value for the extent of the denaturation renders the proteins of the euglobulin-pseudoglobulin zone "salt-insoluble"; the progressive increase in the denaturation causes a corresponding conversion of the fractions of the pseudoglobulin precipitated at successively increasing concentrations of ammonium sulphate and, finally, with the further increases in the extent of the denaturation the albumin fractions of the serum proteins are also rendered "salt-insoluble."

From these observations it is evident that, in the concentration of sera, provided there has been no destruction of antitoxin during the heating of the serum, the losses of antitoxin are regulated by the extent to which the heat-denaturation has converted the antitoxin-bearing proteins into a "salt-insoluble" condition.

My previous work has demonstrated that, in the techniques adopted in the above-mentioned routine methods, the heat-denaturation is sufficient to convert the proteins of the euglobulin-pseudoglobulin zone into a "salt-insoluble" condition. These conclusions, taken in conjunction with the inferences to be drawn from the data recorded above, indicate that the association of anti-

toxin with the unheated proteins of the euglobulin-pseudoglobulin zone is greater in antitetanic than in antidiphtheritic plasma.

In order to furnish further evidence on this point a detailed study has been made of the proportional precipitation of antitoxin and protein by the addition of progressively increasing amounts of sulphate to *antitetanic* and to *antidiphtheritic* plasmas respectively.

It was found that the labour entailed in the investigation was considerably shortened by the substitution of sodium sulphate for ammonium sulphate in the fractionation of the plasma. For, with the use of sodium sulphate in contradistinction to the more toxic ammonium sulphate, there was no necessity for the dialysis of the protein precipitates or of the filtered sulphated plasmas prior to the estimation of their antitoxin content.

The method of procedure was as follows: to separate volumes of unheated antitetanic and antidiphtheritic plasma was added solid anhydrous sodium sulphate in amounts ranging from 9 to 12 per cent., i.e. in amounts sufficient to precipitate the proteins of the euglobulin and of the euglobulin-pseudoglobulin zones. The stoppered bottles containing the sulphated plasmas were heated in a water-bath at 35° until complete solution of the sulphate had been effected. The liquids were then filtered, the respective precipitates being washed on the filters with an aqueous solution of sodium sulphate of the same concentration as that of the sulphated plasma thus filtered.

The antitoxic contents of the filtrates and of the precipitates, the latter being emulsified in a measured volume of water, were estimated in the usual way and, after making the necessary corrections for dilution, etc., data were obtained as regards the percentage precipitation of the respective antitoxins at each stage with the sulphate.

Recent work has shown that the precipitation of the serum proteins and of the antitoxin at a given concentration of ammonium sulphate or of sodium sulphate is somewhat greater from plasma containing cresylic acid than from non-cresylised plasma. I have therefore included in Table I the data obtained from the precipitation of cresylised and of non-cresylised samples of antidiphtheritic and antitetanic plasmas respectively.

Table I. *Showing the percentage precipitation of the antitoxins of antitetanic and of antidiphtheritic horse plasma by anhydrous sodium sulphate.*

* Percentage addition of sodium sulphate to the plasma	Percentage precipitation of antitoxin with the protein precipitated by the sulphate from			
	Non-cresylised plasma		Cresylised plasma	
	Antitetanic	Antidiphtheritic	Antitetanic	Antidiphtheritic
9	16	0	16	0
10	16	5	16	4
11	16	5	27	4
12	30	16	35	21

The study of the results given in the table show that both in cresylised and in non-cresylised plasma the precipitation of antitoxin with the proteins

of the euglobulin-pseudoglobulin zone is greater in antitetanic than in antidiphtheritic plasma.

A preliminary study has also been made of the proportional precipitation of the antitoxins of *antidysenteric* and of *antimeningitic* horse plasma with the various protein fractions, and data have been obtained with regard to the association of the respective antitoxins with the "salt-soluble" globulins from the unheated and from the heat-denatured plasmas.

(a) *The isolation of the "salt-soluble" globulins from the unheated plasmas.*

To a measured volume of plasma was added an equal volume of a saturated solution of ammonium sulphate. The sulphated plasmas were filtered and the precipitates, after having been well washed with a half-saturated solution of ammonium sulphate, drained and well pressed, were emulsified in a small volume of water which was subsequently diluted with a saturated solution of sodium chloride until the volume of the emulsion was approximately twice that of the plasma taken for precipitation. Solid salt was added to the liquid which, after complete saturation with the salt, was allowed to stand at room temperature for 48 hours before filtration. The precipitate, consisting of insoluble protein and salt, was well washed with a saturated solution of salt and to the filtrate and washings was added 0.25 per cent. of glacial acetic acid. The acidified liquid was filtered and the precipitate was pressed and dialysed in the usual way.

(b) *The isolation of the "salt-soluble" globulins from the heat-denatured plasmas.*

Measured volumes of the plasmas used in (a) were adjusted in the usual way so as to ensure, during the heating process, the conversion of the "salt-soluble" proteins of the euglobulin-pseudoglobulin zone into a "salt-insoluble" condition. The adjusted plasmas, contained in stoppered bottles, were heated in a water-bath at 58° for 5 hours. To each of the heated liquids was added an equal volume of a saturated solution of ammonium sulphate and the ensuing precipitate was then subjected to the same treatment as that described above for the corresponding precipitate from the unheated plasmas.

The comparison of the relative antitoxic contents of the original antidysenteric plasma and of the "salt-soluble" globulin fractions isolated therefrom, was made in terms of a laboratory standard toxin prepared by drying Shiga bacilli *in vacuo*. The determinations of the antitoxic values of the original antimeningitic plasma and of the "salt-soluble" globulins were carried out by the method advocated by Hitchens and Robinson [1916].

The values thus obtained for the titre of the respective liquids cannot be relied upon to the same extent as those furnished by the generally accepted methods for the testing of antitetanic and of antidiphtheritic sera. For this reason the data furnished in Table II are open to criticism. However, that a certain amount of reliance can be placed in these results is shown by the fact

that, as was anticipated from our previous knowledge of the heat-denaturation of antitoxic sera, the extent of the association of antitoxin with the "salt-soluble" globulins is considerably less in the heat-denaturated than in the unheated plasmas.

Table II. *Showing the percentage of the total antitoxins precipitated with the "salt-soluble" globulins of antidysenteric and antimeningitic horse sera.*

Plasma	Percentage of the total antitoxin associated with the "salt-soluble" globulins of the	
	Unheated plasmas	Heat-denaturated plasmas
Antidysenteric	50	30
Antimeningitic	30	10

The results depicted in the table are therefore sufficiently striking to lead us to the conclusion that, contrary to our experience with antitetanic and antidiphtheritic plasma, the bulk of the antitoxins in antidysenteric and antimeningitic plasma is associated with the proteins of the euglobulin and of the euglobulin-pseudoglobulin zones.

Attempts to furnish data in regard to the association of the antitoxins of antidysenteric and of antimeningitic sera with the proteins precipitated at successively increasing concentrations of sodium sulphate have been postponed until more exact methods of testing the titre of these sera are forthcoming.

The results of the present investigation are of interest as they show that the various antitoxins are adsorbed by different protein fractions of horse serum, a phenomenon which can be regarded as an indication of differences in the molecular composition of the respective antitoxins.

#### REFERENCE.

Hitchens and Robinson (1916). *J. Immunol.*, **1**, 345.

## V. THE GUANIDINE CONTENT OF FAECES IN IDIOPATHIC TETANY.

BY JOHN SMITH SHARPE.

*From the Department of Physiology, University of Glasgow.*

*(Received December 22nd, 1919.)*

SINCE the condition of tetany has sometimes been ascribed to gastro-intestinal disturbances, and since the work done in this Laboratory has associated it with guanidine intoxication [Noël Paton and Findlay, 1917], the possibility that it may be due to absorption of guanidine from the intestinal tract has to be considered. Against this possibility is the fact observed by Findlay that large doses of guanidine may be administered by the mouth to kittens without producing symptoms. On the other hand it is possible that guanidine may be partly excreted from the intestine. The work of Burns indicates that it is not all eliminated in the urine [Burns, 1917].

I have therefore made some examinations of the faeces of normal children and of those suffering from tetany. The material was procured from The Royal Hospital for Sick Children, and I am indebted to Dr Leonard Findlay.

### METHOD.

Entire specimens of daily excretion of faeces were collected from various normal and tetany cases and weighed. Aliquot parts were subjected to the following process for the extraction of bases, within an hour of being passed, to avoid any possible decomposition.

30 to 50 g. of the moist faeces were mixed thoroughly in a mortar with 100 cc. of water. A little chloroform and thymol were added and the whole placed in a dialyser with a parchment membrane. This was surrounded by 200 cc. of water, put in a cool place and dialysis continued for three days. The dialysate was then washed into a beaker, more water added to the first beaker and dialysis continued for a further two days. The two dialysates were united and evaporated down to a syrup. Ammonium, potassium and excess of sodium were taken out with chloroplatinic acid in the usual way, and the platinum removed by sulphuretted hydrogen. The sulphuretted hydrogen was then evaporated off and the bases precipitated in the filtrate with saturated alcoholic picric acid.

Almost immediate formation of crystals was observed. Small bunches of yellow needle-shaped crystals formed. These were very insoluble in water and gave long needle-shaped forms on recrystallisation from water.

The melting point of these crystals was 230° and the nitrogen content was 25 per cent. These figures agree closely with those for dimethylguanidine.

A loss of about 5 per cent. is incurred by this method, but by using the quantities above-mentioned this is constant and may be corrected for. This loss was determined by adding a weighed quantity of a guanidine salt to normal faeces and proceeding as above. The differences indicated in the tables below are far outside any loss in analysis. The process has the advantage in this case of easily eliminating all the suspended matter without the use of precipitants, which is very troublesome in faeces.

*Tetany faeces.* Age, from 1-2 years.

No.	Daily wt. of fresh faeces	Dimethylguanidine per cent.	Daily excretion of dimethylguanidine
1	25 g.	0.080	0.020 g.
2	30 „	0.074	0.022 „
3	22 „	0.070	0.015 „
4	20 „	0.078	0.016 „

*Normal faeces.* Age, from 1-2 years.

1	15 „	traces	—
2	12 „	0.028	0.003 „
3	13 „	0.018	0.002 „
4	16 „	minute traces	—
5	18 „	„ „	—
6	11 „	„ „	—

CONCLUSIONS.

The average percentage of this base in the four tetany cases examined was 0.075 per cent. of the moist faeces. In the six normals only faint traces were detected, averaging 0.007 per cent.

The average daily excretion of guanidine by the bowel in children suffering from tetany according to the present investigation is 0.018 g. as dimethylguanidine.

The work was done under The Medical Research Committee.

REFERENCES.

- Burns (1917). *Quart. J. Exp. Physiol.*, 10, 3 and 4, pt. 7.  
 Noël Paton and Findlay (1917). *Quart. J. Exp. Physiol.*, 10, 3 and 4, pt. 4.

# VI. ON THE COLOURS OF TWO SEA ANEMONES, *ACTINIA EQUINA* AND *ANEMONIA SULCATA*.

## PART I. ENVIRONMENTAL.

BY RICHARD ELMHIRST.

## PART II. CHEMICAL.

BY JOHN SMITH SHARPE.

*From the Marine Biological Station, Millport, and the Department of  
Physiology, University of Glasgow.*

(Received December 24th, 1919.)

(With Plates III, IV.)

MANY and varied are the theories that hold the field of colour phenomena in nature. This is to be expected, for although a large amount of work has been published on this subject, very little of it takes one further than the surface. There are the theories of Darwin, Poulton, Wallace, Eimer, Simroth, etc., which are all based on a hypothesis of structure in relation to natural selection or environment, the works being so well known that it is unnecessary for us to enter into any detail here. The aim of the present investigation is to endeavour to obtain an interpretation for certain colours.

### Part I.

#### ENVIRONMENTAL.

The commonest British Actinian is *Actinia equina* L., occurring on rocks and stones all around our coasts. Several colour varieties have been described and recorded in varying abundance from many localities. The species is abundant in the Clyde area: the prevailing form being the normal liver-red type, with bright blue "marginal spherules" and an equally blue border round the base, var. *hepatica* [Gosse, 1860, Pl. 6, fig. 2].

#### *Ideal Habitat.*

The ideal habitat for this species, as shown by the accompanying table, is about half-tide mark among the dense growths of *Ascophyllum* and *Fucus serratus*. The luxuriant growth of these weeds provides shelter and moisture during ebb and shade and protection from the force of the waves during flood. The presence of these weeds is, however, not essential because *Actinia* may occur abundantly on shelving rocks where they are replaced by *Chondrus* and *Gigartina*, short crisp weeds some three to four inches high.

In rock pools *Actinia* may occur at any tidal level up to and rarely above high water mark.

Vertical distribution of *Actinia*.

Sea-weed zone	Exposed upper surface of stones	Sheltered
<i>Pelvetia canaliculata</i>	—	Few
<i>Fucus platycarpus</i>	Rare	Few
<i>Fucus vesiculosus</i>	Few	Frequent
<i>Ascophyllum nodosum</i>	Common	Common
<i>Fucus serratus</i>	Common	Common
<i>Laminaria digitata</i>	Few	

*Colour.*

Within the limits of var. *hepatica* there are two shades of colour. (1) A deep red, occasionally tending almost to blackness, which occurs in exposed situations and (2) a paler bright red which occurs in shelter under sea-weed, below stones and in crevices. This agrees with Walton's observations on *Actinia equina* at Aberystwyth and in South Devon [1911, pp. 229 and 239]. Farquhar's on *Actinia tenebrosa* in New Zealand [1898] and Saville-Kent's on the coral *Euphyllia* [1893]. Of the other colour varieties described by Gosse, the brown var. *umbrina* forms less than 1 per cent. of all examined except in a few favoured localities where a pale var. *umbrina* seems to have become established. These varieties (*hepatica* and *umbrina*) merge through red browns so that it is often a matter of considerable difficulty to decide how to describe a particular specimen. Red browns and browns both vary in depth of colour according to the exposure of their surroundings. Two other varieties, the sea-weed coloured *olivacea* and red with green marks *fragacea*, are rare—less than 1 in 5000. Clyde var. *fragacea* are always small, 1 to 1.5 cm., apparently never attaining the inches of the Devon and Cornish specimens.

*Relationship of Colours.*

It is well known that the distinct colour varieties are connected by intermediate shades, which suggests that the pigments are chemically related to each other and probably derivatives from the same source. This is also borne out by several experimental observations of individuals changing colour, notably the fading of brilliant markings. Similar changes have been recorded for *Madrepora prostrata* [Saville-Kent, 1893].

In 1910 a normal *A. equina* was placed in a well-lit glass-sided aquarium. By the next year this specimen had passed from red through red-brown, brown-green to a deep olivaceous-green, i.e., had assumed the coloration of var. *olivacea* and attained a diameter of from 4 to 6 cm. This assumed coloration has persisted through a number of generations from 1911 to 1919. The young are usually pale greyish green about the shade of Gosse's var. *glauca*. A few, however, show a faint reddish tinge but in all cases the final colour has been that of var. *olivacea*.

Experiments are now in progress (1) to repeat the assumption of the green colour and (2) to reverse the process and get the var. *olivacea* to reassume the red of var. *hepatica*. With the former aim, three normal red var. *hepatica* were placed on May 10th under surroundings precisely similar to those in which the change *hepatica* to *olivacea* took place in 1910–11. Now, after five months, they have lost much of their original red and the tentacles are completely brown which, so far, is a repetition, though slower, of the 1910–11 process. With the latter aim a number of var. *olivacea* are being subjected to alternate dryness and submersion, while a second set are being kept in an artificial rock pool cut in the local sandstone. At present, after three months, no very definite results are noticeable but a suggestion of redness is appearing above the blue basal border in two cases.

#### *Inheritance of Colour.*

It is interesting to note that this assumed coloration has so far bred true. Gosse mentions [1860, pp. 178–9] that vars. *cerasum* and *chiococca* breed true. The same is suggested by the frequent occurrence on the shore of the young of any particular variety near an adult of that variety. At times a variety may establish itself in a suitable locality.

There is a rock-sheltered corner on the east shore of Cumbrae at the *Fucus serratus* level where *Metridium dianthus* was always found, both the red and white varieties (1906–15). On visiting this place in 1919 *Metridium* was found to be absent and in its place were a large number of pale brown *A. equina*, in colour between Gosse's vars. *umbrina* and *ochracea*. There were some 250 specimens of this variety which had established itself for a number of yards to the almost total exclusion of the normal red.

#### *Colour Significance.*

The significance of these colour forms has been ably discussed by Walton [1911] who classifies the coloration of actinians as

- (1) Warning;
- (2) Aggressive;
- (3) Protective;
- (4) Colours with some special physiological significance.

In *Actinia equina* the brilliant blue of the "marginal spherules" has been generally accepted as warning coloration. This may well be so, but unfortunately we have no observations bearing on the matter, although the fact that the normal colour often contrasts strongly with the environment may bear this interpretation. Because of this environmental colour contrast it is improbable that the aggressive coloration suggestion holds true although crustacea in aquaria have occasionally been seen to stumble onto an *A. equina*. That the normal colour is protective to any extent will be disproved by a single walk along the shore, despite the redness of the sandstone in several

localities. If the coloration were protective varieties should occur in definite association with environment, e.g. rock pools filled with *Cladophora*, backgrounds of sandstone, basalt or limestone, yet the normal red prevails in all these surroundings. In the *Cladophora* pools, particularly, dark reds which presented a marked contrast to their surroundings have been found occurring abundantly, 800 specimens without a single variety present. This brings us to colours with some special physiological significance, which interpretation is strongly suggested by (1) the variation in colour correlated with exposure already mentioned, and (2) the well-known fact that brightly coloured specimens when kept in aquaria and therefore continually submerged tend to lose their distinctive coloration. The obvious inference seems to be that the pigment is a light screen which has some physiological significance, possibly respiratory, since change of colour often takes place when specimens are kept continually submerged and not exposed to the alternate ebb and flow of the tide.

*Anemonia sulcata* (Pennant)=*Anthea cereus* (Ellis and Sol), occurs abundantly in suitable localities all round our southern and western coasts. In the Clyde, the normal "umber brown" is frequent on *Laminaria*, where it closely resembles the background of sea-weed: a case of aggressive coloration which obtains "much food in the form of deluded crustacea."

The ideal habitat of this species is a bed of *Laminaria digitata* sheltered from heavy waves and having a good exposure to sunlight. The first condition is favourable to the species because it is incapable of strong attachment and the second is essential for the life of the symbiotic algae inhabiting the tentacles. Excellent examples of this ideal habitat are seen in Castle Bay, Little Cumbrae, and between the Eilans in Millport Bay; the species being abundant in both places.

The need of direct strong sunlight is very well illustrated on the walls of the cambers in Gibraltar harbour, where on the walls running east and west, the north sides are practically devoid of *Anemonia* whereas the south sides are covered with thousands of fine specimens of the beautiful rose-tinted variety *smaragdina*.

#### SUMMARY.

1. The ideal habitat is at half-tide level.
2. Intensity of colour varies with exposure to light.
3. Individuals may show (environmental) colour change.
4. Individuals breed true.
5. A given variety may establish itself in suitable environment. In the present absence of evidence of the actual difference in environments it seems that one shade of colour may be as effective for the success of the species as another.
6. The coloration seems to have a special physiological function as a light screen.

**Part II.**

## CHEMICAL.

*Apparatus used.*

A Browning microspectroscope, with an inch objective and condenser, was used. The solutions of the colours were placed in small flat-bottomed glass tubes of about 10 cc. capacity. A compressorium was used for the examination of the fresh tissue which was cleared in glycerol. The positions of the various absorption bands and screens were determined by the Fraunhofer lines on a comparative solar spectrum.

*Actinia equina.*

1. *The ether-alcohol-soluble portion.* In the first part of the chemical work the interest was centred round substances of the nature of lipochromes. The fat solvents were all tried with varying success although the extracts were weak. In all cases the best extract of the ectodermic lining where the colouring matter was contained, was obtained by treatment with a mixture of three parts ether to one of alcohol. This gave in every case strong extractions of the red, brown-red or brown-yellow colours. On evaporation, oily drops were left corresponding in colour with the original coloured solution. These did not mix with water but lay on the surface as an oily layer. No haemoglobin derivatives could be demonstrated in this colouring matter; it is therefore not respiratory in function, at least as an oxygen carrier. Acids produce no change in the colour but on the addition of alkalis, NaOH for example, a brown colour resulted and the spectrum was changed. (See Plate III, A, spectrum 6.) This is important as it involves the possibility of acid or alkali playing a part in the colours of these animals, but whether this is produced by food or light is a question to be considered. There is one point that is significant in the absorption spectra of Plate III, A, that is the screening over the green and red portions of the spectrum.

According to Grotthus' law (1819) photo-chemical action cannot take place unless light is absorbed. Now Engelman has shown that red sea-weeds show the greatest carbon assimilation in the green and by the spectrum they show the greatest absorption. The chlorophyll in these red weeds is modified and acts as an optical sensitiser. This same effect is produced by light of various wave lengths, provided they are absorbed.

Again photo-chemical action seems not to depend on molecular construction, for with colouring matters ranging from red, yellow to blue, the molecules of which are not arranged in the same way, it has been shown that in every case where light is absorbed iodine can be set free by the released oxygen from potassium iodide, independent of absorption wave-length or position on the spectrum [Wager, 1914].

It will readily be seen that there is in these anemones a substance absorbing light in the green and red parts of the spectrum and generally resembling

chlorophyll in its action on light. This substance although not showing the distinctive bands of chlorophyll gives absorption wave-lengths in almost the same position as the maximum absorptions of chlorophyll.

Furthermore it was proved experimentally by Wager's method [1914] that these red and brown substances produce photo-chemical action with the release of active oxygen.

By the use of the fluoroscope a faint fluorescence is seen in the red almost over the lines *B. C.* This is in the position of maximal energy of the chlorophyll system as found by Engelmann and Timiriazev. According to Kerner red fluorescent substances have the power to transform the blue rays to red and ultra-red rays or in other words, rays of light into chemical rays. It is highly probable therefore, that such an action takes place in these anemones and as such may be concerned chiefly in their constructive metabolism.

This has also a bearing on the respiratory pigment which in these animals is very small in amount and must act more as a fixer than a carrier of oxygen.

Thus it will be seen that adequate conservation of the light is all important and that the colouring matter by virtue of the heavy screen towards the blue end affords protection against the harmful ultra-violet light.

In brown specimens a powerful screening of the blue and violet end is again secured by a brown substance soluble in ether-alcohol of the nature of a lipochrome. In spectrum 4, Plate III, A, it will be seen that even with a thin layer the screening towards the violet is very marked. The other parts of the screen behave in very much the same way as those of the red type.

In the lighter sheltered red and brown anemones there is only a variation in the intensity of colour but the photo-chemical nature remains the same.

2. *The respiratory pigment.* Portions of the ectoderm of a red and a brown anemone were cleared in glycerol and examined spectroscopically in a compress. Two bands were visible as indicated in Plate III, B, spectra 1 and 3. It will be noticed that in spectrum 3 the bands are a little further towards the blue end. MacMunn [1914] found a somewhat similar displacement in his brown specimens but he noted a band to the left of the *D* line. This could not be found with sufficient certainty to map. The presence of this band may vary with the state of oxidation of the respiratory pigment, but all our specimens were taken from fresh sea-water and used without delay. The demonstration of reduced alkaline haematin and also of haematoporphyrin is positive, Plate III, B, spectra 2, 4, 5, and in this we confirm MacMunn's findings.

The tests have to be carried out with great care as there is present an extremely small quantity of respiratory pigment. This was further proved by testing the oxygen content of the tissues, which gave spectroscopic evidence of haemoglobin derivatives, by the Barcroft [1914] method. Only 0.25 per cent. of oxygen by volume was given off by the ferricyanide from 5 g. fresh tissue as compared with mammalian blood which gives an average of from 17 to 18 per cent. evolved oxygen.

*Experiment with a young red Anemone.* A young red anemone was placed in fresh sea-water and screened from white light by various coloured glasses. When screened by either red or blue it moved into the yellow or any white light portion within a few hours. This experiment was repeated many times, the animal never failing to seek yellow or white light as rapidly as movement in these animals is possible. When the anemone was left altogether in white light it would remain in the same position for days.

The following are the absorptions of light by the coloured glasses used in this experiment:

*Red.* Nothing in the red, a strong dark band in the yellow and a heavy screen from the green to the end of the visible spectrum.

*Yellow.* A faint shading in the blue end.

*Blue.* A strong screen over the red and orange, a fainter screen from the mid-yellow to the end of the green and nothing in the blue.

Thus with these red and blue glasses the very light that the coloured ectoderm absorbs has been withheld from the anemone, making it necessary for the animal to change its position in order to obtain the rays of light most useful to it.

#### *Anemonia sulcata.*

1. *Ether-alcohol-soluble portion of ectoderm* (free from algae). A faint yellow colour was obtained giving no marked absorption spectrum, only a faint shading in the violet region.

2. *The respiratory pigment.* Alcoholic extracts of the tentacles and mesenteries of this anemone gave a greenish-yellow liquid which showed very characteristic absorption bands. The ether extract gave almost identical bands, the only difference being a change in the intensity of the bands on either side of the *C* line. In the alcohol solution the band to the left of the *C* line, as shown in Plate IV, C, spectrum 1, is very intense. Spectra 2 and 4 (Plate IV, C), the layers of which are only  $\frac{1}{20}$  the thickness of those of 1 and 3, still show this band very markedly. The positions of the bands in ether solution show a very close resemblance to those of  $\beta$ -chlorophyll of the green leaf. The bands on either side of the *C* line occupy the position of the maximum intensity of the chlorophyll system, while in the thin layer the band between *F* and *G* is in the position of the more refrangible band of carotin or xanthophyll. We confirm MacMunn in these findings and also that this chlorophyll-like substance comes from the symbiotic algae contained in the tentacles and mesenteries of this animal. (See Microphotograph, Plate IV, D.)

Further no haemoglobin derivatives could be demonstrated by us in the tissues and it seems conclusive that this chlorophyll is here as an optical sensitiser to supply active oxygen for the use of the anemone. An experiment which may support this is as follows. We screened an anemone from light in a glass vessel containing sea-water. It climbed up the side until it could obtain the maximum daylight available. Whether this is due to a vital

necessity of the anemone or to a stimulus produced by the heliotropic action of the algae on their own behalf are questions which would be difficult to answer. Again Geddes [1882] has shown that *Anemonia sulcata* when placed in bright sunlight evolved oxygen in quantity sufficient for determination by gas analysis apparatus.

#### PREVIOUS WORK.

Moseley [1887] during the "Challenger" expedition made a large series of spectroscopic examinations of the various invertebrate colours. From our present standpoint his work upon the pigments of the Coelenterata whether respiratory or otherwise is of more direct importance. He isolated from certain species of Actiniae a madder red colour. In fresh condition this colour yielded a well defined absorption spectrum. Three bands were visible, one in the green and the others toward the less refrangible end. For this pigment the name polyperthyrin was proposed. MacMunn [1914] found that this polyperthyrin was to all appearances identical with haematoporphyrin although he indicates there is only spectroscopic evidence for this similarity. Moseley's polyperthyrin is very stable even on exposure to light. It is soluble in moderately strong hydrochloric and sulphuric acids. It is insoluble in water, glycerol, alcohol and ether. The colour in strong concentration of the above acids gave a screen from the *D* line extending all the way towards the violet end. When this solution was further weakened two distinct bands appeared close to and on opposite sides of the *D* line. The shading towards the less refrangible end was also lessened. This colouring matter was precipitated on the addition of alkalis as a burnt sienna flocculent deposit which produced on redissolving in acids the three-banded spectrum of the fresh substance.

According to Moseley [1887] polyperthyrin has been found in eleven different species comprising seven genera of Coelenterata. McKendrick [1881] examined the pigments of *Cyanea* from aqueous infusions of the animal. Two bands were observed, one in the orange and the other in the red. This pigment has been termed cyanein.

In Griffiths' *Physiology of the Invertebrata* it is stated: "There is no doubt that the chief mode of respiration of Coelenterates is by means of the ectodermic lining."

MacMunn [1885] devoted much time to the examination of respiratory pigments in Actiniae. The solid portions of *A. equina* were first examined in a compressorium by the microspectroscope. A band was seen which was not unlike that of reduced haemoglobin, and two other bands were noted nearer the violet end. In brown specimens he noted that the prominent band was nearer the violet and also that in some there was a band close to the left side of the *D* line. This pigment has been named by MacMunn actiniohaematin. Actiniohaematin is insoluble in the fat solvents but dissolves in glycerol. It is extracted by treatment with alcoholic potash solution with a change in condition. The spectrum thus seen was very like that of alkaline haematin

which gives a band at *D*. When reduced with ammonium sulphide two bands were observed, being to all appearances the spectrum of haemochromogen or reduced alkaline haematin.

Again the spectrum of haematoporphyrin was obtained by digesting the ectoderm of an *Actinia* in sulphuric acid and filtering through asbestos. This was further proved by the addition of ammonia in excess forming the four-banded spectrum characteristic of alkaline haematoporphyrin.

Moseley [1873] isolated from a *Tealia crassicornis* a substance which he called actiniochrome. MacMunn has proved that Moseley's actiniochrome is not the same as actiniohaematin for the products of decomposition are entirely different; actiniochrome could not be converted to reduced alkaline haematin whereas actiniohaematin very easily could. MacMunn concludes therefore that actiniohaematin is respiratory in function and actiniochrome is ornamental.

Again MacMunn has found certain "yellow cells" (symbiotic algae) in the tentacles and mesenteries of *Bunodes ballii* which give the spectrum of a chlorophyll-like substance.

Actiniochrome was found in this species but haemochromogen could not be demonstrated. He remarks that: "The replacement of actiniohaematin by the colouring matter of these 'yellow cells' is of great interest for it appears that they replace the red pigment of other species."

In the genus *Sagartia* MacMunn found actiniohaematin in many species, also the chlorophyll-like substance (chlorofucin) in *Sagartia bellis*. The "yellow cells" which lie in the endodermal lining of the tentacles were responsible for this spectrum. Further he finds that these "yellow cells" suppressed the respiratory pigment. In *Anemonia* "yellow cells" were also found which yielded the chlorophyll-like substance.

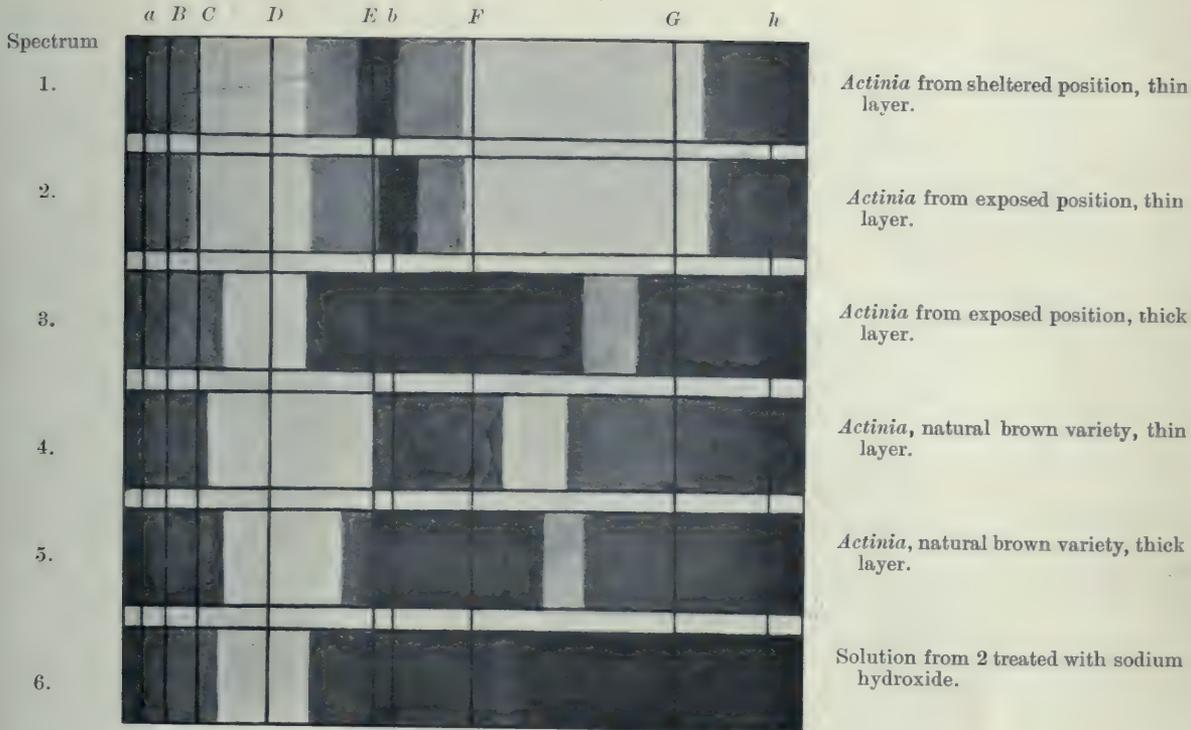
Geddes [1882] proposes the generic name of "philozoon" for the symbiotic algae or "yellow cells." In the same paper he gives a detailed account of chlorophyll-containing animals chiefly Coelenterates. He shows that there is a less amount of oxygen evolved by imprisoned algae, i.e. the symbiotic species, than by free living individuals. He concludes that the avidity for oxygen of the animal protoplasm accounts for this difference.

#### CONCLUSIONS.

1. *Actinia equina*. In view of the small quantity of respiratory pigment along with the alcohol-ether-soluble substances, it is concluded that the red and brown colours of these anemones are not ornamental as MacMunn and others hold, but that they act as optical sensitisers producing active oxygen for the use of the animal through the medium of the respiratory pigment. By virtue of the red screen in the position of the maximum absorption of chlorophyll they act as chemical sensitisers. Further there is reason to believe that these animals can make use of the blue and probably the violet rays.

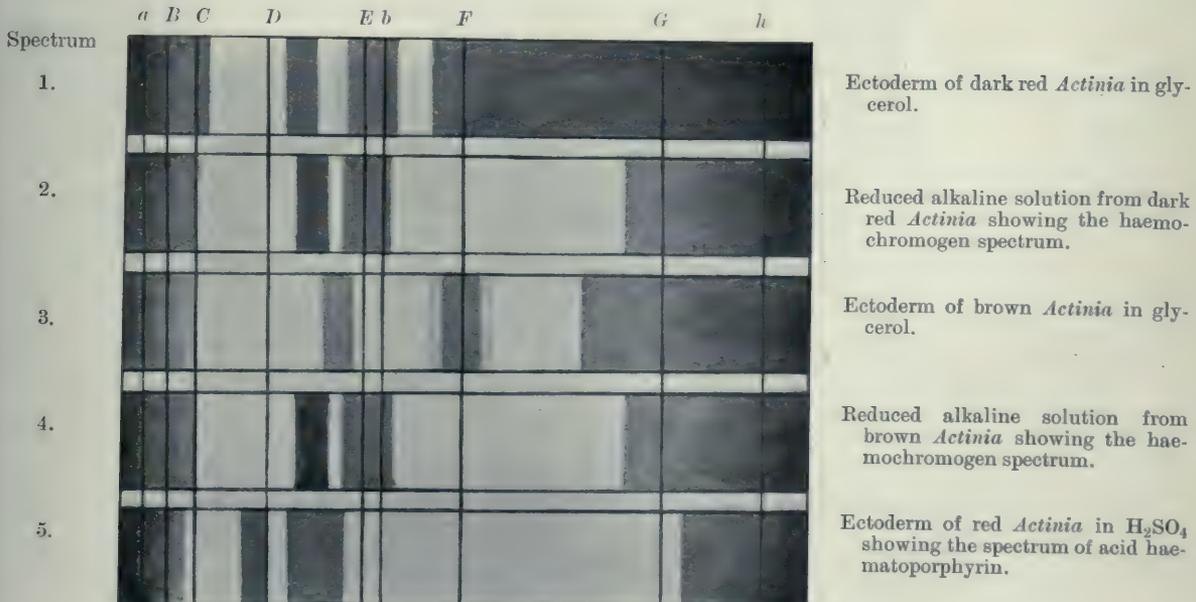
2. *Anemonia sulcata*. In *Anemonia* there is no haemoglobin derivative present, but the animal contains minute algae lining the inside of the tentacles

56



A. *Actinia equina*.

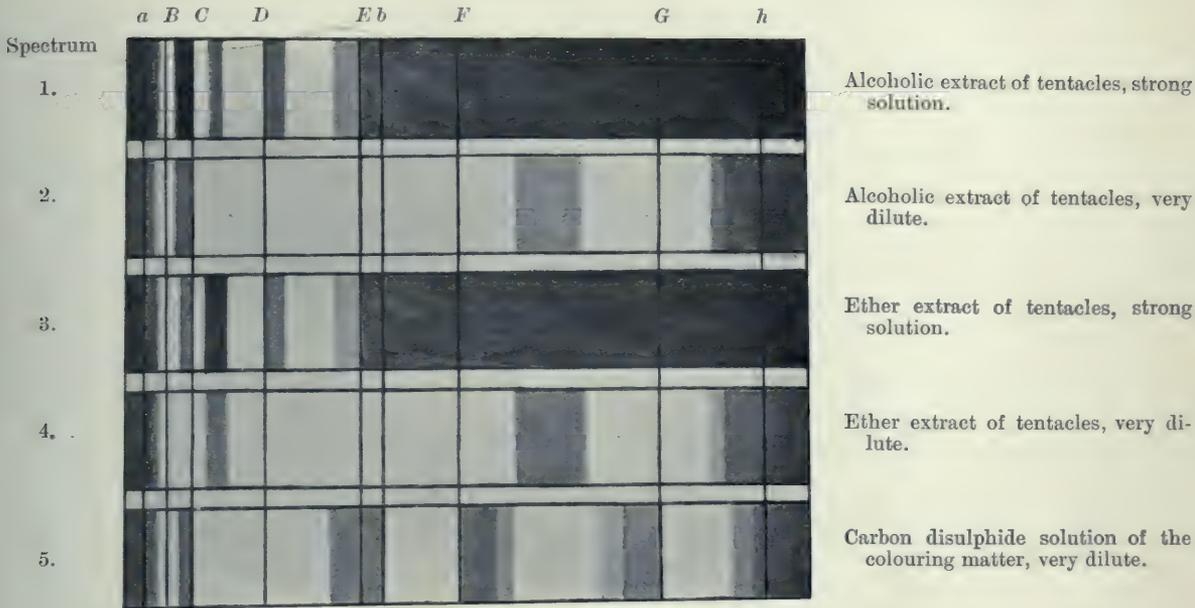
Absorption spectra of ether-alcohol soluble extract of ectoderm.



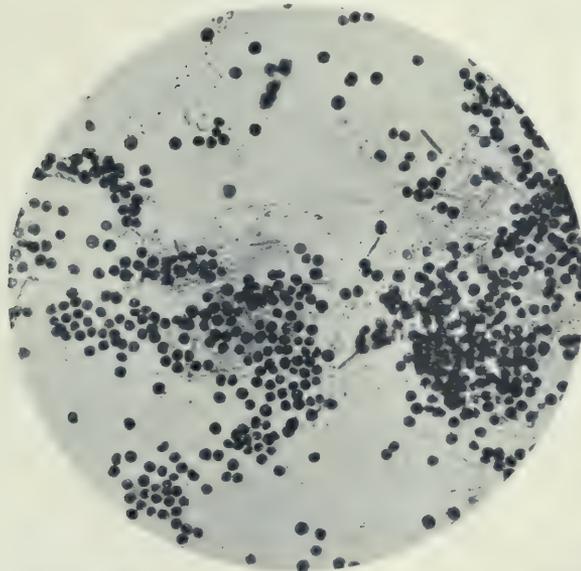
B. *Actinia equina*.

Spectra of respiratory pigment.





C. *Anemonia sulcata*.  
Absorption spectra of solutions.



D. Symbiotic algae from the tentacles and mesenteries of *Anemonia sulcata*. The rod-shaped objects are diatoms.  $\times 150$  diam.



and mesenteries, which contain a chlorophyll-like substance. By its close similarity to the chlorophyll of the green leaf, the action of light on this body seems to be much the same, namely of optical and chemical sensitisation. Active oxygen therefore would be released by the contained algae and used by the animal if need be.

We have to thank Professor D. Noël Paton for help and advice. The expenses were defrayed by the Medical Research Committee.

## REFERENCES.

- Barcroft (1914). *The Respiratory Function of the Blood*.  
Farquhar (1898). *J. Linn. Soc.*, **26**.  
Geddes (1882). *Proc. Roy. Soc. Edin.*, 377.  
Gosse (1860). *British Sea Anemones*.  
McKendrick (1881). *J. Anat. Physiol.*, **15**, 261.  
MacMunn (1885). *Phil. Trans.*, II. 641.  
—— (1914). *Spectrum Analysis applied to Biology and Medicine*, 100.  
Moseley (1873). *Quart. J. Microscop. Soc.*, N.S. **13**, 143.  
—— (1887). *Quart. J. Microscop. Soc.*, N.S. **17**, 1.  
Saville-Kent (1893). *The Great Barrier Reef*.  
Wager (1914). *Proc. Roy. Soc.*, B, **87**, 386.  
Walton (1911). *J. of Marine Biol. Assoc.*, N.S. **9**, 228.

## VII. THE PENETRATION OF ELECTROLYTES INTO GELS.

### I. THE PENETRATION OF SODIUM CHLORIDE INTO GELS OF AGAR-AGAR CONTAINING SILVER NITRATE.

BY WALTER STILES.

*From the Food Investigation Board, Department of Scientific and Industrial Research, and Institute of Physiology, University College, London.*

*(Received December 24th, 1919.)*

#### INTRODUCTION.

"THE manifestly colloidal nature of living protoplasm demonstrated *ad oculos* the significance of studies on colloids for Biology," says a distinguished continental plant physiologist [Czapek, 1911], and indeed the importance of investigations on sols and gels from the point of view of physiology needs no emphasis at the present time. An enquiry into the penetration of salts into gels may help towards the solution of problems of permeability in living and dead plant and animal tissues, and it is from this point of view that the experiments described in this and following papers were made.

A number of investigations on the penetration of electrolytes into gels have been made since the time of Graham. As well as the researches of Graham himself may be mentioned those of de Vries [1884], Coleman [1887, 1888], Chabry [1888], Voigtländer [1889], Durrant [1906], Bechhold and Ziegler [1906], Öholm [1913], Vanzetti [1914], and von Fürth and Bubanović [1918].

In the majority of the cases cited above the method employed to study the diffusion of substances into gels consisted in analysing different layers of the gel after penetration of salt had proceeded for a certain time. Working in this way Graham concluded that diffusion of crystalloids proceeds as rapidly in a gel as in water. Voigtländer investigated the diffusion of a number of chlorides in agar gels of different concentrations and came to the conclusion that the rate of diffusion was independent of the concentration of the gel. Öholm investigated the diffusion of potassium chloride from a solution of normal concentration into 2, 5 and 10 per cent. gelatin, the amount of potassium chloride being determined in four successive layers of the gelatin by titration with silver nitrate. Öholm found a progressive decrease in the rate of diffusion with increasing concentration of the gelatin and concludes that as a first approximation the presence of gelatinous substances affects the

diffusion of an electrolyte in the same way as a non-electrolyte in general, such as sugar or glycerol [cf. Öholm, 1912].

The method used by Coleman, Chabry, Durrant, Vanzetti and von Fürth and Bubanović is similar to that introduced by Lodge for comparing the mobilities of ions. The characteristic of this method is that the diffusion into the gel is followed by means of an indicator in the gel. Thus the penetration of an acid may be followed by means of litmus or other indicator; the diffusion of chlorides by means of silver nitrate. The chief researches by means of this method are those of von Fürth and Bubanović [1918], who have followed the penetration of a large number of salts, acids and alkalies into gels of 5 per cent. gelatin and principally 2 per cent. agar-agar. From their experimental results they draw a number of conclusions in regard to diffusion in gels and water and the various factors determining the rate of diffusion.

While von Fürth and Bubanović are quite right in emphasising the importance of these researches for physiology and physical chemistry, it would appear that they have not realised the complexity of the system with which they deal. As an example of this may be quoted their experiment 7, in which the diffusion of hydrochloric, sulphuric and oxalic acids, sodium chloride, and ammonium, potassium, magnesium and zinc sulphates was followed in gels of 2 per cent. agar containing silver nitrate and barium nitrate. From the rate at which the zone of precipitated silver chloride or barium sulphate moves onward conclusions are drawn as to the abnormal behaviour of sulphates compared with chlorides in regard to the diffusion of these substances in gelatin. Now this can only be the case if the silver and barium salts in the gel are without influence on the rate of diffusion of the salts through gelatin, as the concentration of the indicators in the gel is not mentioned and is evidently regarded as having no influence on the rate of diffusion. But from first principles it is not to be expected that this is the case, and the first step in obtaining data as to penetration into gels by the indicator method is obviously to determine how variation of the different factors in the case of one system influences the experimental results.

In this paper therefore the system consisting of an agar gel which is penetrated by sodium chloride is investigated, the various factors, concentration of sodium chloride in the external solution, concentration of gel and concentration of silver nitrate in the gel being varied and the effect of variation of these factors on the penetration of the chloride determined.

#### METHOD.

The gel was prepared by dissolving ordinary shred agar in distilled water so as to make a solution  $\frac{1}{3}$  times as concentrated as required. A solution of silver nitrate four times as strong as that required in the gel was then added to the warm agar solution the proportions by volume of agar and silver nitrate solution being 3 to 1. The mixture after well stirring was poured into test-tubes to a constant height (generally 6 or 7 cm.) and after the gel had

cooled and set a definite height of the sodium chloride solution was added (this was generally 6 or 7 cm.). The tubes were kept in racks in a dark cupboard in which the temperature was measured by means of a self-recorder. The temperature never varied more than 2° or 3° throughout an experiment.

As the chloride penetrates the gel the silver is precipitated as chloride, the limit of which is sharply marked from the gel below containing no precipitated silver chloride. The distance between the upper surface of the gel and this sharp line of demarcation is called by Voigtländer and by von Fürth and Bubanović the "Diffusionsweg": it will be called here the "penetration," and the movement forward of the silver chloride the "march of diffusion." The penetration of course increases with time, though as we shall see, not at a constant rate. The line of demarcation represents of course a layer of a definite concentration of chloride, namely the saturation concentration of silver chloride in the gel. The saturation concentration of silver chloride in water at 20° is 0.00015 g. of salt in 100 g. of water. It is thus the movement forward of a concentration of sodium chloride of this order of magnitude that is followed by the indicator method.

### EXPERIMENTAL RESULTS.

#### A. *Influence of concentration of sodium chloride.*

In a number of experiments with 2 per cent. agar gels containing some silver nitrate von Fürth and Bubanović have shown that the march of diffusion is more rapid the stronger the solution of salt external to the gel, provided the comparison is made with the same gel.

A large number of experiments have been made in the present research with gels of 0.5, 1, 2, 3 and 4 per cent. agar containing varying quantities of silver nitrate, and the observation of von Fürth and Bubanović as to the influence of concentration in affecting the march of diffusion has been confirmed.

From 30 series of experiments in which the concentration of sodium chloride was the only factor varied three examples will suffice to indicate the influence of concentration of the external salt. The results are shown in the following tables. One series of results is shown graphically in Fig. 1. The others are similar.

Table I. 0.5 per cent. agar gel containing 0.005N AgNO<sub>3</sub>. Each number is the mean of two determinations.

1.2N NaCl		N NaCl		0.5N NaCl		0.2N NaCl		0.1N NaCl		0.05N NaCl	
Time in hrs.	Penet. in mm.										
2.92	13.8	2.92	13.5	2.90	12.4	2.90	10.6	2.88	9.1	2.87	7.9
20.33	38	20.33	37.2	20.32	34.1	20.32	30	20.30	26.2	20.38	22
27.58	44.5	27.58	43.8	27.57	40	27.57	35	27.55	31	27.53	25.8
44.25	56	44.25	54.7	44.23	50.2	44.23	44.1	44.22	38.6	44.2	32.2
70.0	>70	70.0	>70	70.0	64.5	70.0	55.1	70.0	48.1	70.0	40.1

Table II. 2 per cent. agar containing 0.1N AgNO<sub>3</sub>.

5N NaCl		2N NaCl		N NaCl		0.5N NaCl		0.2N NaCl		0.1N NaCl	
Time in hrs	Penet. in mm.										
2.68	10.5	2.55	7.9	2.42	6.3	2.32	4.8	2.18	2.4	2.10	1.0
4.97	14.5	4.83	11.2	4.70	9.1	4.60	7.4	4.47	4.1	4.40	1.0
23.78	39.2	23.65	25.8	23.5	21.3	23.4	17.1	23.3	10.2	23.2	5.0
29.40	45	29.27	29	29.1	24.5	29.0	19.4	29.9	12.1	29.8	5.5
48.42	58.5	48.28	37.5	48.1	31.5	48.0	25	48.9	15	48.8	7
		71.33	45.5	71.2	38.2	71.0	30.5	70.9	18	70.9	8
		95.4	53.5	95.3	44.5	95.2	35	95.1	25	95	8.2

Table III. 4 per cent. agar containing 0.05N AgNO<sub>3</sub>.

5N NaCl		2N NaCl		N NaCl		0.5N NaCl		0.2N NaCl		0.1N NaCl	
Time in hrs	Penet. in mm.										
3.10	13.5	2.98	10	2.83	8.2	2.72	6.5	2.60	2	2.50	1.3
5.27	19.0	5.15	13.2	5.00	11.4	4.88	8.8	4.77	7	4.67	2.5
24.1	39	24.0	29.2	23.8	25	23.7	21	23.6	15.6	23.5	8.5
29.8	43	29.7	33	29.6	28.5	29.4	17.5	29.3	17.5	29.3	10
48.7	52.6	48.6	42.2	48.5	37	48.3	30	48.2	22.2	48.1	13
71.8	65	71.1	52	71.6	45	71.3	37	71.3	27	30.5	16.2
		95.6	64	95.5	53	95.4	42.5	95.2	30.5	95.2	18.4

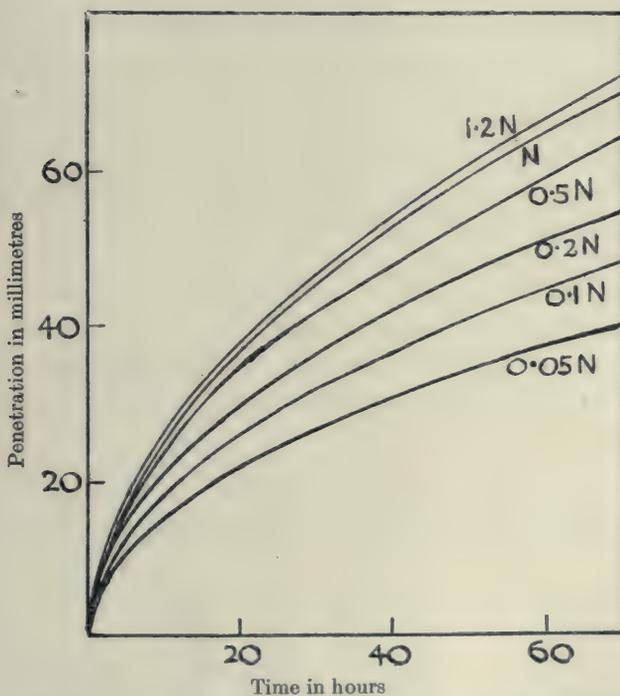


Fig. 1. Penetration of sodium chloride into 0.5 per cent. agar gels containing 0.005N silver nitrate. The initial concentrations of sodium chloride are indicated.

From the data obtained experimentally by von Fürth and Bubanović these authors conclude that the influence of concentration on the march of diffusion can be expressed by the simple relation

$$d = mt^{\frac{1}{2}}c^n,$$

where

$d$  is the distance penetrated,

$t$  ,, ,, time,

$c$  ,, ,, concentration,

and

$m$  and  $n$  are constants.

In their experiments with sodium chloride they obtained  $m = 41.96$  and  $n = 0.12$ ,  $t$  being measured in days,  $d$  in millimetres and  $c$  in normalities.

Put into words this equation states that the march of diffusion is proportional to the square root of the time and to some fractional power of the concentration. The results recorded in Tables I to III show that the march of diffusion is approximately proportional to the square root of the time. This conclusion has also been reached in regard to penetration of acids, alkalis and salts into gels by Coleman [1888], Chabry [1888] and Voigtländer [1889]. How near this approximation is may be seen from the following tables, which show that the march of diffusion is generally approximately proportional to the square root of the time in the case of any particular gel and any particular concentration of penetrating salt. At the beginning of the diffusion the ratio  $P/\sqrt{t}$  is constantly lower, and towards the bottom of a tube generally higher than the approximately constant value for the greater part of the experimental period. The greatest divergences appear when the concentration of the silver nitrate in the gel approaches that of the penetrating salt.

If now we return to von Fürth and Bubanović's equation  $P = mt^{\frac{1}{2}}c^n$ , where the distance penetrated is denoted by  $P$  and the initial concentration of the penetrating salt by  $c$ , we have

$$\frac{P}{\sqrt{t}} = mc^n,$$

or

$$\log \frac{P}{\sqrt{t}} = \log m + n \log c,$$

whence if  $\log P/\sqrt{t}$  is plotted against  $\log c$  a straight line should be obtained.

Table IV. 0.5 per cent. agar gel containing 0.005N  $\text{AgNO}_3$ . (For data see Table I.)  $P$  represents penetration in mm. and  $t$  the time in hours.

1.2N NaCl		N NaCl		0.5N NaCl		0.2N NaCl		0.1N NaCl		0.05N NaCl	
$P$	$\frac{P}{\sqrt{t}}$	$P$	$\frac{P}{\sqrt{t}}$	$P$	$\frac{P}{\sqrt{t}}$	$P$	$\frac{P}{\sqrt{t}}$	$P$	$\frac{P}{\sqrt{t}}$	$P$	$\frac{P}{\sqrt{t}}$
13.8	8.08	13.5	7.90	12.4	7.28	10.6	6.23	9.1	5.36	7.9	4.65
38	8.43	37.2	8.25	34.1	7.56	30	6.55	26.2	5.81	22	4.88
44.5	8.47	43.8	8.34	40	7.62	35	6.66	31	5.91	25.8	4.92
56	8.42	54.7	8.13	50.2	7.55	44.1	6.63	38.6	5.80	32.2	4.84
				64.5	7.71	55.1	6.59	48.1	5.75	40.1	4.79

Table V. 2 per cent. agar containing 0.1N AgNO<sub>3</sub>.  
(For data see Table II.)

5N NaCl		2N NaCl		N NaCl		0.5N NaCl		0.2N NaCl		0.1N NaCl	
<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$
10.5	6.41	7.9	4.94	6.3	4.05	4.8	3.15	2.4	1.63	1.0	0.690
14.5	6.51	11.2	5.09	9.1	4.20	7.4	3.45	4.1	1.94	5.0	0.825
39.2	8.04	25.8	5.31	21.3	4.39	17.1	3.54	10.2	2.06	5.5	1.008
45	8.30	29	5.36	24.5	4.54	19.4	3.68	12.1	2.21	7	1.002
58.5	7.65	37.5	5.40	31.5	4.54	25	3.61	15	2.14	8	0.950
		45.5	5.39	38.2	4.53	30.5	3.62	18	2.14	8.2	0.840
		53.5	5.48	44.5	4.51	35	3.59	25	2.56		

Table VI. 4 per cent. agar containing 0.05N AgNO<sub>3</sub>.  
(For data see Table III.)

5N NaCl		2N NaCl		N NaCl		0.5N NaCl		0.2N NaCl		0.1N NaCl	
<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$	<i>P</i>	$\frac{P}{\sqrt{t}}$
13.5	7.67	10	5.79	8.2	4.87	6.5	3.94	2	1.24	1.3	0.821
19.0	8.27	13.2	5.81	11.4	5.09	8.8	3.98	7	3.20	2.5	1.16
39	7.94	29.2	5.96	2.5	5.12	21	4.31	15.6	3.21	8.5	1.75
43	7.88	33	6.05	28.5	5.24	23.5	4.33	17.5	3.23	10	1.85
52.6	7.54	42.2	6.05	37	5.31	30	4.32	22.2	3.20	16.2	1.92
		64	6.54	53	5.42	42.5	4.35	30.5	3.13	18.4	1.89

In Table VII are given the average values of  $P/\sqrt{t}$  and the corresponding values of *c* in normalities, and Fig. 2 shows the curves obtained when values of  $\log P/\sqrt{t}$  are taken as ordinates and values of  $\log c$  as abscissae. It will be observed that they are certainly not straight lines, and accordingly von Fürth and Bubanović's equation does not hold. A reference to von Fürth and Bubanović's results discloses that these workers only used a very restricted range of concentrations of the penetrating solutions namely from *N* to *N*/4. This corresponds in the curves in Fig. 2 to the portions between the vertical lines *A* and *B*. Even within this very restricted range the curves are only very approximate straight lines when the concentration of silver nitrate in a gel is low. The statement of von Fürth and Bubanović that their law is of wide applicability certainly cannot be accepted.

Table VII.

0.5 per cent. agar containing 0.005N AgNO <sub>3</sub>		2 per cent. agar containing 0.1N AgNO <sub>3</sub>		4 per cent. agar containing 0.05N AgNO <sub>3</sub>	
$\frac{P}{\sqrt{t}}$	<i>c</i>	$\frac{P}{\sqrt{t}}$	<i>c</i>	$\frac{P}{\sqrt{t}}$	<i>c</i>
8.35	1.5	7.38	5	7.83	5
8.15	1	5.28	2	6.05	2
7.54	0.5	4.39	1	5.20	1
6.55	0.2	3.52	0.5	4.23	0.5
5.73	0.1	2.10	0.2	2.92	0.2
4.82	0.05	0.886	0.1	1.61	0.1

The results of a large number of experiments are similar to those recorded above. They indicate that the law connecting the march of diffusion with the square root of the time can be extended with a certain degree of approximation and within certain limits to the case of penetration of an electrolyte into a gel containing a substance with which the penetrating substance reacts. Von Fürth and Bubanović's further extension of this law to include the concentration of the penetrating electrolyte is not of the wide applicability claimed for it, and only holds over very limited ranges of concentration and is then only a crude approximation.

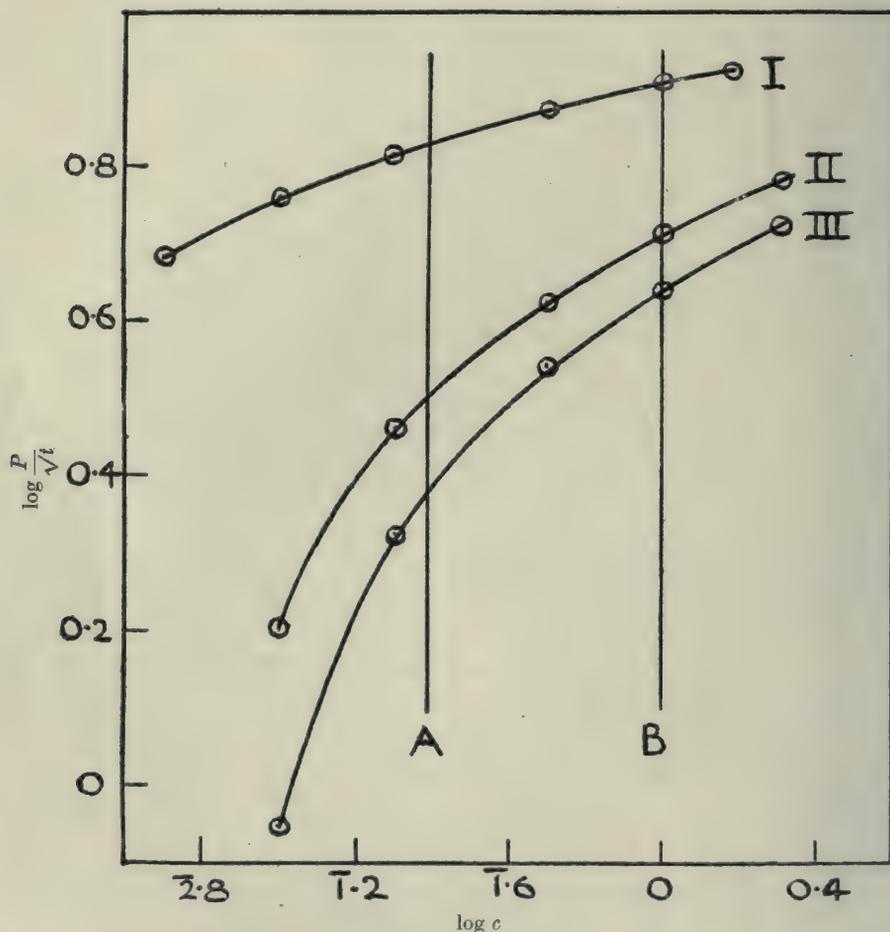


Fig. 2. Curves showing the relation between  $\log P/\sqrt{t}$  and  $\log c$  in the case of three agar-agar gels. I: 0.5 per cent. agar containing 0.005N  $\text{AgNO}_3$ ; II: 2 per cent. agar containing 0.1N  $\text{AgNO}_3$ ; III: 4 per cent. agar containing 0.05N  $\text{AgNO}_3$ . The vertical lines A and B indicate the limits of external concentrations employed in the experiments of von Fürth and Bubanović.

B. *Influence of concentration of silver nitrate in the gel.*

Although it is tacitly assumed by von Fürth and Bubanović that the concentration of silver nitrate in the gel is without influence on the march of diffusion, this is far from being the case. Tables VIII—X show some results representative of a great number which show clearly that increasing the concentration of silver nitrate in the gel reduces the rate at which the sodium chloride penetrates it. The results given in Tables VIII and X are also shown graphically in Fig. 3.

Table VIII. Penetration of sodium chloride of initially normal concentration into gels of 1 per cent. agar containing various concentrations of silver nitrate.

N AgNO <sub>3</sub>		0.5N AgNO <sub>3</sub>		0.2N AgNO <sub>3</sub>		0.1N AgNO <sub>3</sub>		0.05N AgNO <sub>3</sub>	
Time Penet. in hrs in mm.									
None	2.17	3	2.22	5	2.25	6	2.28	6.5	
„	4.55	5.1	4.58	7.6	4.60	9.4	4.62	10	
„	23.32	12.9	23.4	18.5	23.4	22	23.4	23.8	
„	28.90	14	29.0	21	29.0	25	29.0	27	
„	47.93	18.3	48.0	27	48.0	32	48.0	34.5	
„	70.9	21.5	70.9	32.5	70.9	39.5	70.9	42	
„	95.4	25	95.4	37	95.4	45.4	95.4	50	

Similar results have been obtained with these concentrations of silver nitrate in the case of gels of 1, 2, 3 and 4 per cent. agar and of concentrations of sodium chloride ranging from 5N to 0.1N. But the same results are obtained when the range of silver nitrate concentrations is extended in the lower direction to the limiting concentration which is necessary for the silver chloride to be precipitated, as the following tables show.

Table IX. Penetration of 0.5N sodium chloride into gels of 0.5 per cent. agar containing various concentrations of silver nitrate.

0.05N AgNO <sub>3</sub>		0.025N AgNO <sub>3</sub>		0.005N AgNO <sub>3</sub>	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
3.75	10.0	3.75	12	3.75	15
23.0	26.4	23.0	30	23.0	39
28.8	30.0	28.8	34	28.8	43.5
46.9	38.0	46.9	43	46.9	60
71.2	47.1	71.2	54		

Table X. Penetration of sodium chloride of initially normal concentration into 0.5 per cent. gels of agar containing various concentrations of silver nitrate.

0.005N AgNO <sub>3</sub>		0.001N AgNO <sub>3</sub>		0.0005N AgNO <sub>3</sub>		0.0001N AgNO <sub>3</sub>	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
2.92	13.5	2.75	16.0	2.70	16.6	2.65	18.6
20.3	37.2	20.2	44.2	20.07	47.0		
27.6	43.8	27.45	51.8	27.32	55		
44.25	54.7	44.1	66.0	44.1	75		
70	70	69.8	70				

Precipitation of silver chloride not obvious after this time

These results show that in the lowest concentrations usable the concentration of silver nitrate in the gel influences the march of diffusion of sodium chloride through the gel. Thus von Fürth and Bubanović's comparison of the diffusion of chlorides and sulphates into gels containing unknown quantities of silver nitrate and barium nitrate as indicators is not legitimate.

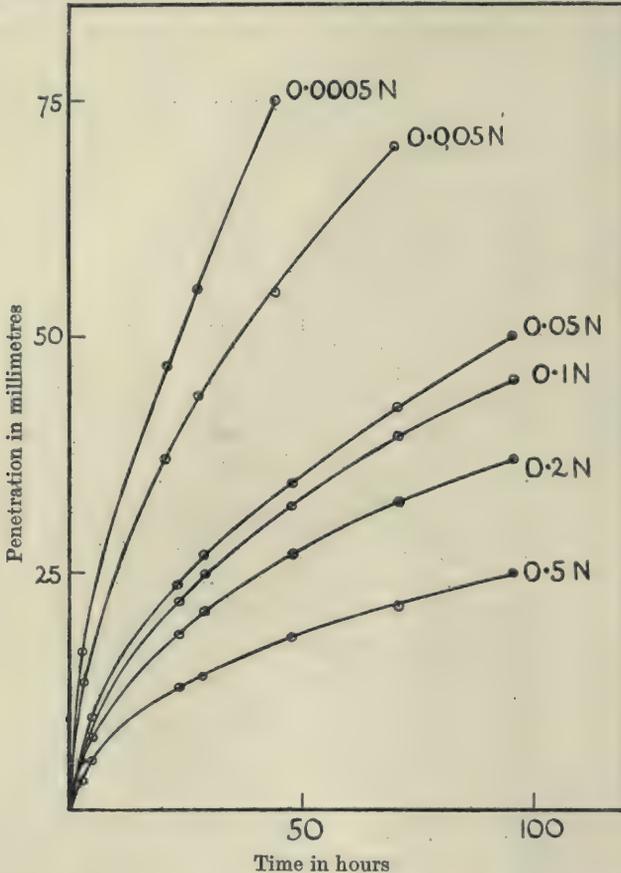


Fig. 3. Penetration of sodium chloride of initially normal concentration into gels containing different concentrations of silver nitrate as indicated.

### C. Influence of the concentration of the gel.

No conclusive evidence could be obtained by the indicator method of any influence of the concentration of the gel on the march of diffusion. The results shown in Table XI are representative of a large number of series of experiments.

In this particular series the march of diffusion was slightly more rapid in the 0.5 per cent. agar than in more concentrated gels. In other series on the contrary it was slightly less rapid in the 0.5 per cent. gel than in others, while in yet other cases the penetration was quicker in gels of intermediate concentration than in gels of either the weakest or strongest concentrations. The

conditions are perhaps complicated by the action between the gel itself and the silver nitrate. If for instance a 2 per cent. agar gel combines with more silver nitrate than a 1 per cent. gel containing the same amount of silver nitrate, the concentration of silver salt able to combine with the chlorion of the sodium chloride may be less, and as has already been shown in the previous section of this paper the concentration of silver nitrate influences the rate of penetration of the sodium chloride into the gel. Loeb [1918] has recently brought forward evidence of the combination of silver nitrate with gelatin, and the colour change which sometimes takes place when silver nitrate is added to agar gels suggests that chemical compounds or adsorption compounds are produced. The method of preparing the gel probably influences the action between the agar and silver nitrate.

Table XI. Penetration of sodium chloride of initial concentration 2*N* into agar gels of various concentrations containing 0.05*N* AgNO<sub>3</sub>.

0.5 per cent. agar		1 per cent. agar		2 per cent. agar		3 per cent. agar		4 per cent. agar	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
2.25	10	2.25	9.5	2.28	9.5	2.28	9.0	2.32	9.5
4.35	14	4.35	13.2	4.38	13.5	4.38	13	4.42	13
22.8	34.2	22.8	31	22.8	30.5	22.8	30.2	22.8	31.5
28.4	38.2	28.4	35	28.6	35	28.6	34	28.6	34
46.8	51	46.8	45	46.8	45	46.9	44	46.9	44.2

Under these circumstances the indicator method cannot be expected to yield very exact information as to the influence of the concentration of the gel on the rate of penetration of sodium chloride into the gel. This is a question which has already been the subject of a number of researches to which attention has been directed in the introduction to this paper. The divergent conclusions of Voigtländer working with chlorides diffusing in agar gels and Öholm on the diffusion of potassium chloride into gelatin are sufficient evidence of the unsettled state of this question.

D. *The influence of the concentration of silver nitrate in the gel considered in relation to the effect of concentration of the penetrating salt on the march of diffusion.*

In part A of the present section of this paper it has been shown that the rate of penetration of the gel by sodium chloride depends on the concentration of the penetrating salt, and in part B it is shown that it is also dependent on the concentration of silver nitrate. It is of importance to consider these two influences in conjunction, that is, how far the differences produced by varying the concentration of sodium chloride are affected by the concentration of silver nitrate in the gel.

In comparing the penetrating power of sodium chloride under different conditions it may be recalled that the penetration (*P*) is approximately proportional to the square root of the time (*t*) so that  $P/\sqrt{t}$  is a constant for the

penetration of sodium chloride into any particular gel. It may be called the penetration factor. In the following tables are shown the values of  $P/\sqrt{t}$  for a number of agar gels containing quantities of silver nitrate varying from  $N$  to  $0.05N$ , with external solutions of sodium chloride of initial concentrations varying from  $5N$  to  $0.1N$ .

Table XII. Values of  $P/\sqrt{t}$  for different concentrations of sodium chloride and silver nitrate.

1 per cent. agar.						
	$2N$ NaCl	$N$ NaCl	$0.5N$ NaCl	$0.2N$ NaCl	$0.1N$ NaCl	
$N$ AgNO <sub>3</sub>	—	—	—	-2.54	-3.75	
0.5 $N$ "	4.802	2.64	1.44	-1.161	-2.64	
0.2 $N$ "	5.16	4.00	2.68	1.44	0.235	
0.1 $N$ "	5.58	4.65	3.56	2.56	1.37	
0.05 $N$ "	6.64	4.97	4.21	2.93	1.93	
2 per cent. agar.						
	$5N$ NaCl	$2N$ NaCl	$N$ NaCl	$0.5N$ NaCl	$0.2N$ NaCl	$0.1N$ NaCl
$N$ AgNO <sub>3</sub>	3.825	—	—	—	-2.37	-4.06
0.5 $N$ "	—	3.416	2.38	1.40	-1.327	-2.56
0.2 $N$ "	—	4.50	3.59	2.65	1.35	0.00
0.1 $N$ "	—	5.35	4.49	3.58	2.16	1.02
0.05 $N$ "	7.08	6.07	5.40	4.49	3.24	2.13
3 per cent. agar.						
	$5N$ NaCl	$2N$ NaCl	$N$ NaCl	$0.5N$ NaCl	$0.2N$ NaCl	$0.1N$ NaCl
$N$ AgNO <sub>3</sub>	4.14	2.46	1.04	0	-2.44	-3.92
0.5 $N$ "	5.16	3.71	2.47	1.51	-0.814	-2.59
0.2 $N$ "	—	4.39	3.69	2.63	1.09	-1.00
0.1 $N$ "	6.87	5.43	4.39	3.52	2.17	0.816
0.05 $N$ "	—	6.05	5.28	4.40	3.23	1.68
4 per cent. agar.						
	$5N$ NaCl	$2N$ NaCl	$N$ NaCl	$0.5N$ NaCl	$0.2N$ NaCl	$0.1N$ NaCl
$N$ AgNO <sub>3</sub>	5.15	2.85	1.34	—	-2.09	-3.44
0.5 $N$ "	6.91	3.70	2.54	1.565	-1.23	-2.64
0.2 $N$ "	6.75	4.56	3.70	2.825	1.395	-0.982
0.1 $N$ "	7.50	5.54	4.93	3.875	2.45	0.663
0.05 $N$ "	7.78	5.98	5.23	4.32	3.21	1.83

An inspection of the numbers in this table indicates that there is no suggestion of differences among them due to variation in the concentration of the gel, and differences in the corresponding numbers in the different tables are in strong probability best described as due to experimental error. The fairest way of considering them is to take the mean values of the corresponding numbers in the four sets of results. These mean values are shown in Table XIII.

In Table XIV are shown the values of the penetration factor obtained in two other series of experiments in which the concentration of silver nitrate

in the gel varied in one case between 0.05*N* and 0.005*N* and in the other between 0.005*N* and 0.0005*N*.

Table XIII. Values of  $P/\sqrt{t}$  for different concentrations of sodium chloride and silver nitrate.

Mean values of the four sets of determinations recorded above.

	5 <i>N</i> NaCl	2 <i>N</i> NaCl	<i>N</i> NaCl	0.5 <i>N</i> NaCl	0.2 <i>N</i> NaCl	0.1 <i>N</i> NaCl
<i>N</i> AgNO <sub>3</sub>	4.37	2.65	1.19	0	-2.36	-3.79
0.5 <i>N</i> "	5.53	3.91	2.51	1.48	-1.13	-0.261
0.2 <i>N</i> "	6.75	4.65	3.745	2.70	1.32	-0.437
0.1 <i>N</i> "	7.18	5.475	4.615	3.63	2.335	0.967
0.05 <i>N</i> "	7.43	6.185	5.22	4.355	3.15	1.89

Table XIV. Values of  $P/\sqrt{t}$  for different concentrations of sodium chloride and silver nitrate.

(a) 0.5 per cent. agar.

	1.2 <i>N</i> NaCl	0.5 <i>N</i> NaCl
0.05 <i>N</i> AgNO <sub>3</sub>	6.08	5.56
0.025 <i>N</i> "	6.96	6.29
0.005 <i>N</i> "	8.44	8.12

(b) 0.5 per cent. agar.

	1.2 <i>N</i> NaCl	<i>N</i> NaCl	0.2 <i>N</i> NaCl	0.1 <i>N</i> NaCl	0.05 <i>N</i> NaCl
0.005 <i>N</i> AgNO <sub>3</sub>	8.44	8.27	6.63	5.82	4.86
0.001 <i>N</i> "	10.02	9.88	8.41	7.72	6.94
0.0005 <i>N</i> "	10.71	9.96	9.17	8.56	7.84

From an examination of these tables certain facts are made clear. In the first place it is evident that the rate of penetration depends very much on the ratio of the concentrations of the penetrating sodium chloride and the silver nitrate in the gel. When the concentration of the latter is higher than that of the former the sodium chloride does not penetrate into the gel, but the silver nitrate on the contrary diffuses out into the sodium chloride solution, and if the experimental tubes remain unshaken, an advancing boundary of silver chloride is seen in contact with the pure sodium chloride solution. This is indicated in the tables by the minus sign. This being the case it might be expected that when the initial concentration of the penetrating liquid is only slightly higher than the concentration of silver nitrate in the gel penetration would only take place to a slight extent, for when withdrawal of sodium chloride by the silver nitrate has taken place to such an extent that its average concentration is reduced to that of silver nitrate in the unaltered part of the gel, no further penetration is possible. This is actually found to be the case. It is perfectly clear that such cases as these are not allowed for in the equation of von Fürth and Bubanović. It is also evident that with decrease of the silver nitrate content of the gel the relative difference in the penetration factor due to differences in concentration of the invading salt rapidly decreases. Thus with 0.1*N* silver nitrate the ratio of the penetration factors of 0.2*N* and

0.1N sodium chloride is 2.335 : 0.967. With 0.0005N silver nitrate this becomes 9.17 : 8.56. Earlier in this paper it has been pointed out that von Fürth and Bubanović's equation connecting time, distance, penetration and initial concentration of the penetrating salt cannot be accepted.

An approximate relation between the concentration, penetration and time becomes clear however on inspection of Fig. 4. Here the data given in Tables XIII and XIV (b) are represented graphically, the values of  $P/\sqrt{t}$  being plotted against the logarithms of the concentrations. It will be observed that the relation between  $P/\sqrt{t}$  and  $\log c$  is approximately linear in the case

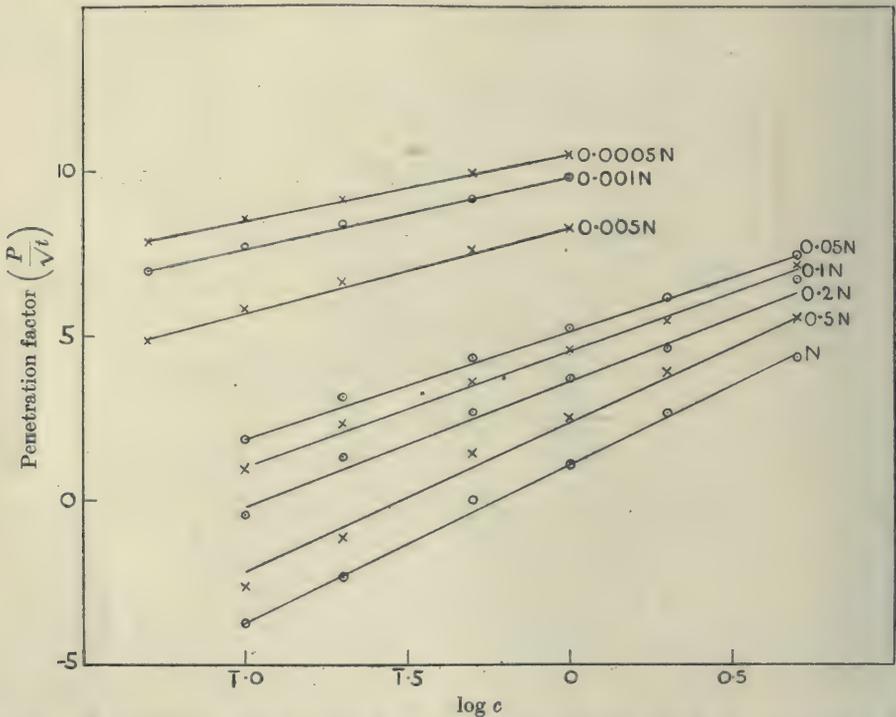


Fig. 4. The relation between the penetration factor ( $P/\sqrt{t}$ ) and the initial concentration ( $c$ ) of the penetrating salt in the case of a number of agar gels containing various concentrations of silver nitrate as indicated on the diagram.

of all the gels examined, these containing quantities of silver nitrate varying from  $N$  to  $0.0005N$  and the initial concentrations of sodium chloride varying from  $5N$  to  $0.05N$ . This approximately linear relation between  $P/\sqrt{t}$  and  $\log c$  is thus of wide applicability. It must however only be regarded as a working approximation.

The equation connecting the penetration (that is the distance a definite concentration of sodium chloride has reached), time, and initial concentration of salt may therefore be written in the form

$$\frac{P}{\sqrt{t}} = k \log c + k',$$

where  $k$  and  $k'$  are constants for any particular gel, in which is involved the factor depending on the concentration of silver nitrate in the gel. It is almost unnecessary to point out that although the penetration factor is dependent on the initial concentration of the penetrating salt this has no bearing on the question whether the coefficient of diffusion is influenced by concentration. For the penetration means no more than the distance between the starting-point of diffusion and a layer of a certain definite, if low, concentration. If the coefficient of diffusion is uninfluenced by concentration, the penetration will obviously be greater in the case of an initially high concentration of penetrating salt than in one of low concentration.

#### SUMMARY.

1. The penetration of sodium chloride into agar gels has been followed by the indicator method. When silver nitrate is present in the gel the entrance of sodium chloride into the gel is marked by the formation of silver chloride, and the progress of the chloride into the gel is indicated by the movement forward of the sharp line of demarcation between the silver chloride in the gel and the unprecipitated silver salt. The distance this line of demarcation has moved forward in any time is called the penetration. It marks the position of a definite concentration of chloride, namely, the saturation concentration of silver chloride.

2. The penetration of sodium chloride into agar gels containing silver nitrate is within wide limits proportional to the square root of the time. If  $P$  is the penetration in a time  $t$ , the value  $P/\sqrt{t}$  is thus a constant for any particular gel and any particular concentration of penetrating salt, and may be called the *penetration factor*.

3. The rate of penetration is dependent upon the initial concentration of the penetrating salt, the higher the concentration of salt the more rapid the penetration.

4. The rate of penetration depends also on the concentration of the silver nitrate in the gel; the higher the concentration of silver nitrate, the lower the rate of penetration.

5. The concentration of the gel itself appears to exercise little influence on the rate of penetration, but owing to the probability of actions between the silver salt and the gel and hence doubt as to the real active concentration of silver salt in the gel, which, as stated in the preceding paragraph, influences the rate of penetration, the influence of concentration of the gel must be left an open question. In any case the influence cannot be great in gels containing between 1 and 4 per cent. agar.

6. The expression obtained by von Fürth and Bubanović to indicate the relation between the penetration, time, and concentration of penetrating salt only holds in very special cases and between very narrow limits, and is then only a crude approximation.

7. An empirical relation between these quantities of more general application is

$$\frac{P}{t^{\frac{1}{2}}} = k \log c + k',$$

where  $P$  is the penetration,  $t$  the time and  $c$  the initial concentration of the penetrating salt. The value of the constant  $k$  depends principally on the nature of the penetrating salt, and also, although to a slight extent, on the nature of the gel contents, while the value of  $k'$  depends principally on the concentration of silver nitrate in the gel.

#### REFERENCES.

- Bechhold and Ziegler (1906). *Ann. Phys.* (4), **20**, 900; *Zeitsch. physikal. Chem.*, **56**, 105.  
 Chabry (1888). *J. Phys.* (2), **7**, 114.  
 Coleman (1887). *Proc. Roy. Soc. Edinburgh*, **14**, 374.  
 — (1888). *Proc. Roy. Soc. Edinburgh*, **15**, 249.  
 Czapek (1911). *Chemical Phenomena in Life*, London.  
 Durrant (1906). *Proc. Roy. Soc. A*, **78**, 342.  
 v. Fürth and Bubanović (1918). *Biochem. Zeitsch.*, **90**, 265; **92**, 139.  
 Loeb (1918). *J. Gen. Physiol.*, **1**, 237.  
 Öholm (1912). *Meddel. Vetenskapsakad. Nobelinstitut*, **2**, No. 22.  
 — (1913). *Meddel. Vetenskapsakad. Nobelinstitut*, **2**, No. 30.  
 Vanzetti (1914). *Zeitsch. Elektrochem.*, **20**, 570.  
 Voigtländer (1889). *Zeitsch. physikal. Chem.*, **3**, 316.  
 de Vries (1884). *Jahresb. Chem.*, **1**, 144.

## VIII. NOTE ON THE USE OF BUTYL ALCOHOL AS A SOLVENT FOR ANTHOCYANINS.

BY OTTO ROSENHEIM.

*From the Physiological Laboratory, King's College, London.*

*(Received January 13th, 1920.)*

IN the purification and preparation of anthocyanins from flowers or fruits, there is need for a solvent, not miscible with water, which will extract the pigments from their solution in dilute acids. Amyl alcohol, the only solvent available so far, has the disadvantage that only mono- and rhamnoglucosides are soluble in it to any extent and even in their case the solubility is so small that as many as twenty extractions have been found necessary. The large bulk of solvent required to work up even small quantities of the colouring matter makes its use impracticable for the purification of the pigment in quantity. Willstätter and Zollinger in their work on the pigment of sloes, have therefore attempted to replace amyl alcohol by other solvents, without, however, finding a suitable one. (Phenol and cyclohexanol were the solvents tried.)

In the course of an investigation into the formation of the pigments in the grape vine, I observed that butyl alcohol removes all the anthocyanins (mono- and rhamnoglucosides as well as normal diglucosides) from their solution in dilute acids. The sugar-free pigments (anthocyanidins) are still more soluble in this solvent. On repeatedly washing the butyl alcoholic layer with fresh dilute acid, the non-glucoside pigments remain in the butyl alcohol, whilst the glucosidic anthocyanins pass again into the acid aqueous layer as oxonium salts. Butyl alcohol behaves therefore in this respect exactly like amyl alcohol.

As this observation promised to be useful for the purification of anthocyanins, I made some experiments with typical representatives of each type and compared quantitatively their solubility in butyl alcohol, taking their solution in amyl alcohol as a standard. In order to make the conditions comparable to those obtaining during their purification, the anthocyanin solutions were prepared by extracting the petals of flowers or skins of fruits with ethyl alcohol containing 1 per cent. hydrochloric acid and precipitating the filtered extracts with ether. After washing the pigments with ether and removing the latter with a current of air, they were dissolved in  $N/2$  sulphuric acid and the solutions diluted to a uniform arbitrary tint. They were then divided into two parts, of which one was shaken out with an equal volume of

butyl alcohol and the other with amyl alcohol under identical conditions. The alcohols were previously saturated with the dilute acid employed, in order to keep the volume of the solutions constant. The relative strength of the first extract with each solvent was determined colorimetrically in a Hellige-Autenrieth's Colorimeter. The extractions were then continued until all but traces of the pigment was removed. The figures given under (1) in the following table refer to butyl alcohol only.

	Mono-glucosides			Rhamnoglucosides	
	Chrysanthememin	Oenin	Idaein	Prunicyanin	Violanin
1. Number of extractions	2-3	3-4	3-4	4-5	4-5
2. Solubility (amyl alcohol = 1)	4	4	10	16	25

The diglucosides examined were pelargonin, cyanin, and delphinin. They required from 7-8 extractions for the complete removal of the pigment. No comparative figures for their solubility in butyl alcohol can be given, as they are practically insoluble in amyl alcohol. It will be seen that even the less soluble anthocyanins possess a solubility in butyl alcohol four times as great as that in amyl alcohol, whilst that of the most soluble examined is twenty-five times as great. The unidentified anthocyanins of numerous flowers and fruits were, without exception, found to be soluble in butyl alcohol<sup>1</sup>.

It would appear from these results that butyl alcohol, which is now a commercial product, would be of advantage in the preparation of mono- and rhamnoglucosides especially. The solubility of the diglucosides in the solvent, whilst not sufficient for purposes of preparation, is of such an order as to make it useful in those cases where their removal is essential, e.g. for the study of the colourless substances accompanying them in the plant.

I have successfully used butyl alcohol in the preparation, on a relatively small scale, of oenin (from grapes) and of idaein (from cranberries), the well-crystallised picrates of which may be easily obtained from the butyl alcohol extracts by the usual methods. Butyl alcohol may also replace ethyl alcohol and glacial acetic acid for the direct extraction of anthocyanins, especially from dried petals or leaves. In the latter case the pigment is only soluble in butyl alcohol when present as the oxonium salt of a mineral acid. This has the advantage that by a preliminary treatment of the dry powder with the solvent, such substances as chlorophyll, flavones, etc., are removed. The powder is then treated with butyl alcohol containing 2-3 per cent. hydrochloric acid, which extracts the anthocyanin as oxonium salt. From this solution the pigments are precipitated by light petroleum and purified by appropriate methods.

<sup>1</sup> It is interesting to note that the pigment of beetroot, which differs in many respects from anthocyanins, is insoluble in butyl alcohol.

751

# IX. AN INVESTIGATION OF THE METHODS EMPLOYED FOR COOKING VEGETABLES, WITH SPECIAL REFERENCE TO THE LOSSES INCURRED.

## PART II. GREEN VEGETABLES.

BY HELEN MASTERS AND PHYLLIS GARBUTT.

*From the Household and Social Science Department,  
King's College for Women.*

*(Received January 15th, 1920.)*

THESE investigations were undertaken, as indicated in Part I [Masters, 1918], primarily with a view to obtaining some comparison of the different methods of cooking vegetables, with special references to the losses incurred and the time required for cooking. It was hoped that in this manner some method of cooking might be devised, which would be economical both as regards food material and as regards fuel, and that some further information concerning the changes which take place during the cooking process might be obtained.

Katherine Williams [1892] gives the results of a number of analyses of cooked and uncooked vegetables, but does not indicate how these results would be modified by different methods of cooking.

Other observers [Harden and Zilva, 1918; Delf, 1918] have shown that the antiscorbutic properties of vegetables may be destroyed by the action of dilute alkaline solutions or of heat. This is a physiological problem and the question whether or not it is advisable from a dietetic point of view that heat or alkaline solutions should be used in the preparation of vegetables hardly comes within the scope of this paper. It is hoped, however, that these investigations may prove of some assistance in arriving at a decision on such matters.

Numerous kinds of green vegetables are made use of for edible purposes, but the varieties available vary according to the season of the year and it was found impossible to work with the same variety throughout. To obviate this difficulty as far as possible, a number of experiments were made with each variety, and the nature of the vegetable used is stated in each case; but this variation in the nature of the material has hampered the work considerably and increased the difficulty of obtaining reliable results. The vegetables principally employed were cabbage, spring greens and Brussels sprouts.

In cooking green vegetables it is necessary not only to consider the losses on cooking and the time of cooking, but also the extent to which it is possible to preserve the original green colour of the vegetable, since no method of cooking can be considered satisfactory from a practical point of view unless the vegetables are a "good colour."

Some investigations were therefore first made of the colour changes which occur when green vegetables are cooked under various conditions.

#### COLOUR CHANGES PRODUCED IN GREEN VEGETABLES ON COOKING.

It appears to be fairly generally accepted that the preservation of the colour of green vegetables during the process of cooking can best be effected in the following ways:

(1) By cooking in a considerable volume of rapidly boiling water in an *open* vessel, *i.e.* in a vessel without a lid.

(2) By the addition of a small amount of alkali, usually sodium bicarbonate, to the cooking water.

Preliminary experiments indicated that there was considerable foundation for these ideas. Vegetables cooked in a covered vessel were undoubtedly inferior in colour to those cooked in an open vessel. The addition of ordinary salt to the cooking water appeared to improve the colour slightly, but the difference was not very marked. The cooking water was slightly acid to litmus.

The addition of small amounts of sodium bicarbonate to the cooking water had a marked effect on the colour of the vegetables. A bright green colour, more pronounced than that of the uncooked vegetables, was produced in most cases. It was also noticed that the alkaline cooking water was dark in colour, and that the veins and stems of the leaves of the vegetable, which are usually white, were coloured green, as though the colouring matter had been spread or developed by the action of the alkali.

Conversely, it was noted that if a little acid, *e.g.* acetic acid, were added to the cooking water, the vegetables rapidly changed in colour and became brown, but that the cooking water was only slightly coloured.

Chlorophyll, it is stated [Allen, 1909], is soluble in alkaline solutions and is rapidly decomposed after the death of the cell, a transformation to a brownish green chlorophyllan occurring unless alkali is present; the brown colour of badly cooked vegetables is attributed to the formation of this substance.

A preparation of chlorophyll was examined and was found to be insoluble in water, but readily soluble in water containing a little sodium bicarbonate; the solution thus obtained gave no colour change on treatment with dilute acids, and it was thought possible that the chlorophyll had been specially treated to render the colour stable.

On extracting some of the fresh green vegetable with alcohol, however, a solution which was deep green in colour was obtained, and this colour was

rapidly changed to brown on the addition of a few drops of acetic acid. The same effect was produced with other acids, but with sulphuretted hydrogen (see below) the reaction was slow, the colour changing gradually on standing for a few minutes at the room temperature.

Thus, in order to preserve the green colour of the vegetables, the presence of any acid reacting substance in the water should be avoided. This may, of course, be effected by rendering the water alkaline by the addition of sodium bicarbonate. The other effects observed on the addition of the bicarbonate, *i.e.* the dark colour of the cooking water and the stained appearance of the white portions of the leaf, may be attributed to the solubility of the chlorophyll in the alkaline solution. (For the effect of the addition of sodium bicarbonate on the time of cooking, etc., see pp. 82, 83.)

The action of acid on chlorophyll, taken in conjunction with the fact that the cooking water was found to be acid unless sodium bicarbonate was added, seemed to indicate that acid substances might be produced from the vegetables on cooking. Such acids, if volatile in steam, would, to a great extent, be removed if the vegetables were cooked in an open vessel of rapidly boiling water, but would be mostly retained if a covered vessel were used, and this might afford an explanation of the differences in colour observed with vegetables cooked in open and in covered pans.

Further information as regards the production of acid substances on cooking therefore appeared to be needed, and experiments were made to investigate the nature of the acids produced.

#### *Acid substances produced on cooking green vegetables.*

In order to determine whether volatile acids were produced on cooking, green vegetables were cooked in a large distilling flask attached to a condenser, and the distillate was collected in portions of about 100 cc.

It was found that the distillate was at first acid but on continuing the distillation it afterwards became slightly alkaline.

#### *Examination of the distillate.*

Since the distillate was only slightly acid, it appeared hardly feasible to identify the acids present without some further process of separation and concentration. It was, therefore, proposed to separate the acids in the form of their lead salts, by adding lead carbonate to a considerable volume of the distillate. On the addition of lead carbonate, however, a brown coloration was obtained which seemed to indicate the presence of sulphide.

This was confirmed by adding a few drops of a dilute solution of sodium nitroprusside to another portion of the distillate, which had previously been rendered *slightly* alkaline by the addition of ammonia. The characteristic violet colour produced by sulphides was obtained.

*Determination of volatile sulphide in the cooking water.*

Since it seemed probable that the amount of volatile sulphide produced might vary with the nature of the vegetable, and also with the method of cooking, it appeared of interest to try and devise some method for obtaining a measure of the amount of sulphide present in the cooking water, and a number of experiments were made with this object in view. The amount of sulphide produced is very small in comparison with the weight of vegetable cooked. The composition of the vegetable itself is by no means constant even when the same type of vegetable is used, and it is difficult to obtain any very consistent results.

After several methods had been tried and found unsuitable, a method was finally devised which gave results of sufficient accuracy to be of value for practical purposes, but before describing this method and the results obtained, a brief summary of the other methods and the difficulties met with may be given here for the benefit of other observers.

In the first experiments, 100 g. of cabbage were cooked in water in a flask attached to a condenser and the distillate was collected in portions of 100 cc. in Nessler cylinders. The amount of sulphide in each portion was determined colorimetrically.

The amount of sulphide in the first portion of the distillate was small, increased as distillation proceeded and then decreased again becoming fairly constant, but it was impossible to obtain any definite end-point, and after a time the distillate became cloudy.

It seemed probable that sulphide would continue to be evolved as long as the cooking was continued, and it was noted that sulphide was produced even when the distillate was no longer acid.

In further experiments, the vegetables were cooked for a definite time only under a reflux condenser and the cooking water distilled after straining off the solid matter. It was found that the sulphide could not be completely separated from the cooking water by distillation and that some of the sulphide underwent decomposition during this process.

There was also a considerable loss of sulphide from the condenser during the first part of the experiment in spite of a device arranged to minimise such loss. These observations were confirmed by some experiments made with dilute solutions of hydrogen sulphide of known concentration, which showed that a large proportion of the sulphide was decomposed when such solutions were heated and distilled under conditions similar to those employed in the experiments.

It appears hardly possible to obtain a reliable measure of the amount of sulphide produced, during cooking, but it was thought that if some method, which did not involve a distillation process, could be devised, it might be possible to obtain a measure of the amount of sulphide left in the cooking water at the end of the experiment, and that if all experiments were carried

out under the same conditions such information might be of value for comparative purposes, even though it did not represent the total amount of sulphide produced during cooking. For this purpose, a modification of the method described by Winkler [1917] was adopted, and was found to give more consistent results than the methods previously described.

*Method used for the determination of the volatile sulphide in the cooking water of green vegetables.*

The vegetable was cooked as before under a reflux condenser for one hour. Carbon dioxide was bubbled through a measured volume of the cooking water carrying with it the volatile sulphide, which was absorbed in a solution of hydrogen peroxide and thus oxidised to sulphuric acid, the amount of which was determined by titration with sodium hydroxide after all the carbon dioxide had been removed by boiling.

The determination was made as follows: 100 g. of the vegetable were cooked in 750 cc. water under a reflux condenser for one hour. The cooking water was made up to a litre and 250 cc. placed in a flask connected with a carbon dioxide Kipp and an absorption apparatus consisting of three conical flasks, each containing 150 cc. of a 0.15 % hydrogen peroxide solution. Carbon dioxide was bubbled through the cooking water, carrying with it the hydrogen sulphide, which was absorbed by the hydrogen peroxide and oxidised to sulphuric acid. The contents of the absorption flasks were washed into a beaker, and after the carbon dioxide had been expelled by boiling and the solution evaporated to about one-eighth of its original bulk, the sulphuric acid formed was determined by titration with *N*/100 sodium hydroxide solution. Correction was in each case made for the acidity of the hydrogen peroxide solution.

Several experiments were made and the average results obtained were as follows:

Table I.

Nature of vegetable	g. of hydrogen sulphide in cooking water from 100 g. of vegetable
Cabbage I	0.0017
" II	0.0015
Green leaf of cabbage	0.0049
White " "	0.0011
Brussels sprouts I	0.0094
" " II	0.0084

The results indicate that there is considerable variation in the amount of sulphide produced from the two types of vegetable used in the experiments, the average results for the Brussels sprouts being considerably higher than those obtained for cabbage.

Some further experiments were made in this connection, in which the green portions of the cabbage were separated from the white portions, and

cooked separately, the amount of sulphide left in the cooking water being determined in each case (see Table I above).

These results show that sulphide is liberated from both portions, but that less is liberated from the white leaf than from the green. Thus the relatively high results obtained with Brussels sprouts may be partly accounted for by the relatively small portion of white leaf found in this vegetable.

In addition to determining the sulphide present in the cooking water, determinations of the nitrogen and sulphur contents of the vegetables and portions of the vegetables were made.

#### *Determination of sulphur in cabbage.*

The sulphur was determined by fusing the dried vegetable with sodium peroxide, the sulphate thus formed being subsequently precipitated and determined as barium sulphate [see the method described in the Official and Provisional Methods of Analysis issued by the United States Department of Agriculture].

#### *Determination of protein.*

The nitrogen was determined by the Kjeldahl process, on about 3 g. of the dried vegetable, and the results were calculated as protein on 100 g. of uncooked vegetable and not on the dried vegetable.

Table II.

	Nature of vegetable	g. sulphur present in 100 g. uncooked vegetable	g. protein in 100 g. uncooked vegetable
I.	Cabbage (whole)	0.160	1.9
	„ green portion	0.162	2.2
	„ white „	—	1.6
II.	Cabbage green portion	—	1.9
	„ white „	—	1.5
I.	Brussels sprouts	0.135	3.9
II.	„ „	—	3.5

These results, when compared with those of Table I, indicate that there is no definite correspondence between the sulphur content and the volatile sulphide in the cooking water.

As regards the nitrogen content, the results obtained appear to be in agreement with those arrived at by other observers.

Dugast [1882] found that generally speaking the protein in the leaf of the vegetable was greater than that in the stalk and that in the former case it varied from 11.9–21.2 % and in the latter from 7.3–13.3 % calculated on the *dried* vegetable.

Comparison with Table I shows that the yield of volatile sulphide is greatest where the protein content is high and *vice versa*. This suggests the possibility that the sulphide is produced by the decomposition of protein material, and it may be noted in this connection that Rettger [1906] showed that volatile

sulphide is liberated from heated milk and attributes this to the partial decomposition of the proteins.

The possibility of the sulphide being produced from alkyl sulphides<sup>1</sup> was also considered and attempts have been made to isolate such bodies by extracting some of the dried vegetable with ether in a Soxhlet extractor; so far no indication of their presence has been obtained, but further investigations in this connection are needed.

*Other volatile acids produced on cooking.*

In the experiments thus far described, the production and determination of volatile sulphide has been principally considered, but the possibility of the liberation of other volatile acids during the cooking process was also borne in mind and in some of the preliminary experiments the acidity of the distillate from the cooking water as well as the amount of sulphide present were determined. The results obtained for the acidity showed considerable variation even when samples of the same vegetable were used, the differences being still more marked when the experiments were made with different vegetables. It has been shown in the foregoing that such variations are almost inevitable in experiments of this character. In spite of this variation in the determined acidities, some evidence was obtained that the acidity of the distillate was greater than could be entirely accounted for by the volatile sulphide present.

Other observers have shown the presence of such acids as acetic and formic in vegetables. Börgmann [1883] found that a great number of plants, both in the presence and in the absence of chlorophyll, contained formic and acetic acids, and that in the case of chlorophyll-bearing plants the percentage of acids rises when assimilation is repressed.

Nelson and Beck [1918] showed that propionic and acetic acids could be isolated from fermenting cabbage (*sauerkraut*) and that in two cases formic acid had also been detected.

It, therefore, appeared by no means improbable that volatile organic acids might be liberated from green vegetables during the process of cooking. In order to verify this, it was first necessary to devise some method of removing the volatile sulphide in such a manner that other volatile acids would be left in solution.

Some experiments made with dilute solutions of formic and acetic acids containing hydrogen sulphide showed that, if the sulphide were expelled by bubbling carbon dioxide through the solution, in the manner previously described, the excess of carbon dioxide could be removed without loss of the acetic and formic acids, by heating the solution under a reflux condenser for about half-an-hour. If the time of heating exceeded one hour there was a slight loss of volatile acid, but heating for half-an-hour was found to be sufficient to expel the carbon dioxide. This method was next applied to the examination of the cooking water in which green vegetables had been cooked.

<sup>1</sup> Tibbles [1912] states that most of the Cruciferae contain allyl sulphides.

*Examination of the cooking water for acids other than hydrogen sulphide.*

The vegetable was cooked in a flask attached to a condenser for one hour. The distillate was diluted to a measured volume with distilled water and divided into two portions. The acidity of one portion was determined directly by titration with standard acid. The volatile sulphide was removed from the other portion and the acidity of the solution then determined by titration.

It is, perhaps, necessary to point out that such determinations do not, for the reasons already stated, give any exact measure of the volatile acids produced on cooking, but it was thought that they might furnish some evidence as to the presence or absence of volatile acids other than hydrogen sulphide.

The acid in the distillate from 100 g. of cabbage was found on the average to require 0.014 g. of sodium hydroxide for complete neutralisation, whilst after the removal of volatile sulphide the sodium hydroxide required for neutralisation averaged 0.005 g. Thus the solution is still slightly acid after the removal of the volatile sulphide, but the amount of acid present is extremely small and it is difficult to obtain consistent results.

Qualitative tests for formic and acetic acids were also carried out with this distillate, and some indication of the presence of these acids was obtained, but in dealing with such small quantities these tests can hardly be regarded as conclusive.

*Total acidity of the cooking water.*

In addition to determining the volatile acidity of the cooking water, some determinations of the total acidity were made. Owing to the dark colour obtained on the addition of alkali, phenolphthalein could not be used as indicator, but it was found that if turmeric paper were used as an outside indicator a moderately good end-point could be obtained. The acid in the cooking water from 100 g. of cabbage was found to require, on the average, 0.055 g. of sodium hydroxide for complete neutralisation. Thus the total acidity of the cooking water is considerably greater than the volatile acidity measured as described above. This may be partly accounted for by the loss of volatile sulphide on distillation and also the difficulty of separating the volatile acids completely by distillation, but it is also possible that non-volatile acid substances are present in the cooking water and further investigations are in progress.

*The addition of sodium bicarbonate to the cooking water.*

If sodium bicarbonate is added to the cooking water, any acids produced during the cooking process are neutralised and the cooking water at the end of the experiment is alkaline. It was found that if this alkaline cooking water were distilled, using a trap as in a Kjeldahl determination to prevent non-volatile alkali passing over, an alkaline distillate which contained appreciable quantities of sulphide was obtained.

It was observed that the vegetables cooked and disintegrated more rapidly in the presence of alkali. This is possibly because the cell walls are composed of a hydrated form of cellulose, which is more or less soluble in alkaline solutions [Cross and Bevan, 1903]. It also seemed possible that the amount of sulphide produced would be greater than when water only was used, but owing to the difficulty of determining such small quantities of sulphide in an alkaline solution, it is not possible at present to make any definite statement on this point.

As regards the alkalinity of the distillate from the cooking water when sodium bicarbonate is used, it should be noted that it was observed that the distillate from the cooking water became alkaline after a time, as the cooking proceeded, even when bicarbonate was not employed.

It was thought that this alkalinity might possibly be due to the evolution of ammonia or substituted ammonias formed as decomposition products during cooking, and that, if this were the case, the ammonia would probably be liberated more rapidly from a solution containing alkali.

Some preliminary experiments appear to give support to this theory, as some evidence has been obtained that the amount of alkali liberated is considerably greater when sodium bicarbonate is employed. These results, however, need further confirmation.

#### SUMMARY AND CONCLUSIONS.

The changes in colour observed on cooking green vegetables appear to be chiefly due to the action of acids liberated during cooking on the chlorophyll, the characteristic green colour of which is destroyed by the action of acids. During cooking, hydrogen sulphide and probably also volatile organic acids are liberated; these acids may to some extent be neutralised by other products of decomposition, but unless sodium bicarbonate is added, the cooking water is acid at the end of the experiment.

Green vegetables are usually cooked in an open vessel containing rapidly boiling water, and in this way there is a maximum loss of volatile acids, but if, on the other hand, they are cooked in a covered vessel then these volatile acids are retained in the vessel for a time at least. Although the volatile acidity is comparatively small, it is possible that the action of acids on the chlorophyll may be specially harmful during the earlier stages of cooking, and hence conditions which tend to prevent a free and rapid loss of volatile acids have a deleterious effect on the colour of the cooked vegetable, and this probably explains the poor colour of green vegetables cooked in a covered vessel.

It may also be noted that many cooks advocate the use of a large volume of water for cooking in order to preserve the colour; this would have the effect of diluting the acids produced.

If sodium bicarbonate is added, the cooking water does not become acid although volatile sulphide is produced. The neutralisation of the acids pro-

duced during cooking does not, however, entirely explain the action of the bicarbonate. The alkali has a solvent action on the chlorophyll, and also, probably because of its action on the plant cellulose, it causes a decrease in the time required for cooking. This latter effect also tends to improve the colour, since prolonged cooking renders the vegetables a bad colour.

In dealing with the action of bicarbonate, it is perhaps necessary to draw attention to a popular fallacy. It is frequently stated that the bicarbonate is added to soften the water and that this renders the vegetables a good colour. Careful observations, however, show that the colour of green vegetables cooked in really soft water, *i.e.* distilled water, compares unfavourably with that of those cooked in ordinary London tap water. This difference is, in all probability, due to the alkalinity of the tap water.

#### LOSSES INCURRED DURING COOKING AND TIME REQUIRED FOR COOKING.

The question of colour having been dealt with, a comparison of the different methods of cooking as regards losses incurred and time of cooking was next considered.

The methods most frequently employed for cooking green vegetables appear to be the following:

- I. Boiling in water, with or without the addition of sodium bicarbonate and salt.
- II. Steaming.
- III. Conservative method.

Vegetables were cooked by each of the above methods and the time of cooking, loss of solids, and loss of mineral matter compared.

As mentioned in Part I, it is difficult in carrying out experiments of this type to obtain consistent results, owing to variations in the nature of the material used for the experiments, the absence of any very definite end-point, and the difficulty of working under exactly similar conditions without adopting precautions which would not ordinarily be employed, and which, if introduced, might render the results less valuable from a practical point of view.

In the case of fresh green vegetables, the variation in composition is much more marked than with the dried vegetables dealt with in Part I; also, as already stated, the type of vegetable available varies according to the season of the year. Thus it is often impossible to repeat experiments, or confirm results obtained at an earlier period of the investigations. For these reasons, it was deemed advisable to carry out several experiments in each case and to take an average and also, in comparing the different methods of cooking, to make a control experiment with each sample of vegetable, by boiling a portion in water and comparing the results with those already obtained.

The methods adopted for cooking were as follows.

### I. *Boiling in water.*

(a) 100 g. of the vegetable were washed with cold water in a strainer and were then immersed in 1000 cc. of boiling water, heated in an open pan and the boiling continued until the vegetables were quite soft as indicated by gently piercing with a fork. The time of cooking was noted. The vegetables were strained off, the cooking water evaporated to dryness on a water-bath and the residue heated in a steam-oven until a constant weight was obtained. This gave the weight of solid matter lost during the cooking process. The residue was then ignited over a flame until completely ashed, to obtain the weight of mineral matter.

#### (b) *The addition of sodium bicarbonate to the cooking water.*

The above experiments were repeated with the addition of 0.5 g. of sodium bicarbonate to the cooking water. The weights of residue and mineral matter were determined as before, a correction being made for the sodium bicarbonate added.

#### (c) *The addition of salt to the cooking water.*

The above experiments were repeated with the addition of 2.0 g. of salt to the cooking water. The weights of residue and mineral matter were determined as before, a correction being made for the sodium chloride added.

### II. *Steaming.*

100 g. of the vegetable were washed with cold water in a strainer, and placed in a steamer, covered with a lid, over a pan of boiling water. The cooking was continued until the vegetables were soft, and the time of cooking noted.

The vegetables were allowed to drain for a few minutes over the pan and were then removed from the steamer. The steamer was washed down with a little distilled water, and the washings added to the cooking water. The solids and mineral matter in the cooking water were determined as described above.

### III. *Conservative method.*

In the so-called "conservative" method of cooking vegetables, the vegetables are cooked in a very small quantity of water, so that the water is all absorbed by the vegetables during the cooking process. The vegetables are served without straining and there is practically no loss on cooking except volatile matter. 100 g. of the vegetable were covered with about 50–60 cc. of water and gently heated in a covered pan until quite soft. The time of cooking was noted.

The average results obtained for the different methods are given below (Table III). It should be noted, however, that the uncooked vegetable used for the experiments contains a large proportion of water, on the average about 89 %, the residues obtained therefore represent the loss on 100 g. of uncooked vegetable or on 11 g. of solid matter on the average, and the results

have been expressed as the loss both on 100 g. of vegetable and also on 100 g. of solids.

Table III.

Nature of vegetable	Method of cooking	Time of cooking	Loss of solids		Loss of mineral matter	
			(a) on 100 g. of raw vegetable	(b) on 100 g. of solids	(a) on 100 g. of raw vegetable	(b) on 100 g. of solids
I. Cabbage	Boiling	30 mins.	4.45	40.45	—	—
"	" + 0.5 g. sodium bicarbonate	20 "	3.37	30.64	—	—
"	Boiling	30 "	3.19	26.94	0.689	5.82
"	" + 0.5 g. sodium bicarbonate	20 "	2.26	19.10	0.438	3.70
"	Boiling	35 "	3.81	39.64	0.437	4.55
"	" + 0.5 g. sodium bicarbonate	22 "	3.41	35.48	0.421	4.38
II. Spring greens	Boiling	20 "	5.17	37.68	1.023	7.49
"	" + 2 g. salt	20 "	4.50	32.77	0.800	5.83
"	"	25 "	3.39	33.50	0.803	7.95
"	" + 2 g. salt	30 "	3.49	34.55	0.892	8.83
III. Cabbage	"	35 "	3.81	39.64	0.437	4.55
"	" + 2 g. salt	30 "	3.55	36.94	—	—
IV. Cabbage	Boiling	45 "	3.80	41.99	0.428	4.75
"	Steaming	1½ hrs.	0.88	9.72	0.224	2.48
"	Boiling	30 mins.	3.56	39.33	0.555	6.13
"	Steaming	45 "	1.44	15.91	0.304	3.35
"	Boiling	35 "	3.81	39.64	0.437	4.55
"	Steaming	1 hr.	0.92	9.61	0.303	3.15

These results show that the addition of sodium bicarbonate to the cooking water decreases the time required for cooking (compare I, Table III) and that the loss in solid matter is slightly less. This latter effect is probably due to the shorter time required for cooking. (Compare the action of ammonium carbonate in steaming vegetables.)

The addition of salt does not appear to have any marked effect either on the losses or on the time of cooking.

As would be expected, the loss in solid matter when the vegetables are steamed is considerably less than when they are cooked in boiling water, though the time of cooking is somewhat greater. It should be noted, however, that in cooking with steam it is necessary to use a covered vessel in order to retain the steam, and this applies also to the conservative method of cooking. The vegetables have, therefore, in both cases a bad colour, this effect being probably enhanced by the relatively long time required for cooking, and both methods are, for this reason, open to objection from a practical point of view.

*The addition of ammonium carbonate to the water used for steaming green vegetables.*

The experiments previously described, in connection with the action of alkali on the colouring matter of green vegetables, seemed to indicate that if the steam could be rendered alkaline this difficulty might possibly be obviated. Some experiments were, therefore, made in which green vegetables were steamed over boiling water to which a little ammonium carbonate had been added.

Preliminary experiments showed that the colour of the vegetables was greatly improved in this way. The experiments also showed that, provided only small quantities of the carbonate were employed, the cooked vegetables did not taste or smell of ammonia. The addition of the alkali appeared, as in the case of the addition of sodium bicarbonate to the water used for boiling, to decrease the time required for cooking, and if too much were added the vegetables rapidly became slimy and disintegrated.

Several experiments were made in order to determine the most suitable proportions of ammonium carbonate to use.

These experiments showed that some vegetables disintegrated more rapidly than others, but that on the whole the best results were obtained when 0.5–1.0 g. of ammonium carbonate was added to 1000 cc. of water for steaming, and when 0.1–0.2 g. was added to 100 g. of vegetable for the conservative method.

The losses of solid and mineral matter when 100 g. of the vegetable were steamed by this method were determined and the average results obtained were as follows:

Table IV.

Nature of vegetable	Method of cooking	Amount of ammonium carbonate added to 1000 cc. water	Time of cooking	Loss of solids		Loss of mineral matter	
				(a) on 100 g. of raw vegetable	(b) on 100 g. of solids	(a) on 100 g. of raw vegetable	(b) on 100 g. of solids
I. Cabbage	Boiling	—	34 mins.	3.81	39.64	0.437	4.55
„	Steaming	—	1 hr.	0.92	9.61	0.303	3.15
„	„	0.5 g.	18 mins.	0.48	5.01	0.157	1.63
II. Cabbage	Steaming	—	1 hr.	0.90	8.17	0.261	2.37
„	„	2.4 g.	15 mins.	0.47	4.26	0.148	1.34
III. Cabbage	Steaming	—	30 mins. (not completely cooked)	0.52	4.72	—	—
„	„	2.4 g.	30 mins. (slightly overcooked)	0.66	5.96	—	—

*Conservative method.*

Nature of vegetable	Method of cooking	Amount of ammonium carbonate added to 60 cc. water	Time of cooking
I. Cabbage	Boiling	—	30 mins.
„	Conservative	—	1 hr.
„	„	0.14 g.	30 mins.
II. Cabbage	Conservative	—	1 hr.
„	„	0.3 g.	12 mins.

The results show that the losses of solid and mineral matter are less than in the case of vegetables steamed in the ordinary manner without the addition of ammonium carbonate, and this in spite of the fact that the vegetables appear to disintegrate more rapidly when ammonium carbonate is used. The explanation of this result probably lies in the shorter time required for cooking in the presence of the alkali.

In order to illustrate this point some experiments were carried out in which portions of the same cabbage were cooked (*a*) over water, (*b*) over water containing excess of ammonium carbonate (2.4 g. of ammonium carbonate per litre). In the first experiment, the cabbage was cooked completely in both cases, the time of cooking noted and the loss of solid matter determined. In the second experiment, the cabbage was cooked for the same length of time in both cases and the loss of solid matter determined.

These results (see Table IV) show that if the time of cooking is increased, the losses increase, and if the vegetables are cooked for the same length of time only, the loss when ammonium carbonate is added is slightly greater than when water only is used.

Although the preliminary experiments indicated that the cooked vegetables had no appreciable taste or smell of ammonia, if only a small quantity of ammonium carbonate were used, it was thought advisable to carry out a few experiments in order to determine whether any considerable quantity of the ammonium salt were retained in the vegetable, as if this were the case its addition to the cooking water might be considered undesirable.

*Determination of the proportion of the ammonium carbonate retained by the cooked vegetable.*

The vegetables were cooked in a steamer of wire gauze suspended in the neck of a wide necked flask. The flask was connected with an upright condenser, the end of which dipped below the surface of a measured volume of standard acid contained in a beaker. 35 g. of the vegetable were used for each experiment. A weighed quantity of ammonium carbonate was added to the water in the flask and the water boiled for half-an-hour. The amount of ammonium carbonate left in the flask and the amount absorbed by the acid in the beaker were determined, and the difference between the sum of

these amounts and the amount of ammonium carbonate originally added was taken as a measure of the amount of ammonium carbonate absorbed by the vegetable (see Table V).

Table V.

Weight of cabbage	Volume of cooking water	Weight of ammonium carbonate added	Weight of ammonium carbonate left in flask	Weight of ammonium carbonate in beaker	Total ammonium carbonate in flask and beaker	Loss of ammonium carbonate
35	500	0.248	0.154	0.041	0.195	0.053
35	500	0.246	0.151	0.055	0.206	0.040
					<i>Mean</i> ...	0.046

The results indicate that the cooking water and distillate together contain most of the ammonium carbonate originally added, but a small proportion is lost and is presumably absorbed by the vegetable. It is possible that, by the time the vegetable has been cut up and dished ready for table, some of the ammonia at first absorbed will have escaped and that the proportion finally retained by the vegetable is less than that indicated by the experimental results.

Delf [1918] in dealing with the loss of antiscorbutic properties on cooking cabbage states that in general the loss is greater the longer the time of heating and that slow cooking at a low temperature is more deleterious than more rapid cooking at a higher temperature. It would be interesting if information could be obtained in order to show whether cooking for a short time in steam rendered slightly alkaline is less or more deleterious than cooking in steam without the addition of alkali for a longer period.

#### SUMMARY.

A comparison of the different methods of cooking green vegetables shows that the addition of a small quantity of alkali to the cooking water, in addition to having a marked effect upon the colour, also decreases the time required for cooking, and slightly decreases the losses incurred; this latter effect being probably due to the decrease in the time of cooking.

The addition of salt to the cooking water appears to have no appreciable effect either on the time of cooking or on the losses incurred.

The losses of solid and mineral matter when the vegetables are steamed are less than when they are cooked in boiling water. Cooking by steaming and also by the conservative method in which there is practically no loss is, however, open to the objection that the vegetables are in both cases cooked in a covered vessel, and have, therefore, usually a bad colour; also the time required for cooking is greater than when the vegetables are boiled in water.

Both these difficulties may be obviated, and in the case of steamed vegetables the losses incurred reduced by the addition of a small amount of ammonium carbonate to the water from which the steam is generated. The

addition of excess of alkali, either of ammonium carbonate in steaming or of sodium bicarbonate in boiling vegetables, is to be avoided or the vegetables become slimy and tend to disintegrate. (See also Summary, p. 83.)

This work has been made possible owing to the receipt of a research grant by one of us (P. G.) from the Department of Scientific and Industrial Research, for which we desire to express our thanks.

#### REFERENCES.

- Allen (1909). *Commercial Organic Analysis*, 4th Edition.  
Borgmann (1883). *Bied. Centr.* **65**.  
Cross and Bevan (1903). *Cellulose*, 2nd Edition.  
Delf (1918). *Biochem. J.* **12**, 416.  
Dugast (1882). *Annales Agronomiques*, 226.  
Harden and Zilva (1918). *Lancet*, September 7.  
Masters (1918). *Biochem. J.* **12**, 231.  
Nelson and Beck (1918). *J. Amer. Chem. Soc.* **40**, 1001.  
Retteger (1906). *Amer. J. Physiol.* **6**, 450.  
Tibbles (1912). *Foods, their Origin, Composition and Manufacture*.  
U.S. Dept. of Agriculture, Bureau of Chemistry, *Bulletin* No. 107.  
Williams (1892). *J. Chem. Soc.* **61**, 226.  
Winkler (1917). *Analyst*, **12**, 26.

## X. ON THE THEORY OF GELS. II. THE CRYSTALLISATION OF GELATIN.

By SAMUEL CLEMENT BRADFORD.

(Received January 27th, 1920.)

IN a previous paper [1918] it has been shown that the reversible gelation of the natural emulsoids is a crystallisation process resulting from the viscosity of these substances and from their very small diffusion constants which greatly retard the crystallisation of the cooling sols, allowing the accumulation of a large excess concentration. Thus the two factors  $K$  and  $P$  in von Weimarn's formula are both so large that, with a small value of  $L$ , a very great number of crystallisation centres are produced, and a gel is formed. Detailed examination of the theory showed that it was sufficient to explain the numerous properties of gels. It was also pointed out that complex organic substances and those which are highly aggregated in solution tend to crystallise in the form of spherites, built up of a great many crystalline needles arranged radially, the interstices being filled with liquid giving the appearance of a globular crystal. The inference was drawn that organic gels are usually composed of submicroscopic spherites bathed in the mother-liquor from which they have crystallised, though in certain cases the ultimate crystalline particles may be of other forms.

This conception of gelation is in complete agreement with recorded experimental results: the variation in intensity of the Tyndall cone observed by Arisz [1915]; the development of submicrons seen by Menz [1909] in gelating 2% gelatin, which increased from 4 or 5, showing Brownian movement, in a square division of the field with a side of  $9\mu$ , to 80 or 100, at rest, in the same space; the actual observation by Hardy [1912] of microscopic spherites, *i.e.*  $10\mu$ , in reversible carbon tetrachloride gels of 5-dimethylaminoanilo-3:4-diphenylcyclopentene-1:2-dione; and the globulitic ultramicroscopic structure of natural organic hydrogels from which the water had been expressed, recorded by Bachmann [1911]. Moreover it was shown that gelatin can readily be precipitated by alcohol in the form of spherites.

The utility of von Weimarn's theory is not confined to the explanation of the colloid state. It indicates, further, the conditions necessary to obtain a given substance in any desired form. If his formula explains the usual occurrence of gelatin in the colloid condition, it should be possible, by suitable modification of the factors, to reduce the number of crystallisation centres sufficiently to obtain microscopic crystals of gelatin from water. The form in which such crystals would be expected to occur is that of spherites.

Since Bütschli [1896] found that the liquid expressed from 5 % gelatin gels contains from 0.16 to 0.46 % of gelatin, the solubility of this substance in water must be about this value. To obtain larger crystals of gelatin it should be necessary merely to reduce the concentration of the crystallising sols to about this degree. Hydrosols of Nelson's "Crystal" leaf gelatin were made of 0.5 and 0.3 %. These were quickly heated to 100° and filtered into small crystallising dishes which had been previously cleaned with chromic acid mixture. The dishes were covered with lids and the liquid allowed to evaporate spontaneously at room temperature. The sols were only very slightly opalescent at first, but, particularly in the case of the 0.5 % sols, the opalescence soon developed to an extraordinary degree, reaching a dense milky, and almost opaque, appearance by the third day. After a few days the cloudiness disappeared and a faint light buff-coloured precipitate was seen on the base of the vessels. The amount of this precipitate slowly increased.

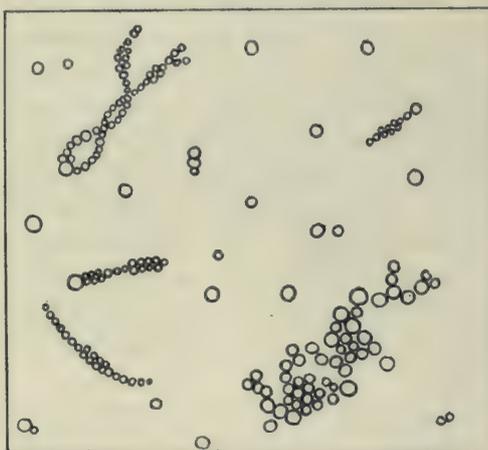


Fig. 1.

Gelatin spherites.  $\times 800$ .

After about 30 days the supernatant liquid was poured off and the preparations examined under the microscope. In addition to numerous spherites there were, however, many bacteria present. The experiments were, therefore, repeated in the presence of 0.1 % mercuric chloride<sup>1</sup> with similar results, except that bacteria were entirely absent and the spherites perhaps more numerous.

The buff-coloured precipitate was examined most conveniently on the base of the dish with a  $\frac{1}{15}$ " oil-immersion objective and  $8\times$  eyepiece giving a magnification of about 800. That from the 0.3 % sols usually gave the best results. The diagram shows the appearance. Numerous single spherites were seen, mostly of apparently about 2.0 to 2.2  $\mu$ , or actually from 0.25  $\mu$  to 0.28  $\mu$  in diameter. In addition there were many small and some large aggregates of spherites. A number of typical ones are shown in Fig. 1,

<sup>1</sup> The molecular theory of solution [Bradford, 1919] gives reasons for supposing that dilute  $\text{HgCl}_2$  would have little effect on the crystallisation of gelatin from water.

usually not more than two or three would be visible at once. The smaller spherites appear to prefer aggregation into filaments of which the individual elements were fairly distinct, but perhaps not quite as marked as in the diagram. The aggregates of larger spherites were usually in the form of colonies. Frequently the filaments appeared to originate from a large spherite, as shown, very much in the same way, on a smaller scale, as filaments often appear to have grown from spherites of silver dichromate and other inorganic substances. Except for the filaments and the more numerous detached spherites, the whole appearance is very similar to that of gelatin precipitated from a dilute sol by alcohol, or to the globulitic appearance observed ultra-microscopically by Bachmann, and to the spherites of mastic and gamboge obtained by Perrin.

Having regard to the appearance at first of the remarkable opalescence and its diminution as the precipitate appears, it cannot be doubted that gelatin crystallises from water exactly as indicated by von Weimarn's theory. The only possible complication in the cooling of the hot sols is that gelatin may melt in contact with water at a comparatively low temperature, so that the first separation may be into two liquid phases, one rich and the other poor in gelatin. Subsequent crystallisation from the more concentrated phase would result in still smaller submicrons.

In conclusion the writer wishes to express his indebtedness to Prof. J. B. Farmer for the kind loan of a high power microscope.

## REFERENCES.

- Arisz (1915). *Koll. Chem. Beihefte*, **7**, 1.  
Bachmann (1911). *Zeitsch. anorg. Chem.* **73**, 150.  
Bradford (1918). *Biochem. J.* **12**, 351.  
— (1919). *Phil. Mag.* **38**, 696.  
Bütschli (1896). *Ueber die Bau quellbarer Körper*. Göttingen.  
Hardy (1912). *Proc. Roy. Soc. A.* **87**, 29.  
Menz (1909). *Zeitsch. physikal. Chem.* **66**, 137.

# XI. A NOTE ON THE EFFECT OF PURGATION ON THE CREATININE CONTENT OF URINE.

BY DAVID BURNS.

*From the Department of Physiology, the University of Glasgow.*

*(Received February 16th, 1920.)*

ANDERSON and BOSWORTH [1916] found that the administration of inositol to man, in doses of about 0.5 g. per kilogram of body weight, produced an increased excretion of creatinine, both immediately after the initial dose and during the period succeeding the administration of this substance. They offer no explanation of this increase. (It is regrettable that they employed a diet containing meat as it has been demonstrated that the amount of creatinine excreted has no definite ratio to the amount of flesh ingested [Burns and Orr, 1916].) From their results two points bearing on this subject may be noted:

(a) Inositol is only to a slight extent absorbed by man and has no appreciable effect (except creatinine increase) on metabolism.

(b) The ingestion of inositol was invariably followed by diarrhoea.

Burns and Orr [1914], in studying the effect of copious ingestion of water on the nitrogen metabolism of man, came to the conclusion that the ingestion of water in large amounts either had no appreciable effect on the creatinine excretion or caused (one experiment) a temporary increase. Most of their subjects of experiment had, on the first day of increased water ingestion, rather loose stools. In one instance, the administration of three litres of water at one time caused such severe diarrhoea that the experiment had to be abandoned. The results of this experiment are appended (Table I), from which it will be seen that the creatinine excretion rose from 1339 mg. to 1385 mg. per day.

Table I.

Effect of diarrhoea induced by an excessive intake of water.

Total N.	Creatinine	Creatine	Notes
gm.	mg.		
12.8	1339	Nil	
13.6	1339		
13.1	1385	„	3 litres of water extra

The following experiments were designed to show how far the purging effect of inositol might be answerable for the creatinine increase.

Two subjects, B. and W., were employed. They had been often on experimental diets and could, therefore, be relied on to adhere strictly to the routine laid down. The diets prescribed (Table II) were creatine-creatinine-free. Urine was collected in 24 hour periods, made up to a volume of 2000 cc.,

and analysed immediately. After a suitable pre-period the subjects took three teaspoonfuls of Rochelle salts (or in Exp. 5 sodium bicarbonate and in Exp. 6 acid sodium phosphate) in warm water at 11 a.m. and 2 p.m. on one day. The collection of urine was continued for a day or two thereafter.

*Results.* Detailed results are appended in tabular form (Tables III, IV and V).

*Nitrogen.* The amount of nitrogen excreted by the kidneys varied but slightly. On the day after the purge the amount was somewhat less than on the preceding days, due probably to a retention of nitrogen succeeding the loss of nitrogen by the bowel on the day before.

*Creatinine.* In the first four experiments the creatinine output was increased on the day of the purge. In Exps. 1 and 2 (Table III) this increase

Table II.

## Diets used during experiments.

<i>Expt. 1.</i>	Breakfast:	Tea—two cups with milk and sugar Bread—one slice brown and one white, butter Fruit
	Lunch:	Four slices bread and cheese 250 cc. milk, 50 cc. water (hot) Essence of coffee
	Tea:	As breakfast
	Supper:	Porridge and milk (250 cc.)
<i>Expt. 2.</i>	Higher nitrogen intake	Same as for <i>Expt. 1</i> with the following exceptions: Breakfast: Cocoa instead of tea and one egg added Dinner and Supper: 300 cc. milk instead of 250 (no added water)
<i>Expt. 3.</i>	Higher calorie intake	Bread 650 g. Potatoes 300 Rice 200 Fruit 100 Margarine 50 Jelly 50 Porridge and milk, two eggs, chocolate (measured) Tea and cocoa (measured)
<i>Water Expt.</i>	As <i>Expt. 1</i> , <i>i.e.</i>	Protein 110 g. Carbohydrate 325 Fat 67

Table III.

## Effect of Rochelle salts per os on urinary creatinine.

<i>Expt. 1.</i>	Subject B.				
	Day	Reaction	Total N. (g.)	Creatinine (mg.)	Notes
	1	Neutral	12.5	1680	
	2	Neutral	12.8	1675	
	3	Neutral	12.7	1680	
	4	Alkaline	12.7	1690	Rochelle salts in warm water
	5	Slightly alkaline	11.9	1810	
<i>Expt. 2.</i>	Subject B.				
	1	Neutral	15.1	1680	
	2	Alkaline	15.1	1700	Rochelle salts in warm water
	3	Alkaline	12.9	1780	
	4	Neutral	14.98	1690	
	5	Neutral	14.7	1700	
<i>Expt. 3.</i>	Subject W.				
	1	Acid	12.96	1152	
	2	Acid	10.83	1142	
	3	Alkaline	10.33	1362	Rochelle salts in warm water
	4	Acid	9.83	1100	

was followed by a still further increase. In Exp. 3 the creatinine excretion on the day following the administration of Rochelle salts was slightly sub-normal. After  $\text{NaH}_2\text{PO}_4$  a decreased creatinine elimination was observed (Exp. 5, Table V).

Table IV.

Effect of  $\text{NaHCO}_3$  per os on urinary creatinine.

Expt. 4.	Subject W.	Day	Reaction	Total N. (g.)	Creatinine (mg.)	Creatine	Notes
		1	Neutral	10.4	1640	Nil	
		2	Neutral	10.4	1640	"	
		3	Acid	10.5	1680	"	
		4	Alkaline	10.6	1640	"	
		5	Alkaline	10.89	1634	"	8 g. $\text{NaHCO}_3$
		6	Acid	10.89	1640	"	(no purging effect)

Table V.

Effect of  $\text{NaH}_2\text{PO}_4$  per os on urinary creatinine.

Expt. 5.	Subject W.	Day	Reaction	Total N. (g.)	Creatinine (mg.)	Creatine	Notes
		6	Acid	10.89	1640	Nil	
		7	"	12.18	1630	"	16 g. $\text{NaH}_2\text{PO}_4$
		8	"	11.4	1550	"	
		9	"	11.01	1620	"	

To summarise; diarrhoea induced by:

1. excessive water ingestion,
2. irritation of intestinal wall by an inert substance (inositol—Anderson and Bosworth),
3. inrush of fluid into the intestine by exosmosis from the tissues (Rochelle salts),

is accompanied by an increase in the creatinine content of the urine.

This alteration in the creatinine output might at first sight seem to be associated with an alteration in the H<sup>+</sup> concentration of the urine. Underhill [1916, 1] has reported a series of experiments on rabbits and dogs in which he demonstrates a causal connection between acidosis<sup>1</sup> and creatinuria, and indisputably proves that relief of the acidosis by the administration of alkali leads to a decrease or even to a cessation of the elimination of creatine. The apparent connection between creatine and creatinine is apt to cause one to take for granted that on a creatine-fixed diet the appearance of creatine in the urine is accompanied by a fall in the amount of creatinine excreted—a totally gratuitous assumption. Underhill [1916, 2] in a second paper proves that while the administration of alkali prevents the elimination of creatine in the earlier period of a starvation acidosis, it does not materially affect the creatinine output. In Exp. 4, W. took 8 g. of  $\text{NaHCO}_3$  on one day. No diarrhoea resulted and there was no appreciable alteration in the creatinine output. Acid sodium phosphate in a dose of 16 g. reduced the creatinine content of the urine but did not cause the appearance of creatine.

<sup>1</sup> Acidosis is in this paper used in the clinical sense of the term.

Two reasons may be put forward as explanation of this increased creatinine excretion after diarrhoea:

(a) Increased tone of the visceral musculature.

The evidence of the connection of tone of striped muscle with the creatine content of muscle and with the creatinine content of urine seems conclusive [Pekelharing, 1911; Pekelharing and Harhink, 1910; Pekelharing and van Hoogenhuyze, 1916, 1, 2; Cathcart, Henderson and Paton, 1918; Riesser, 1916; Stenvers, 1917].

No evidence has however been brought forward to show that visceral muscle, under sustained contraction, leads to the elimination of increased amounts of creatinine. Further, the increased peristalsis of diarrhoea does not necessarily indicate increased tone of the visceral musculature. It has been proved that muscular work, apart from sustained contraction, does not cause an increased creatinine excretion.

(b) The sensation of well-being experienced after a purge leads one to the conclusion that the organism, having been freed from by-products and residues, is in better working order, and, as a whole, improved in tone. The experiment in which sodium biphosphate was given cannot in fairness be brought in evidence against this, as the acidosis produced would cause a decreased creatinine elimination [Underhill, 1916, 1].

#### SUMMARY.

Purgation by Rochelle salts or copious water ingestion leads to a slight increase in the amount of creatinine eliminated.

Administration of an alkali which does not induce purgation causes no appreciable alteration in the creatinine output.

The exhibition of an acid purgative ( $\text{NaH}_2\text{PO}_4$ ) leads to a slight decrease in the creatinine content of urine (acidosis phenomenon).

I have to thank Prof. Noël Paton for helpful guidance and kindly criticism.

The expenses were defrayed by a grant from the Carnegie Trust for which I express my thanks.

#### REFERENCES.

- Anderson and Bosworth (1916). *J. Biol. Chem.* **25**, 398.  
 Burns and Orr (1914). *Brit. Med. J.* **505**.  
 — (1916). *Biochem. J.* **10**, 495.  
 Cathcart, Henderson and Paton (1918). *J. Physiol.* **52**, 70.  
 Pekelharing (1911). *Zeitsch. physiol. Chem.* **75**, 207.  
 — and Harhink (1910). *Proc. Amster. Akad.* **14**, 310.  
 — and van Hoogenhuyze (1916, 1). *Kon. Akad. v. Wetensch. Natuurk. Afd.* **24**, 1577.  
 — (1916, 2). *Kon. Akad. v. Wetensch. Natuurk. Afd. Proc.* **18**, 1564.  
 Riesser (1916). *Arch. exp. Path. Pharm.* **80**, 183.  
 Stenvers (1917). *Ned. Tijdsch. v. Geneesk.* **61**, 85.  
 Underhill (1916, 1). *J. Biol. Chem.* **27**, 127.  
 — (1916, 2). *J. Biol. Chem.* **27**, 141.

## XII. THE PREPARATION OF SÖRENSEN'S PHOSPHATE SOLUTIONS WHEN THE PURE SALTS ARE NOT AVAILABLE.

By CHARLES JAMES MARTIN.

*From the Department of Experimental Pathology, Lister Institute.*

*(Received February 17th, 1920.)*

I HAVE found the following method useful and satisfactory when away from the resources of a well-equipped laboratory during the war, and it may be useful to others at the present time.

Recrystallise  $\text{NaH}_2\text{PO}_4$ ,  $\text{H}_2\text{O}$  of the B.P. Dry in air between filter paper. Make a supposed  $M/7.5$  solution of the recrystallised salt. Titrate 10 cc. of this against  $N/7.5$   $\text{NaOH}$ , adding 0.05 cc. of 0.5% solution of phenolphthalein as indicator. Add the soda until, at  $18^\circ\text{C}$ ., the colour is of the same intensity as that of a 0.0025% sol of  $\text{KMnO}_4$ .

If exactly 10 cc. of the  $N/7.5$   $\text{NaOH}$  is required, a  $M/15$  solution of  $\text{Na}_2\text{HPO}_4$  results. I have usually found, however, that slightly less than 10 cc. is required owing to imperfect drying of the recrystallised salt. We will call  $a$  the cc.  $\text{NaOH}$  required.

To make a litre of each phosphate solution proceed as follows:

(1) For  $M/15$   $\text{NaH}_2\text{PO}_4$  dissolve  $\frac{138.1}{15} \times \frac{10}{a}$  g. of the recrystallised salt and make up to 1 litre.

(2) For  $M/15$   $\text{Na}_2\text{HPO}_4$  dissolve  $\frac{138.1}{15} \times \frac{10}{a}$  g. of the recrystallised salt in about 500 cc., add 66.66 cc. of  $N$   $\text{NaOH}$  and make up to a litre.

The proportion in which these two solutions should be mixed to obtain any hydrogen ion concentration between  $\text{P}_\text{H} = 6$  and  $\text{P}_\text{H} = 8$  (within which range they are usable) is given by Sørensen [1909] and by Walpole [1911, 1914].

Moulds grow in phosphate solutions readily and are particularly troublesome in hot climates. Most antiseptics are not permissible. I have obviated this source of trouble by shaking the solutions with a little calomel for a few minutes and then filtering.

### REFERENCES.

- Sørensen (1909). *Compt. rend. Lab. Carlsberg*, 8, 1.  
Walpole (1911). *Biochem. J.* 5, 207.  
— (1914). *Biochem. J.* 8, 628.

### XIII. THE INFLUENCE OF REACTION ON COLOUR CHANGES IN TYROSINE SOLUTIONS.

BY ELFRIDA CONSTANCE VICTORIA VENN.

*From the Research Institute in Dairying, University College, Reading.*

*(Received February 24th, 1920.)*

THE work which is described in this paper is a continuation of that which was previously carried out on colour changes produced by two groups of bacteria on caseinogen and certain amino-acids [Cornish and Williams, 1917]. In that paper it was demonstrated that certain groups of bacteria which had been isolated from discoloured Stilton cheese were capable of producing changes of colour on certain media, consisting of simple suspensions or solutions in water of caseinogen and certain amino-acids, which are among its degradation products and many of which are known to be present in ripe Stilton cheese. In the course of that work it had been frequently found that the constitution and reaction of the media upon which the organisms were grown appeared to exercise a very considerable influence upon the degree of discoloration, which tended to vary with the nature of the amino-acids present and to diminish with increased acidity.

Further tests were therefore carried out to study the influence of these two factors. Water solutions or suspensions which were either neutral or slightly acid were made of caseinogen, tyrosine, tryptophan, histidine, alanine and cystine and, in addition, agar media containing these substances were prepared and standardised to a reaction of  $-1$  and  $-3$  according to Eyre's method. The growths upon these media produced some striking colours which are recorded in Cornish and Williams' paper.

The present paper is concerned with further investigations designed to discover the effect of varying degrees of acidity and alkalinity upon the colours produced by one strain upon a single amino-acid. For this purpose a member of the Gram-negative Alk. group [Cornish and Williams, 1917]  $C_{22} 4^B$  was chosen, and its colour-producing effects upon tyrosine were studied. This strain  $C_{22} 4^B$  had been found capable of producing colour in histidine water, alanine water, caseinogen water, tyrosine water, tryptophan water and upon agar media containing respectively these amino-acids. Tyrosine was selected as the amino-acid partly because it is present in very considerable quantities in ripe cheese and is prepared with relative ease, and partly because it is a medium upon which colour changes are easily produced.

As a preliminary experiment a solution of 0.1 % tyrosine was made, and from this solutions of varying reaction from approximately + 15 to - 35 (Eyre's standard) were prepared by the addition of the requisite quantities of sodium hydroxide or hydrochloric acid. After inoculation, colours were produced in those media, of which the reaction lay between + 5 and - 35. In the latter case the colour was black, and in the former there was only slight darkening of the solution. The tubes which contained solutions of

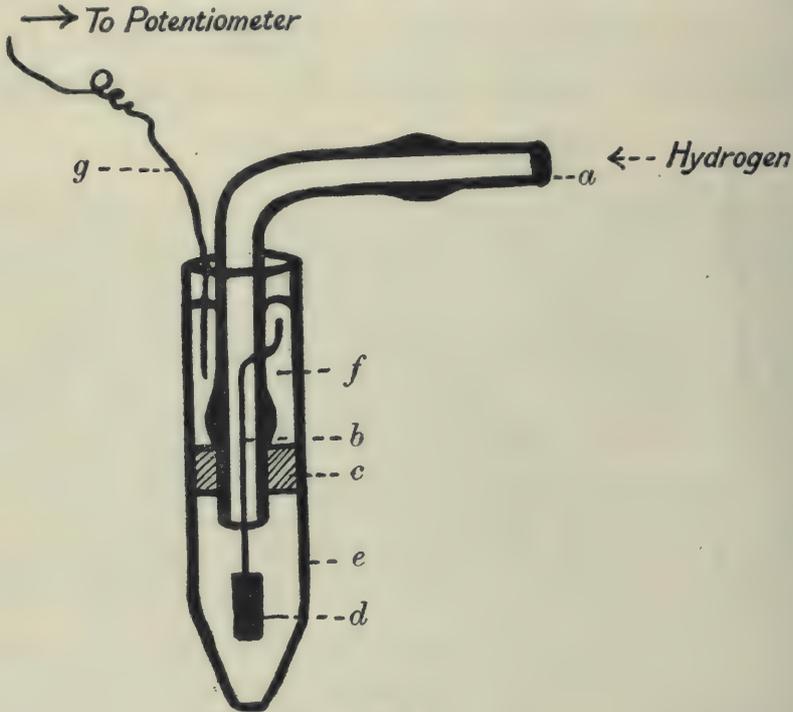


Fig. 1.

a = Glass tubing.

b = Platinum wire.

c = Rubber band.

d = Platinum foil.

e = Glass cover.

f = Mercury cup.

g = Wire leading to potentiometer.

greater acidity than + 5 showed no change in colour and the controls remained unchanged. In view of these observations the series was then extended and solutions were made up with reactions which varied between - 35 and - 85. At the end of 27 days no colour change was found in those solutions with a greater alkalinity than - 45. These results seemed to indicate that there was a certain range of reaction within which this strain of bacilli was capable of acting upon tyrosine with production of colour, but beyond which colour changes were not produced.

In order to obtain a more accurate measure of the range within which colour changes may be produced, it was decided to carry out electromotive force determinations on a further series of inoculated and control tyrosine solutions.

The apparatus used in these investigations was a Tinsleys's universal potentiometer, a very sensitive d'Arsonval galvanometer, and as a calomel half electrode the saturated cell described by Barendrecht [1915].

For some time an endeavour was made to use the small hydrogen electrode also described by Barendrecht, but it was found very difficult to obtain consistent results with this type of electrode, owing no doubt to the nature of the solutions under investigation and the difficulty of cleaning the electrode. The simple hydrogen electrode described below was therefore devised, and for this particular piece of work has been found to give consistently satisfactory results.

The hydrogen electrode is shown slightly larger than actual size in the accompanying diagram (Fig. 1). (*a*) is a piece of glass tubing bent at right angles and slightly thickened as shown. (*b*) is a length of platinum wire, one end being fused through the glass and the other one carrying a platinum plate (*d*). The outer glass covering (*e*) is held in position by the rubber band (*c*) and the space above is filled with mercury forming the cup (*f*) by which contact is made to the potentiometer by wire (*g*) by simply inserting the wire into the mercury.

The advantages of this simple electrode are two. There is no dead space or side tube, and it is very easily and quickly cleaned and platinised. The disadvantage is the use of rubber tubing, but if this ring is placed in the position shown, the liquid under investigation need never come into contact with the rubber. The electrode is used in the same way as that of Barendrecht [1915] and shares its advantage of being small and therefore needing less time for saturation than a larger one. When a reading has been completed, the mercury is tipped out, the outer tube and rubber ring are taken off, and the electrode is cleaned and replatinised. This is done between each determination. It is found that there is no danger of leakage if the sizes of the glass and rubber tubings are carefully adjusted.

The results obtained by this method are given in the following table from which it appears that there is a definite range of reaction over which the organism studied is capable of producing darkening in tyrosine solutions, and beyond which such discoloration fails to appear.

In all cases except Nos. 30 and 31, the  $P_H$  was determined within a month of the date of inoculation of the solution, and a control uninoculated solution was also tested in 31 cases. Solutions Nos. 30 and 31 were kept for 14 months after inoculation before the  $P_H$  was determined, but at the end of that time no colour had developed and the  $P_H$  values were those recorded.

No explanation is offered of the fact that the inoculated portion of solution No. 29 with a  $P_H$  of 1.95 shows a slight colour. Apart from this single instance there appears to be a gradual increase of colour from  $P_H$  3.23 passing through a maximum and gradually disappearing as the  $P_H$  approaches a value of about 9.7. The gradations in colour are not a perfect sequence, but if the whole series be considered it is found that, except in the case of No. 29, no colour

was produced at a  $P_H$  below 2.28; at 3.23 a lightish brown colour appeared which became a deep brown or reddish brown from 5.83 to 9.47. Above this point the colours are for the most part of a yellowish type until the limit is reached at 9.71.

Table of colour reactions produced in 0.1 % tyrosine solutions of varying  $P_H$ .

No.	Solution	$P_H$	Colour	No.	Solution	$P_H$	Colour
1	Inoculated	9.67	No colour	17	Inoculated	8.68	D.B.
	Control	9.71			Control	8.74	
2	Inoculated	9.63	V.SI.Y. with	18	Inoculated	8.43	V.D.B.
	Control	9.59	V.SI.P.		Control	8.57	
3	Inoculated	9.61	V.SI.Y.	19	Inoculated	8.32	D.B.
	Control	9.63			Control	8.38	
4	Inoculated	9.61	V.SI.Y.	20	Inoculated	8.20	D.P.B.
	Control	9.63			Control	8.60	
5	Inoculated	9.61	Cinnamon	21	Inoculated	7.51	D.B.
	Control	9.63			Control	7.63	
6	Inoculated	9.55	SI.P.Y.	22	Inoculated	6.69	D.B.
	Control	9.60			Control	7.15	
7	Inoculated	9.55	D.Y. with	23	Inoculated	5.83	V.D.B. with
	Control	9.58	P. tinge				SI.P.
8	Inoculated	9.54	V.V.V.SI.P.	24	Inoculated	3.23	L.B.
	Control	9.56			Control	(?) 2.30	
9	Inoculated	9.54	V.SI.Y.	25	Inoculated	2.59	No colour
	Control	9.53			Control	2.35	
10	Inoculated	9.50	P.B.	26	Inoculated	2.28	No colour
	Control	9.45			Control	2.47	
11	Inoculated	9.40	R.B.	27	Inoculated	2.26	No colour
	Control	9.40			Control	2.05	
12	Inoculated	9.39	V.V.SI.	28	Inoculated	2.09	No colour
	Control	9.45			Control	2.05	
13	Inoculated	9.14	V.D.B.	29	Inoculated	1.95	L.P.B.
	Control	8.87			Control	1.93	
14	Inoculated	8.91	D.R.B.	30	Inoculated	1.72	No colour
	Control	9.20			Control	1.71	No colour
15	Inoculated	8.87	R.B.	32	Inoculated	1.46	No colour
	Control	8.68			Control	1.63	
16	Inoculated	8.75	P.V.SI.B.	33	Inoculated	1.32	No colour
	Control	8.43			Control	1.30	

Abbreviations: B=Brown; D=Dark; L=Light; P=Pink; R=Red; SI=Slight; V=Very; Y=Yellow.

#### REFERENCES.

- Barendrecht (1915). *Biochem. J.* 9, 66.  
 Cornish and Williams (1917). *Biochem. J.* 11, 180.

## XIV. BARGER'S MICROSCOPICAL METHOD OF DETERMINING MOLECULAR WEIGHTS.

### PART I. THE PRINCIPLE OF THE METHOD WITH REFERENCE TO THE MOLECULAR AND IONIC ATTRACTION OF SOLUTE FOR SOLVENT.

BY KUMAO YAMAKAMI.

*From the Department of Experimental Pathology,  
The Lister Institute, London.*

*(Received February 24th, 1920.)*

A VERY reliable and convenient method has been described by Barger [1904] for determining molecular weights, which is explained by the author to be based upon the vapour pressure.

Its usefulness for studying the degree of association of acids, phenols, etc., when dissolved in mixtures of an associative and a non-associative liquid, has been proved by his interesting work which was published in the following year.

The technique of this method is quite simple. Series of drops, taken from two solutions, the one the standard, the other a given solution, of which the molecular weight of the solute is to be determined, are alternately introduced into a capillary tube, and the change in the length of the drops is studied by means of a micrometer. If one series of drops is observed to have increased while those alternating with them have decreased, it is to be assumed that the solution from which the former series of drops was taken has a higher molecular concentration than the latter.

The given solution is thus compared with a series of standard solutions of known molecular concentrations and in this way two limits are reached for the unknown molecular concentration.

The mechanism of this method is explained by Barger in the following way: "Each drop is placed between two others of a different solution and can evaporate on either side into a small air chamber. This closed air chamber is soon saturated with vapour, which can condense freely on the drops. If the vapour pressures of the two solutions are equal, the evaporation will equal the condensation, and there will be no change in the drops. If, on the other hand, the vapour pressures are unequal, there will be a gradient of the vapour pressure in the air spaces; some drops will therefore be in contact with an atmosphere, the vapour pressure of which is greater than their own.

Condensation will take place on these drops and they will increase. The others, alternating with them, have a vapour pressure greater than that of the adjoining air spaces; these drops will evaporate and thus decrease. Hence there is distillation from drops of the one series to those of the other series, although all are at the same temperature.”

In the course of an investigation of caseinogen solutions by this method, I have observed some facts which made me think it impossible to ascribe the mechanism of this method solely to vapour pressure.

I was determining the molecular and ionic concentration of alkali caseinogenate solutions, employing urea solutions as the standard. But it was feared that urea might combine with the alkali contained in the caseinogenate solutions. As Barger states in his paper, substances which may combine with the solute of the solutions employed are not to be used as standards because a certain amount of mixing of the two solutions is inevitable. All the drops of both series are successively taken in from one end of the capillary tube, so that the wall of the tube is wetted by each drop alternately. Therefore, not only the drops of one series are contaminated with the other solution when they are taken in, but also it cannot be denied that the drops remain in contact with each other by means of a thin layer of a mixture of the two solutions. Hence it becomes necessary that the standard substance should not combine chemically with the substance of unknown molecular weight.

Under these circumstances, I tried to modify the method in such a way that this limitation in the choice of the standard substance might be removed.

At first sight this appeared to be very easy because I thought a satisfactory result would always be obtained if the two solutions were brought into a closed air space avoiding contact with each other.

But this was not the case. The volume change of solutions thus confined in an air-tight chamber was found to proceed extraordinarily slowly, if they were not allowed to touch each other, even when the two solutions were very close together. Such fairly regular and rapid change in volume of the solutions, as is seen in Barger's method, was very difficult to obtain.

The protocol of the experiments described in the following section illustrates this fact.

*Experiment I.* In the capillary tubes which are generally employed in Barger's method two small drops of solutions of different molecular concentrations were introduced from either end of the tube, so that the wall of the tube between the drops was left partly unwetted by the solutions. The lengths of the tubes were all 8 cm. The first drop was introduced in the usual way and allowed to slide down to the middle of the tube and then the other drop was introduced from the other end and allowed to slip down for a distance of 2 cm., the first drop, accordingly, going back to a point 2 cm. away from the other end. Each drop therefore stood 2 cm. away from its respective end and 4 cm. away from the other; 2 cm. of the wall of the tube between the drops remained dry, while the other 2 cm. were wetted by the first drop.

The ends of the tubes were then carefully sealed by heating. The tubes were then placed under water in Petri dishes, and the change of the drops at room temperature,  $12^{\circ}$ - $16.5^{\circ}$ , was studied every 24 hours, after the initial length of the drops had been measured immediately after the preparation of the tubes.

The results are given in Table I, in tenths of scale divisions, as explained below after Table III.

Table I.

No. of tube	Diameter of tube	Change in length of drop No. 1 (0.5 M urea solution)							
		Initial length	1st 24 h.	2nd 24 h.	3rd 24 h.	4th 24 h.	5th 24 h.	6th 24 h.	7th 24 h.
1	480	520	-10	-10	-5	-3	—	—	—
2	443	530	-12	-12	-3	-2	—	—	—
3	450	420	-6	-6	-10	-8	—	—	—
4	400	413	-8	-7	-5	-5	—	—	—
5	510	212	-13	-5	-4	-2	0	0	+2
6	467	242	-8	-3	-3	+2	+5	0	+3
7	492	356	-11	-13	-0	-0	-2	+1	-1

No. of tube	Diameter of tube	Change in length of drop No. 2 (0.1 M urea solution)							
		Initial length	1st 24 h.	2nd 24 h.	3rd 24 h.	4th 24 h.	5th 24 h.	6th 24 h.	7th 24 h.
1	480	462	-46	-42	-40	-21	—	—	—
2	443	236	-41	-42	-15	-40	—	—	—
3	450	436	-48	-30	-35	-15	—	—	—
4	400	630	-42	-30	-18	-16	—	—	—
5	510	322	-47	-41	-24	-19	-15	-8	-17
6	467	760	-30	-42	-25	-17	-6	-15	-9
7	492	449	-43	-27	-5	-16	-18	-7	-5

It will be easily realized how quickly and regularly the change of drops takes place in Barger's method, if the above protocol is compared with the change of drops observed under normal conditions, as is shown in Table II.

*Experiment II.* 0.5 M solution of urea was compared with 0.1 M solution. In Table II the drops indicated by odd numbers are those of 0.5 M solution, and the even numbers denote the drops of 0.1 M solution. The lengths of the air spaces between the drops were also measured in order to make the relation of each drop clear (they are placed in brackets).

Table II.

No. of tube	Drops					Diameter of tube
	1	2	3	4	5	
1 (860)	585 (925) +13	550 (1150) -12	528 (840) +8	600 (870) -15	601 (1200) +9	460
2 (950)	683 (1080) +14	540 (847) -15	522 (705) +8	680 (824) -14	521 (915) +14	445
3 (1045)	487 (1210) +10	479 (920) -17	545 (965) +17	569 (1030) -12	500 (995) +11	515

The initial measurement was made immediately after the preparation of the tubes, and the second determination was made 2.5 hours afterwards at room temperature, 15°.

As shown in Table II, the change in the lengths of the drops is quite decisive, the drops of the stronger solution increasing regularly and the drops of the weaker solution decreasing, only 2.5 hours after the preparation of the tubes, whereas such a change does not occur even after 100 hours at the same temperature and with the same solutions if the drops are 4 cm. apart and 2 cm. of the wall between the drops is left dry, as described in Exp. I, Table I. The difference between the drop-changes in these two cases seems too great to be attributed solely to the greater distance between the drops in Exp. I. That vapour pressure plays only a subsidiary part results also from experiments which were made with solutions of which an accurately known volume (about 0.4 cc.) was delivered on to weighed dry filter papers (3 × 12 cm.) placed parallel and face to face 6–7 mm. apart in stoppered glass cylinders at 37°. After 24 hours the cylinder was weighed before and after the removal of each paper separately, thus giving the weight of the papers by difference. Both papers invariably lost weight (0.06–0.15 g.) and no sort of regularity could be discerned unless the papers were allowed to touch after the solutions had been uniformly distributed in them. In that case it was possible to distinguish between 0.1 and 0.166, and between 0.1 and 0.05 molar concentrations, by the differences in the loss of weight.

These experiments with tubes and filter papers compelled me to doubt the importance of the vapour pressure in causing the change of drop-length in Barger's method.

The next experiment seems to prove definitely the existence of a factor other than the vapour pressure, the action of which appears to depend on the fact that the two solutions are in contact.

*Experiment III.* 0.5 *M* and 0.25 *M* aqueous solutions of urea, boric acid and cane sugar were prepared and the drop-change according to Barger's method was investigated in the two series.

Now, if the change of volume of the drops is produced solely by the difference in vapour pressures of neighbouring drops, this change must stop when the concentrations have all become equal, owing to the dilution of the drops of the stronger solution, and the concentration of the drops of the weaker solution by evaporation. The decrease in volume of drops taken from 0.25 *M* solutions must cease before their original length is halved, because their molecular concentration must be 0.5 at this point, if the decrease has taken place only by evaporation of the solvent.

If the drops of 0.25 *M* solution had ever been shown to have become just half of their initial volume, it would mean that distillation had proceeded from the stronger solution to the weaker, for the concentration of the 0.25 *M* drops would then be 0.5 *M*, while that of the drops of 0.5 *M* solution would be correspondingly weaker than 0.5 *M*.

This remarkable result has, indeed, been observed. The decrease in length of drops taken from 0.25 *M* solution actually proceeded until they were less than one-third of their original length, as shown in Table III (a), (b), (c).

It is impossible to explain this experimental observation without assuming that the drop-change occurred because of a transference, not only of vapour, but also of liquid from the weaker to the stronger solution, that is, the stronger solution attracted the solvent of the weaker solution through the film furnished by the liquid membrane which extended between the two drops. As the liquid membrane, differing from the semipermeable membrane which is generally employed in osmotic pressure experiments, is perfectly permeable for both solute and solvent, so the dissolved solute molecules have accompanied the solvent molecules to the stronger solution and consequently the 0.25 *M* drops were not correspondingly concentrated when diminution of their volume occurred.

Similarly, the dilution of the stronger solutions did not go parallel with the increase of their volume, which would be expected if the increase of the drops were caused by condensation of vapour only.

This seems to be the most probable explanation for the further increase of the volume beyond the point at which it would stop if the change depended solely on vapour pressure.

Table III (a)

		30 hours at 35° C.					Diameter
		1	2	3	4	5	of
		(0.25 <i>M</i> )	(0.5 <i>M</i> )	(0.25 <i>M</i> )	(0.5 <i>M</i> )	(0.25 <i>M</i> )	tube
Urea	(850)	270 135	(750) 532 640	(1070) 264 140	(960) 600 740	(910) 254 110	(750) 455
"	(850)	304 140	(845) 684 890	(1020) 270 125	(860) 642 765	(1200) 230 101	(710) 515
Boric acid	(1000)	145 35	(850) 635 725	(990) 172 55	(888) 650 780	(940) 210 70	(775) 465

(b)

		30 hours at 35° C.					Diameter
		1	2	3	4	5	of
		(urea)	(sugar)	(urea)	(sugar)	(urea)	tube
Sugar, 0.5 <i>M</i>	(890)	193	(680) 521	(810) 274	(800) 630	(850) 290	(1000) 480
Urea, 0.25 <i>M</i>	(890)	35	(680) 670	(810) 95	(800) 800	(850) 110	(1000) 480
"	(890)	430 280	(1110) 360 510	(900) 395 240	(1030) 380 520	(1150) 460 310	(1300) 525
"	(1150)	304 151	(1000) 740 890	(860) 325 170	(1090) 695 850	(980) 220 55	(1060) 510

(c)

		5 days at 13–16° C.					Diameter
		1	2	3	4	5	of
		(0.25 <i>M</i> )	(0.5 <i>M</i> )	(0.25 <i>M</i> )	(0.5 <i>M</i> )	(0.25 <i>M</i> )	tube
Urea	(895)	383 173	(945) 781 1004	(1005) 423 210	(1100) 867 1060	(975) 447 235	(1000) 445
"	(1030)	445 218	(965) 817 1030	(749) 390 177	(1010) 720 930	(940) 464 261	(920) 430
Boric acid	(910)	350 169	(744) 606 806	(780) 266 94	(772) 640 865	(916) 340 126	(850) 525

The measurement of the drop-length was made with the ocular micrometer 0.1 mm. No. 2 and Leitz objective No. 2.

One scale division of this micrometer corresponded to  $\frac{1}{37}$  mm. when tested by means of a Carl Zeiss objective micrometer, 0.01 mm. The readings were made and are recorded throughout this paper in tenths of scale divisions. As the length of the intervals between the drops varied from 750 to 1400 tenths of a scale division, so the actual lengths of the intervals in the experiments were 2–4 mm., that is, a little greater than those indicated by Barger.

The fact that the 0.25 *M* drops decrease until they are less than one-third of their original length shows how small is the contribution of vapour pressure to the change of drops compared with the other factor.

In the following experiments the velocity of evaporation, which takes place from the surfaces of drops confined in glass tubes, was investigated in the case of aqueous solutions of various concentrations, by measuring the decrease in length after standing for some time at constant temperature.

The object of these experiments was to gain information as to the relative parts played by the vapour pressure and the second factor in changing the length of the drops in Barger's method.

*Experiment IV.* The leading idea of this experiment was that, if the vapour pressure is solely responsible for the change of drop-volume, then the difference between the velocity of evaporation of the weaker solution and that of the stronger solution must be equal to the magnitude of the volume-change in drops according to Barger's method, but if, on the other hand, the difference of these two velocities is not equal to the magnitude of the change, then the ratio of these two must represent the relative contribution of the vapour pressure and the other factor in bringing about the change.

The velocity of evaporation from the surface of the drops was found by measuring the decrease in their length when singly confined in capillary tubes.

The results thus obtained are presented in Table IV (a), (b), (c), (d).

*Explanation of Table IV (a), (b).* One small drop of distilled water was introduced into a capillary tube, both ends were carefully sealed and it was mounted on a glass slide, put in a Petri dish, and the decrease in length of the drop was observed at a constant temperature of 35°.

It was found that the drop thus confined in the capillary tube decreases in length fairly regularly with lapse of time. The length of the water drops decreased 80–96 tenths of a scale division during the first 24 hours. This appeared to be approximately the maximal amount which can evaporate from the surface of drops at this temperature and during this period, since increasing the length of the capillary tube did not result in a more rapid decrease of the drop-length. In an 8 cm. capillary tube, the amount of evaporation was not appreciably affected by the presence of another water drop near the other end of the tube.

This fact shows that the humidity of the air produced by evaporation during this period is not sufficiently great to influence appreciably the velocity

of evaporation. This conclusion is supported furthermore by the fact that the decrease in volume proceeds with almost the same speed during the next 24 hours.

In this experiment the approximate diameter of the capillary tube was measured, because it was feared that the surface dimension of the drops might not be exactly directly proportional to the diameter; in other words, the evaporating surface of a drop in a narrow tube might be relatively greater than that of a similar drop in a wider tube. But this apprehension proved to be unfounded. The amount of decrease was not influenced to any great extent by the difference of diameter within the variation of diameter of the tubes which we employed.

Table IV (a)

Single water drops in capillary tubes of varying length at 35° C.

No. of tube	Length of tube	Diameter of tube	Initial length of drops	Change in the 1st 2 hours	Change in the 1st 24 hours	Change in the 2nd 24 hours
1	6 cm.	450	726	- 4	-91	-85
2	8	460	962	- 8	-87	-90
3	10	455	453	- 6	-83	-88
4	12	400	775	- 5	-92	-94
5	14	430	680	-15	-98	-93

(b)

Two water drops in one tube (8 cm. long) at 35° C.

No. of tube	Diameter of tube	Drop No. I				Drop No. II			
		Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours	Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours
1	480	370	-86	-86	-79	466	-82	-80	-74
2	420	642	-85	-91	-69	440	-89	-81	-82
3	475	469	-86	-82	-67	303	-78	-76	-60
4	510	332	-84	-77	-74	496	-91	-85	-60
5	455	518	-87	-80	-71	421	-83	-83	-75

*Explanation of Table IV (c), (d).* When a substance was dissolved in the water, the velocity of evaporation expressed in terms of the diminution in length of the drops decreased with the concentration of the solution, as shown in Table IV (c), (d).

The amount of evaporation from drops of 0.5 *M* urea solution at a constant temperature of 35° in the first 24 hours varied from 28-39 tenths of a scale division, the mean decrease in length being 32.

Table IV (c)

Single drop and two drops of urea solution (0.5*M*) in one tube at 35° C.

No. of tube	Diameter of tube	Drop No. I				Drop No. II			
		Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours	Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours
1	480	469	-32	-12	8	—	—	—	—
2	410	332	-38	-23	3	—	—	—	—
3	505	308	-39	-21	18	—	—	—	—
4	445	242	-31	-21	4	533	-39	-28	- 4
5	492	440	-34	-16	2	442	-28	-18	-14
6	465	428	-30	-22	11	415	-29	-24	- 8
7	460	302	-29	-14	6	320	-34	-16	- 2

The amount of evaporation from 0.25 *M* drops was found to be 53 tenths of a scale division as is shown in the Table IV (*d*).

Table IV (*d*)

Two drops of urea solution (0.25*M*) in one tube at 35° C.

No. of tube	Diameter of tube	Drop No. I				Drop No. II			
		Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours	Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours
1	420	244	-55	-36	-23	267	-44	-50	-32
2	440	501	-56	-35	-21	486	-52	-37	-20
3	510	320	-55	-37	-19	352	-57	-37	-11
4	435	382	-51	-42	-21	340	-54	-34	-26

Now if the change (increase and decrease) of volume of two drops in Barger's method is solely due to a difference of their vapour pressures, such volume-change should be equal to the difference between their decreases, when they are investigated separately as in Table IV (*a*)–(*d*), at the same temperature and for the same length of time.

The magnitude of change would, therefore, be expected to be about  $87.4 - 32.0 = 55.4$  when 0.5 *M* urea solution is compared with distilled water.

In the same way, the drops would change their length 34.4 ( $87.4 - 53.0$ ) when 0.25 *M* urea solution is compared with water, and 21.0 tenths of a scale division when 0.5 *M* solution is compared with 0.25 *M* solution.

But the change of drops actually observed at 35° for 24 hours was far greater than these calculated values (Table IV (*e*), (*f*), (*g*)).

The amount of change of the drops was 249, 148.5, 128.8 (mean values) respectively in the cases of 0.5 *M* : water; 0.25 *M* : water; and 0.5 *M* : 0.25 *M* (Table IV (*e*), (*f*), (*g*)).

The amount of change in the drops when compared at the same temperature for the same length of time was thus found to be about 5–6 times greater than the value calculated from the velocity of evaporation of each solution. It must be assumed therefore that the proportion of change due to the vapour pressure is only  $\frac{1}{5}$ – $\frac{1}{6}$  of the entire change.

This ratio was also obtained in the case of boric acid solutions; when, however, lactose solution was investigated in this way, the ratio was a little smaller. The mean amount of evaporation from a single drop was respectively 0.5 *M* = 30, 0.25 *M* = 50, 0.1 *M* = 70.2, while the mean change of drops according to Barger's method was respectively

0.5 <i>M</i> : water	...	...	175
0.25 <i>M</i> : water	...	...	90
0.1 <i>M</i> : water	...	...	68
0.5 <i>M</i> : 0.25 <i>M</i>	...	...	68
0.5 <i>M</i> : 0.1 <i>M</i>	...	...	112
0.25 <i>M</i> : 0.1 <i>M</i>	...	...	53

Table IV (e)

The amount of volume change of drops in Barger's method, at 35° C., in the first 24 hours, 0.5M urea compared with water.

No. of tube	Diameter of tube	1 (urea)	2 (water)	3 (urea)	4 (water)	5 (urea)
1	470	(1040) 635 + 246 (1100)	674 - 224 (1200)	593 + 297 (1210)	676 - 231 (1095)	781 + 260 (1100)
2	415	(1070) 614 + 271 (1120)	586 - 201 (1180)	707 + 213 (1150)	770 - 220 (1200)	680 + 270 (1280)
3	575	(980) 620 + 265 (1085)	640 - 235 (1060)	914 + 238 (990)	680 - 252 (1170)	663 + 220 (1300)

The mean amount of volume change = 249 tenths of a scale division.

(f)

The amount of volume change of drops in Barger's method, at 35° C., in the first 24 hours, 0.25M urea compared with water.

No. of tube	Diameter of tube	1 (urea)	2 (water)	3 (urea)	4 (water)	5 (urea)
1	470	(1510) 484 + 136 (1090)	599 - 129 (1070)	425 + 160 (830)	520 - 150 (1330)	610 + 150
2	510	(1315) 450 + 160 (940)	462 - 147 (1400)	365 + 130 (1250)	520 - 133 (1150)	431 + 159
3	440	(1225) 465 + 148 (985)	437 - 153 (1210)	442 + 138 (1345)	415 - 129 (980)	514 + 146

The mean amount of volume change = 148.5 tenths of a scale division.

(g)

The amount of volume change of drops in Barger's method, at 35° C., in the first 24 hours, 0.5M compared with 0.25M urea.

No. of tube	Diameter of tube	1 (0.5M)	2 (0.25M)	3 (0.5M)	4 (0.25M)	5 (0.5M)
1	490	(860) 585 + 132 (925)	550 - 95 (1150)	520 + 114 (840)	471 - 124 (870)	720 + 110 (1200)
2	425	(950) 683 + 121 (1080)	540 - 102 (845)	522 + 140 (705)	525 - 150 (624)	676 + 140 (1190)
3	415	(980) 593 + 128 (985)	564 - 115 (725)	492 + 127 (965)	530 - 141 (910)	588 + 148 (1080)

The mean amount of volume change = 128.8 tenths of a scale division.

(The numbers in brackets denote the lengths of the air spaces between the drops.)

The ratio between the calculated value and the actual amount for lactose solutions was respectively

0.5M : water	...	...	3.0 : 1
0.25M : water	...	...	2.4 : 1
0.1M : water	...	...	4.0 : 1
0.5M : 0.25M	...	...	3.5 : 1
0.5M : 0.1M	...	...	2.8 : 1
0.25M : 0.1M	...	...	2.6 : 1

This small ratio probably does not indicate a greater part played by the vapour pressure here than in the case of urea or boric acid solutions, because

the evaporation of the single drops also takes place with a smaller speed than in the case of these latter solutions.

The probable explanation for this smaller ratio in the former case is that the molecules of sugar have a greater weight than those of urea and boric acid, hence the speed with which the former move in the solution is smaller than that of the latter. Now, in the case of the single drops, the surface of the drops becomes concentrated when evaporation proceeds, and this is also the case with the drops of weaker solutions in Barger's method, while the surface of the drops of stronger solutions becomes diluted by the entering of solvent.

It will take some time before these concentrated or diluted surface layers of the drops mix completely with the interior portions, causing the whole drop to become homogeneous. The slower the molecule moves the more time this diffusion will take, and thus the surface layer of the drops containing heavier molecules will remain longer in a concentrated or diluted condition than the surface of drops with lighter molecules.

This will result in slower evaporation of a solution with large molecules (this does not necessarily mean that the vapour pressure of such a solution is smaller). The smaller amount of evaporation, obtained in the single drop experiment, must have been due to this process. On the other hand, the volume-change of drops in Barger's method will be lessened by the slow mixing of the surface layer with the interior.

Thus the calculated amount and that actually observed are both influenced by the slowness of the movement of the molecules, and their ratio is affected doubly by this cause.

These ratios did not change when the same experiment was performed at  $0^{\circ}$ , although the amount of evaporation as well as the amount of the drop-change in Barger's method was reduced to  $\frac{1}{6}$ — $\frac{1}{7}$  of the value measured at  $35^{\circ}$ . It is of course evident that these ratios will change according to the length of the intervals between the drops. The part played by the streaming of solvent through the contact film of the drops will become smaller with the increase of the distance between them, so that the change of drops will be produced finally only by the difference of vapour pressure when some limit of distance has been exceeded. But under ordinary conditions, as directed by Barger (2–3 mm.), the amount of change ascribable to vapour pressure seems to be very small, and his method becomes quite unpracticable if the distance between the drops is so great that the drop-change is produced solely by the difference of vapour pressure.

The next question to be solved is what is the other factor which causes the volume-change of drops in this method. This cannot be other than the molecular and ionic attraction of solute for solvent, namely the force which is known also to give rise to the phenomenon of osmotic pressure. In this connection it seems to me that the "bombardment" theory of osmotic pressure, as represented in the text-books of Nernst, Lewis, Hedin, etc., is

less acceptable than van't Hoff's conception, particularly as explained more precisely by Mathews [1916].

"Osmosis" should be explained as a phenomenon of the wandering of the solvent molecules from a point where a weaker attractive power of solute molecules exists towards a point where a stronger attractive power is exerted. It does not matter whether there is a semipermeable membrane or not.

The force which produces the change of drops in Barger's method should thus be considered as the molecular-ionic attraction of solute for solvent.

Moreover, the change of the drops themselves seems to be due both to osmosis and to distillation, which are both caused by the same internal energy of solution, namely, the molecular-ionic attraction.

#### SUMMARY.

The principle upon which Barger's microscopical method of determining molecular weights is based has been studied, and it has been proved that the force which effects the volume-change of the drops is not simply the vapour pressure, but also the osmosis through the thin film of liquid between the drops. This latter factor plays the greater part in producing the change.

If the length of the intervals between the drops is 2-3 mm.,  $\frac{5}{8}$  to  $\frac{6}{7}$  of the volume-change of the drops is attributable to osmosis.

Further, as osmosis as well as the lowering of vapour pressure are equally the results of one and the same internal energy of solution, that is, the molecular and ionic attraction of solute for solvent, which is of the same magnitude for all kinds of molecules and ions in the same solvent at the same temperature, it is possible to measure molecular weights or the degree of association or dissociation by investigating the volume-change of drops which are brought in contact with drops of standard solutions of known molecular concentrations.

In conclusion I wish to express my heartiest thanks to Dr C. J. Martin, the Director of the Lister Institute, who has kindly supplied me with material and apparatus. Prof. G. Barger, who has read this paper in manuscript, informs me that he is in general agreement with my conclusions. He has frequently observed the effect of Exp. III, when using organic solvents. Like myself in my experiments with filter papers he has entirely failed in repeated attempts to bring about a regular isothermic distillation in a closed space, when there was no film of liquid between two solutions differing only slightly in vapour pressure.

#### REFERENCES.

- Barger (1904). *J. Chem. Soc.* **85**, 286.  
Mathews (1916). *Physiological Chemistry*, 2nd ed., William Wood & Co.

## XV. THE FORMATION OF FERROUS SULPHIDE IN EGGS DURING COOKING.

BY CHARLES KENNETH TINKLER AND MARION CROSSLAND SOAR.

*From the Chemical Laboratory, Household and Social Science  
Department, King's College for Women.*

*(Received February 27th, 1920.)*

It is a matter of common experience that on prolonged cooking of an egg in its shell (*e.g.* when it is immersed in boiling water for 15 minutes or longer) a greenish black coloration is produced on the surface of the yolk.

It is also well known that if a so-called "hard-boiled egg" be immersed in cold water immediately after cooking, the green colour is either not apparent at all, or is much less marked than is the case when the egg is allowed to cool slowly.

In view of the fact that this coloration is only on the *surface* of the yolk, it would appear that in its production some chemical action takes place, either between some constituent of the membrane which surrounds the yolk and some constituent of the yolk itself, or that some substance which is produced from the egg white, and which can penetrate this membrane, interacts with some constituent of the yolk.

### *The Nature of the Coloured Substance.*

It appeared probable at the outset of the investigation that the greenish black coloration was due to ferrous sulphide in a fine state of division, and the various experiments which have been made seem to confirm this view.

Thus the coloured substance is readily soluble in dilute acids, the solution so obtained giving positive reactions for iron and evolving hydrogen sulphide on warming, whereas cooked egg yolk showing no greenish colour does not give these reactions. Moreover, the coloured egg yolk is decolorised on prolonged exposure to air or on treatment with hydrogen peroxide. This is also the case with the greenish coloured precipitate of ferrous sulphide when, for example, this substance is precipitated in a fine state of division on a piece of filter paper by the interaction of a solution of ferrous sulphate and ammonium sulphide.

It is well known that egg white on prolonged heating evolves considerable quantities of hydrogen sulphide and that the yolk of egg contains iron, probably in combination with proteins. The amount of iron in egg yolk, according to Sherman [1914], is 0.0085 % by weight. A solution of ferrous

ammonium sulphate containing iron equivalent to that in egg yolk was made up and portions of the solution poured on to filter papers. On the addition of colourless ammonium sulphide to these filter papers, colorations due to ferrous sulphide were obtained, which were almost identical with those obtained on the yolks of eggs when unbroken eggs are immersed for 15 minutes or more in boiling water.

Pollacci [1904] has investigated the losses of sulphur which take place on heating yolk and white of egg separately, and also the effect of keeping uncooked whole eggs for 24 hours in an atmosphere of hydrogen sulphide. The chocolate brown colour produced in the yolk of the uncooked egg in this case is said to be due to the formation of iron sulphide.

*The Effect of Prolonged Heating on Yolk and White of Egg separately.*

Evidence that the green coloration was due to interaction between constituents of both the yolk and the white was obtained in the following way. Two yolks in their membranes, separated completely from egg white and washed with distilled water, were heated in boiling water for 20 minutes. One was then cooled quickly by washing in a stream of cold water, and the other was allowed to cool slowly. In neither case was the slightest green colour observed, but on placing portions of these yolks in a gas jar containing hydrogen sulphide to which a few drops of ammonia had been added, green colours were obtained both in the portions still covered with membrane and on those taken from the inside of the yolk.

It was found that the green colour was produced only very slowly by the action of hydrogen sulphide alone, but was immediately formed on the addition of ammonia.

Yolk of egg, separated from egg white, was also heated alone in a test tube for 90 minutes in boiling water, and even in this case no green coloration was apparent. Cooked egg white treated in the same way with hydrogen sulphide and ammonia gives no coloration until a considerable time has elapsed, and even then the colour produced is very faint. It should be noted in this connection that the iron content of egg white (0.0001 %) is only about one-eighty-fifth that of egg yolk [Sherman, 1914]. According to Pollacci [1904], egg white is free from iron.

From the results of these experiments it may be concluded that some constituent of the white of egg plays a part in the formation of the green layer of ferrous sulphide. Additional evidence that the yolk alone does not give rise to both constituents necessary for the formation of the ferrous sulphide is afforded by the fact that the latter is only produced as a thin layer on the surface of the yolk. If both the iron and sulphur were provided by the yolk, the green coloration would probably extend through the whole of the yolk, diminishing in intensity on approaching the centre, owing to the fact that the temperature at the centre of the egg does not rise as rapidly on cooking as that of the outside of the yolk.

It can also be shown that whereas neither white nor yolk of egg on heating alone shows any green coloration, when heated in contact with one another the characteristic green coloration is produced (see below).

*Experiments relating to the Membrane round the Yolk.*

In order to determine whether or not any constituent of the membrane round the yolk takes any part in the reaction, an experiment was made as follows. Egg white was heated in a test tube until coagulated, and egg yolk, free from membrane, was then poured into the test tube to form a layer above the white. On heating the test tube for about 15 minutes in boiling water, a green ring was obtained at the junction of the yolk and white.

On examining a "hard-boiled" egg, the colourless yolk membrane can be seen adhering to the inside of the white of the egg, and does not become absorbed by the yolk as appears at first.

It would seem, therefore, that the membrane round the yolk does not play any part in the formation of the green coloration. It must, however, be permeable to hydrogen sulphide.

*The Effect of Time of Heating on the Coloration obtained.*

Two eggs were heated in boiling water for 15 minutes and two for 30 minutes. In both cases one of the eggs was cooled down quickly by removing the shell and placing in a stream of running water, while the other was allowed to cool with the water in which it had been heated.

In the case of the eggs heated for 15 minutes practically no green colour was obtained on the yolk of the egg which was cooled quickly, whereas a slight green colour was apparent on the other.

In the case of the eggs heated for 30 minutes, very little difference was apparent in the amount of green colour developed, whether the egg was cooled quickly or allowed to cool with the water; but a much more intense coloration was produced round the yolks of these eggs than round the yolks of those heated only for 15 minutes.

It appears, therefore, as would be expected, that the amount of green coloration depends upon the length of time the egg is heated, and that the effect of the cold water in preventing the formation of the green colour is simply that, on cooling the egg quickly, the preliminary decomposition necessary for the formation of ferrous sulphide is checked. The act of placing a hard-boiled egg in cold water does not therefore lead to the *removal* of the green coloration already formed, but prevents its formation.

This view is supported by the following observation. Two eggs were heated in boiling water for 15 minutes, the shell of one was removed at once and the white of the egg removed. No green colour was observed. The white was then replaced round the yolk and secured with cotton to prevent the access of air. The egg was allowed to cool slowly, and when cold, the white of the egg was again removed. The green coloration was then apparent.

The other egg was allowed to cool in the water in which it had been cooked and, on examination when cold, was found to show the usual green coloration round the yolk. In addition to the effect of the cold water in checking the decomposition of the egg white, the rapid cooling on the outside of the egg will lead to the diffusion of the hydrogen sulphide from the white away from the yolk, and in this way, also diminish the amount of ferrous sulphide formed.

In order to investigate further the effect of prolonged heating on the formation of the colour, two eggs were heated in boiling water, one for two hours, when still only a thin film of green round the yolk was apparent, and the other for seven hours. In the latter case the colour of the white after cooking was buff and the green layer was thicker than in the previous case, especially on one side where its thickness was about one millimetre.

Subsequent experiments showed that if the yolk of an egg after cooking is not surrounded by a layer of white of uniform thickness, the thickness of the layer of ferrous sulphide is greater where the yolk has been surrounded by a greater thickness of white.

The object of heating one of the eggs for seven hours was also to ascertain, if possible, whether the hydrogen sulphide from the white could penetrate the thin film of ferrous sulphide at first formed, and produce in the yolk layers of ferrous sulphide as in the Liesegang phenomenon. No such layers were, however, apparent, either in the yolk of this egg cooked in boiling water for seven hours, or in an egg yolk which, completely surrounded by its membrane, washed free from adhering white, and cooked in boiling water for 20 minutes, was then placed in a gas jar with hydrogen sulphide and ammonia.

It was found that if an uncooked egg is kept for a day in an atmosphere of hydrogen sulphide, as in Pollacci's experiments, and then heated in boiling water for 20 minutes, the greenish black coloration due to ferrous sulphide extends throughout the whole of the yolk.

#### *The Effect of the Age of the Egg upon the Amount of Green Coloration.*

It was thought that the age of the egg might determine to a considerable extent the amount of green coloration formed on prolonged cooking. To ascertain if this were so, three eggs, (1) an egg laid one day previously, (2) an egg laid five weeks previously, (3) an egg laid six weeks previously, were immersed in boiling water for 20 minutes and allowed to cool in the water. The yolks were then separated from the whites and treated in exactly the same way, each being immersed in dilute iron-free sulphuric acid. After ten minutes the acid liquid was filtered and the solutions diluted to the same volume in each case. There was certainly a difference in the amount of iron contained in the solutions; the extract from the yolk of the fresh egg containing least iron, and that from the six week old egg most, but the difference was not very great. This was in accordance with the appearance of the yolks before treatment with the acid. Only slightly more green coloration was

apparent in the case of the egg laid six weeks previously, than in the case of the one day egg.

*The Liberation of Hydrogen Sulphide from Egg White and Yolk.*

Although the sulphur content of the yolk of an egg—0.157 %—is not very much less than that of the white—0.196 % [Sherman 1914]—this element is evidently in less stable combination in the latter than in the former. On heating white and yolk of egg separately in a test tube immersed in boiling water, hydrogen sulphide is evolved (shown by its action upon lead acetate paper) from the white after about three and a half minutes, while no trace of the gas is obtained from the yolk in this time.

An approximate comparison of the amounts of hydrogen sulphide obtained on distilling equal weights of egg white and yolk (1 g.) with water (400 cc.) was obtained by the addition of standard lead acetate solution to the distillates, and comparing the colours obtained with those formed with known amounts of the lead acetate solution to which hydrogen sulphide solution had been added.

In the case of the egg white the first 100 cc. of the distillate was matched by 2.5 cc. of standard lead acetate solution (1 cc. = 0.0001 g. Pb), whereas a similar volume of the distillate from the egg yolk was free from hydrogen sulphide.

On continuing the distillation 2 cc. of the standard lead solution were required for the next 100 cc. of the distillate from the egg white and 0.7 cc. from a similar volume of distillate from the yolk.

Sulphuretted hydrogen is thus more readily obtained from egg white than from egg yolk. [See also results obtained by Pollacci, 1904.]

*The Reactions of Egg White and Yolk towards Litmus.*

Uncooked egg white has an alkaline reaction, and uncooked egg yolk an acid reaction, towards litmus. It was at first thought possible that ammonia, or a substituted ammonia, might be produced by the decomposition of the egg white. With the hydrogen sulphide this ammonia or amine might pass through the membrane, and by neutralising the acid of the yolk facilitate the production of ferrous sulphide. Small quantities of ammonia and substituted ammonia are produced both from the white and yolk on distillation with water, but this ammonia is perhaps not necessary for the production of the ferrous sulphide, as it was noticed in the course of the experiments that the yolk of egg, which is originally acid to litmus, becomes alkaline on heating.

On heating a mixture of egg yolk and water containing blue litmus the indicator is seen to change colour at approximately 70°.

When an egg is heated in boiling water in the ordinary process of "boiling" for three to five minutes, the temperature of the yolk probably does not reach 70°, so that if hydrogen sulphide were present it might possibly not react immediately with the yolk to form ferrous sulphide, owing to the acid

reaction of the yolk at a temperature below 70°. On prolonged heating, however, the yolk will become alkaline and the conditions for the formation of ferrous sulphide will be favourable.

*The Formation of Ferrous Sulphide in preserved and dried Eggs.*

Eggs which have been preserved in water glass appear to behave normally with regard to the formation of ferrous sulphide on cooking. Thus two eggs which had been preserved for ten months in water glass were boiled for 20 minutes. One was cooled quickly and the other allowed to cool slowly. In the case of the former no formation of ferrous sulphide was apparent, but in the case of the latter the usual appearance of the substance on the surface of the yolk was noted.

Eggs which have been dried appear to be altered in some way which largely prevents the formation of iron sulphide on prolonged cooking. Thus if the white and yolk of an egg be beaten together and some of the mixture heated in a test tube in boiling water for 20 minutes, a slight green colour is apparent, whereas if the beaten egg is dried in a vacuum desiccator and then mixed with water immediately after drying, only a very faint coloration is observed on cooking. If the dried egg is kept for some time, the formation of ferrous sulphide on cooking does not take place. Some commercial preparations of dried eggs also failed to show the formation of ferrous sulphide on cooking.

SUMMARY.

The greenish black coloration observed on the surface of the yolk of a "hard-boiled" egg appears to be due to the formation of ferrous sulphide, the hydrogen sulphide necessary for the formation of the substance being produced by the decomposition of a sulphur compound of the egg white.

The non-formation of the coloration when a "hard-boiled" egg is placed in cold water immediately after cooking is due to the checking of the decomposition owing to cooling.

REFERENCES.

- Pollacci (1904). *Gazzetta*, 34 (i), 278.  
Sherman (1914). *Food Products* (Macmillan).

## XVI. ON THE PRESENCE OF AMYLASE IN MILK AND CHEESE.

BY MASAYOSHI SATO.

*From the Columbia University, New York.*

*(Received February 28th, 1920.)*

THERE has been little investigation regarding enzymes in raw milk, especially amylase. In 1883, according to the literature, Béchamp [1883] isolated from human milk an enzyme which liquefies starch and converts it into sugar, and named it galactozymase. He claimed that the enzyme was not present in cow's milk. His work was recognised by Bouchut [1885], Moro [1898], Biolchini and Luzzati [1902], Hippus [1905], and Spolverini [1902]; the last named found very little amylase in dog's, ass's and human milk and none in cow's and goat's milk.

Zeitscheck [1904] found evidence of the existence of slight amylolytic activity in all the samples of human, ass's, horse's, goat's, buffalo's and cow's milk examined by him. Spontagh [1905] also proved the presence of amylase in cow's and other milk which he examined.

Wohlgemuth and Strich [1910] were unable to obtain any evidence of the presence of amylase in cow's milk but found it in dog's, rabbit's and guinea-pig's milk, dog's milk containing most and guinea-pig's the least.

Koning [1907] recognised, on all occasions, evidence of amylase in cow's milk; thus he found that about 15–20 mg. of starch were decomposed by 100 cc. of milk in half-an-hour at 37°; and Giffhorn [1909] also found that 100 cc. of milk will decompose from 10–25 mg. of starch in one half-hour at 37°. Lane-Clayton [1913] carried out investigations upon the amylase content of sterile cow's milk collected by means of a collecting tube. Amylase was found in each experiment, but the amount present was so small that the 10 cc. of milk used were able to hydrolyse only from 0.001–0.002 g. of starch in three hours at 37°.

Regarding the activity of amylase in milk Koning found that the optimum temperature was 45° and that it was destroyed by heating the milk for 45 minutes at 68°, but Giffhorn found that the enzyme action was destroyed by heating at 65° for 30 minutes.

In regard to the source of this ferment in cow's milk there are some diversities of opinion among investigators.

According to Spolverini this ferment was obtained from cow's and goat's milk, which did not generally contain amylase, when the animals were fed with fodder containing a large quantity of starch, such as malt, and in such cases he was of opinion that a part of the amylase of milk may be derived from the udder and illustrated it by Dupony's experiment in which he proved that the amount of amylase was increased in the blood, liver and kidney by feeding with carbohydrates. But this opinion was denied by van der Velde and Landtscher [1909] who got negative results by the same experiment.

Koning showed that amylase was present in milk of animals with mastitis and that in normal milk the first and middle milks were richer than the strippings and also that certain bacteria were capable of producing amylase, but he believed that the amylolytic action obtained by him with cow's milk was not due to contamination by bacteria. Giffhorn also believed that amylase is increased considerably in milk by the pathological condition of the udders.

Wohlgemuth and Strich insisted that a part of the amylase of milk may be derived from the blood, but the milk generally contains at least 100 times as much amylase as the blood, the great part of this being a product of the activity of the mammary glands. Grimmer [1913] obtained evidence of its presence by his experiment on the gland tissue of animals of which the milk showed only little or no trace of amylase. Upon this experimental basis he suggested that amylase in milk was the product of the mammary gland.

As an application of the detection of the existence of amylase in milk, Koning carried out an experiment with the object of discovering whether milk has been boiled before being sold, and also stated that the presence of milk from a mastitis cow can be discovered by testing for this ferment. The former is generally used as a supplementary test to detect whether milk has been boiled or not, but the latter observation is not, at present, recognised as a reliable aid in the diagnosis of mastitis.

As to the degree to which starch is broken down by the amylase in milk, Moro [1902] (working with human milk) believed that some of it was converted into dextrin and maltose but that most of it remained in the stage of dextrin. The difficulty of estimating the terminal point of this reaction in milk is, however, very great, since it is almost impossible to detect the formation of minute quantities of maltose.

In short, at the present time nothing definite is known regarding the existence, origin and biological action of this enzyme in cow's milk. We also lack knowledge of any literature on amylase in cheese.

In the present investigation, as the first step, we examined raw milk for the existence and activity of amylase, and likewise the water-extract of fat-free cheese, determining in each case the power of liquefying potato- and also rice-starch, to see what difference if any exists in the effect of the amylase of raw milk and of cheese in liquefying these two starches.

*Materials.*

Milk and cheese.—The grade *A* raw certified milk and American Cheddar cheese used in the experiment were supplied by the Sheffield Farm Company in New York city.

Distilled water.—The distilled water which was used in the experiment was twice redistilled, first from alkaline permanganate solution into a reservoir whence it siphoned into a second flask from which it was again distilled. Phosphoric acid was added to this portion in order to retain any ammonia that might have come from the first distillation. Tin condensers were used and the final distillate was caught in "Nonsol" bottles, contact with the air being avoided as far as possible.

Substrates.—The substrates used in the experiment were eight samples, five of potato- and three of rice-starch, prepared by the following methods:

No. 1. Commercial potato-starch as purchased and further purified by repeated washing with distilled water.

No. 2. Potato-starch was prepared in the laboratory from mature August potatoes, care being taken to prevent contamination by organisms, dust, fumes, or water containing electrolytes.

No. 3. Potato-starch was prepared from potato-starch No. 2 by washing with 0.3 % NaOH.

No. 4. Lintner soluble potato-starch purchased from Merck and further purified with distilled water.

No. 5. Soluble potato-starch prepared from potato-starch No. 2 in the laboratory by Brown and Morris' method of treatment with dilute hydrochloric acid.

No. 6. Rice-starch prepared in the laboratory from the commercial product in the same way as potato-starch No. 2.

No. 7. Rice-starch prepared from rice-starch No. 6 by washing with 0.3 % NaOH.

No. 8. Soluble rice-starch prepared from starch No. 6 in the laboratory in the same way as the soluble potato-starch (No. 5).

NOTE:—For convenience, the different preparations of starch are designated by the following figures:

P.S. I = Starch No. 1	P.S. V = Starch No. 5
P.S. II = " " 2	R.S. I = " " 6
P.S. III = " " 3	R.S. II = " " 7
P.S. IV = " " 4	R.S. III = " " 8

*Apparatus.*

Thermostat.—The digestion of starch by the amylase was carried out in a bath which contained water kept at 40° by means of carbon filament lamps. The water in the bath was stirred constantly to ensure an even temperature throughout the experiment.

Glassware.—All glassware used in the experiment was treated to avoid any condition which could effect enzyme action.

*Analysis of the starch.*

The moisture, amylose and acidity of the various samples of starch were determined by means of the following methods:

*Moisture.* From 0.2 to 1.0 g. was dried in an air-oven at 100°–110° for four hours, allowed to cool in a desiccator over sulphuric acid for half-an-hour, and weighed.

*Amylose.* Amylose was determined by hydrolysing to glucose and estimating the glucose by Fehling's volumetric method.

*Acidity.* Acidity was determined by titration of the starch paste and solution with 0.001 *N* sodium hydroxide or sulphuric acid, using rosolic acid as indicator.

The results obtained are given in Table I.

Table I.

Substance	Moisture %	Amylose %	Acidity (cc. of <i>N</i> /1000 acid or alkali, per 1 g. amylose)
P.S. I ...	14.64	84.59	9.21 (H <sub>2</sub> SO <sub>4</sub> )
P.S. II ...	13.79	85.88	7.19 "
P.S. III ...	16.65	89.29	14.22 "
P.S. IV ...	9.73	90.00	3.83 (NaOH)
P.S. V ...	13.84	85.55	2.44 (H <sub>2</sub> SO <sub>4</sub> )
R.S. I ...	8.43	91.09	3.49 "
R.S. II ...	11.69	86.54	7.89 "
R.S. III ...	9.26	90.36	4.21 "

*Preparation of potato- and rice-starch paste and solution.*

The amount of starch equivalent to 1 g. of amylose was placed in an Erlenmeyer flask and 80 cc. distilled water added. The liquid was then heated slowly, with constant shaking, until it boiled, and the boiling was continued during three minutes. The flask was then cooled and the alkalinity or acidity of the starch was corrected by adding to each starch paste or solution sufficient *N*/1000 H<sub>2</sub>SO<sub>4</sub> or *N*/1000 NaOH to make it exactly neutral, after which the volume was made up to 100 cc. with distilled water and the sample kept in an ice-box at 7°–8° until used.

*The amylase activity of raw milk.*

As soon as the milk sample reached the laboratory, it was well mixed and, after the estimation of its acidity with *N*/10 NaOH, was divided into two portions, one of which was kept raw, the other pasteurised by heating for 30 minutes at about 80°. The former was used for the enzyme examination, and the latter as control.

The following procedure based upon the method of Koning with slight modification was used.

Two series of test-tubes were very carefully cleaned and dried, placed on the test-tube-stand and the whole set in a bath of ice-water. Then known

quantities of starch paste or solution were carefully measured in increasing amounts, *e.g.* 0.001 cc., 0.002, 0.003, ..., into each of the tubes by means of a long 1 cc. pipette (divided into 1/100 cc.) reaching to the bottom of the test-tube, thus avoiding the lodging of any of the solution on the side of the tube.

10 cc. of raw or pasteurised milk, which had been kept in the ice-box at 7°–8°, were very accurately measured and introduced into each tube and agitated to ensure a perfect mixture.

The test-tube-stand was transferred from the ice-water to the thermostat and after various lengths of time was taken out and placed in the ice-water to stop the action. After a few minutes exactly 1 cc. of iodine solution (1 g. iodine, 2 g. potassium iodide + 300 cc. water) was added to each tube, and the contents thoroughly mixed. The tube in which the blue or violet colour had entirely disappeared, giving place either to red, orange or yellow, was noted and the power of the enzyme was calculated as the number of grams of starch which is digested to this stage in a given time by 100 cc. of raw milk.

### Results.

The quantity of starch decomposed by nine samples of milk in half-an-hour and by four samples in 24 hours<sup>1</sup> is given in Table II.

In the control experiment with pasteurised milk it was found that 10 cc. of the milk was not able to hydrolyse more than 0.002 g. of starch (0.001 g. of starch in 10 cc. of milk cannot be detected by this method).

Table II.

(1) The quantity of starch decomposed by 100 cc. raw milk in 30 minutes at 40°.

	P.S. I	P.S. II	P.S. III	P.S. IV	P.S. V	R.S. I	R.S. II	R.S. III
	g.	g.	g.	g.	g.	g.	g.	g.
Average of 9 experiments	0.0092	0.0093	0.0096	0.0151	0.0154	0.009	0.010	0.0081
Maximum	0.011	0.011	0.011	0.021	0.021	0.010	0.012	0.010
Minimum	0.005	0.005	0.005	0.012	0.012	0.005	0.005	0.004

(2) The quantity of starch decomposed by 100 cc. of raw milk in 24 hours at 40°.

	P.S. I	P.S. II	P.S. III	P.S. IV	P.S. V	R.S. I	R.S. II	R.S. III
	g.	g.	g.	g.	g.	g.	g.	g.
Average of 4 experiments	0.0233	0.0235	0.0245	0.034	0.035	0.0233	0.0243	0.023
Maximum	0.026	0.027	0.027	0.035	0.036	0.025	0.026	0.025
Minimum	0.022	0.022	0.022	0.034	0.034	0.022	0.023	0.022

From Table II it will be seen that the eight preparations of starch employed can be arranged in the following order, according to the readiness with which they were digested:

#### *Digestion for 30 minutes.*

P.S. V; P.S. IV; R.S. II; P.S. III; P.S. II; P.S. I; R.S. I; R.S. III.

#### *Digestion for 24 hours.*

P.S. V; P.S. IV; P.S. III; R.S. II; P.S. II; (P.S. I; R.S. I); R.S. III.

In all cases, preparation P.S. V was decomposed most readily, and the digestion of R.S. III was the slowest. Preparation R.S. II which was washed with alkali was decomposed somewhat faster than P.S. III which was treated

<sup>1</sup> 0.3 cc. of toluene was added in these cases per 10 cc. of milk as antiseptic.

in the same way, in the course of digestion during 30 minutes. But, on the contrary, the digestion of the former preparation was rather less than that of the latter in the course of 24 hours.

We undertook the next experiment, choosing three preparations. In order to make these relationships clearer the digestion of three of the preparations P.S. III, P.S. V and R.S. II was carried out for 30 minutes, 1, 3, 5, 7, and 24 hours respectively; the results are given in Table III.

0.3 cc. of toluene was used in each experiment per 10 cc. of milk.

Table III.

The quantity of starch decomposed by 100 cc. of raw milk.

	<i>In 30 minutes.</i>			Acidity of milk N/10 alkali per 100 cc. cc.
	P.S. III g.	P.S. V g.	R.S. II g.	
Average of 8 experiments	0.0074	0.0164	0.0083	23.53
Maximum ... ..	0.010	0.018	0.010	23.92
Minimum ... ..	0.005	0.015	0.007	22.88
<i>In 1 hour.</i>				
Average of 8 experiments	0.0094	0.0244	0.0099	
Maximum ... ..	0.012	0.027	0.012	
Minimum ... ..	0.007	0.022	0.008	
<i>In 3 hours.</i>				
Average of 8 experiments	0.0123	0.0296	0.0126	
Maximum ... ..	0.016	0.031	0.016	
Minimum ... ..	0.009	0.028	0.010	
<i>In 5 hours.</i>				
Average of 8 experiments	0.0144	0.033	0.0145	
Maximum ... ..	0.019	0.035	0.019	
Minimum ... ..	0.010	0.031	0.010	
<i>In 7 hours.</i>				
Average of 8 experiments	0.015	0.0327	0.0151	
Maximum ... ..	0.020	0.034	0.020	
Minimum ... ..	0.010	0.032	0.020	
<i>In 24 hours.</i>				
Average of 8 experiments	0.0182	0.0363	0.0165	
Maximum ... ..	0.025	0.037	0.023	
Minimum ... ..	0.011	0.035	0.010	

As shown in Table III, the amount decomposed during 30 minutes was the largest in the case of P.S. V, the order being P.S. V—R.S. II—P.S. III.

This order was also maintained for the digestions of one, three, five and seven hours respectively, but was disturbed in the digestion of 24 hours by P.S. III which now came ahead of R.S. II, the order being accordingly changed to P.S. V—P.S. III—R.S. II. P.S. V was always decomposed most readily. Thus we could ascertain the fact that, at the beginning of digestion, rice-starch is decomposed a little faster than potato-starch, though the difference cannot be said to be very remarkable.

## THE PRESENCE OF AMYLASE IN CHEESE.

*Composition of the cheese.*

The composition<sup>1</sup> of the samples of cheese used in this experiment was as follows (Table IV):

Table IV.

		Cheese sample I %	Cheese sample II %
Water	... ..	34.720	36.883
Fat	... ..	29.623	31.175
Protein	... ..	31.175	27.175
Lactic acid, etc.	... ..	1.450	1.262
Ash	... ..	3.032	3.504

*Preparation of a water-extract of fat-free cheese.*

The following method was used for the preparation of a water-extract of fat-free cheese.

The crusts of the cheese were removed with a knife and it was then well ground in a mortar and dehydrated with alcohol. The ground cheese was extracted with ether in a Soxhlet apparatus for about three days or longer, in order to remove the fat completely. The fat-free material was then spread on a porous plate, dried at the room temperature and ground to a fine powder. 10 g. of this material were transferred to a stoppered flask containing distilled water, kept cold (7°-8°), and allowed to stand in the ice-box with frequent shaking for 1½ or 24 hours. In the latter case 1 cc. of toluene was used per 50 cc. of water as antiseptic. The solution was then filtered by suction through a Buchner funnel, containing a thin mat of long-fibred asbestos, and made up to 500 cc. with distilled water.

*Method of experiment.*

The experiment was carried out in the same way as that with milk, 0.3 cc. of toluene being added for 10 cc. water-extract of fat-free cheese.

As the first step, the activities of the amylase of the 24 hours' water-extract with toluene and 1½ hours' water-extract without toluene were determined, using potato soluble starch as substrate.

The results are given in Table V.

Table V.

Time	1½ hours' water-extract (without toluene)		24 hours' water-extract (with toluene)	
	Exp. I g.	Exp. II g.	Exp. I g.	Exp. II g.
30 minutes ... ..	0.008	0.0095	0.003	0.006
1 hour ... ..	0.010	0.012	0.004	0.008

<sup>1</sup> The analyses were made by Richmond's method.

It will be seen from Table V that when the 24 hours' extract with toluene is compared with the  $1\frac{1}{2}$  hours' extract without antiseptics, the latter is found to be much more active than the former. Further experiments on this point were therefore considered unnecessary, and in studying amylase in cheese the  $1\frac{1}{2}$  hours' extract without addition of toluene was used throughout the investigation.

*Results of experiments.*

We investigated the digestion of the four kinds of starch P.S. III, P.S. V, R.S. II, R.S. III, comparing the quantity decomposed by cheese samples I and II during the lapse of 30 minutes, 1, 3, 5 and 24 hours respectively, and obtained the results shown in Tables VI, VII and VIII.

Table VI.

*Cheese Sample I.*

Grams of starch decomposed by 100 cc. of the water-extract of 2 g. fat-free cheese (=5.835 g. of original cheese at 40°).

	<i>In 30 minutes.</i>				Acidity of water-extract of fat-free cheese N/10 alkali per 100 cc.
	P.S. III g.	P.S. V g.	R.S. II g.	R.S. III g.	
Average of 19 experiments	0.0062	0.0073	0.0079	0.0054	6.60
Maximum ... ..	0.009	0.009	0.009	0.007	
Minimum ... ..	0.005	0.006	0.006	0.004	
<i>In 1 hour.</i>					
Average of 11 experiments	0.0081	0.0096	0.0108	0.0077	
Maximum ... ..	0.009	0.011	0.011	0.009	
Minimum ... ..	0.007	0.008	0.009	0.007	
<i>In 3 hours.</i>					
Average of 12 experiments	0.0141	0.019	0.0158	0.0146	
Maximum ... ..	0.015	0.021	0.018	0.016	
Minimum ... ..	0.013	0.018	0.014	0.013	
<i>In 5 hours.</i>					
Average of 5 experiments	0.0206	0.025	0.0202	0.0174	
Maximum ... ..	0.022	0.026	0.021	0.018	
Minimum ... ..	0.019	0.024	0.019	0.017	
<i>In 24 hours.</i>					
Average of 3 experiments	0.0303	0.036	0.0293	0.022	
Maximum ... ..	0.031	0.037	0.030	0.024	
Minimum ... ..	0.030	0.035	0.029	0.021	

Table VII.

*Cheese Sample II.*

Grams of starch decomposed by 100 cc. water-extract of 2 g. fat-free cheese (=6.519 g. of original cheese at 40°).

<i>In 30 minutes.</i>				
	P.S. III	P.S. V	R.S. II	R.S. III
	g.	g.	g.	g.
Average of 7 experiments	0.0109	0.0126	0.0119	0.009
Maximum ... ..	0.012	0.013	0.013	0.010
Minimum ... ..	0.010	0.012	0.011	0.008
<i>In 1 hour.</i>				
Average of 7 experiments	0.0156	0.0174	0.0146	0.0124
Maximum ... ..	0.017	0.018	0.015	0.014
Minimum ... ..	0.015	0.016	0.014	0.012
<i>In 3 hours.</i>				
Average of 7 experiments	0.021	0.026	0.020	0.0189
Maximum ... ..	0.022	0.027	0.021	0.020
Minimum ... ..	0.020	0.025	0.019	0.018
<i>In 5 hours.</i>				
Average of 7 experiments	0.024	0.031	0.022	0.0209
Maximum ... ..	0.025	0.032	0.024	0.022
Minimum ... ..	0.023	0.030	0.022	0.019

Calculating the amount of starch decomposed by 100 g. of the original cheese from the averages given in Tables VI and VII, the following results were obtained (Table VIII):

Table VIII.

*Cheese Sample I.*

Time of digestion	P.S. III	P.S. V	R.S. II	R.S. III
	g.	g.	g.	g.
30 mins. ... ..	0.1063	0.1251	0.1357	0.0925
1 hr. ... ..	0.1388	0.1645	0.1851	0.1320
3 hrs. ... ..	0.2416	0.3256	0.2691	0.2485
5 ,, ... ..	0.3530	0.4284	0.3464	0.2982
24 ,, ... ..	0.5193	0.6170	0.5021	0.3770

*Cheese Sample II.*

30 mins. ... ..	0.1672	0.1902	0.1825	0.1381
1 hr. ... ..	0.2393	0.2669	0.2240	0.1902
3 hrs. ... ..	0.3068	0.3988	0.3068	0.2899
5 ,, ... ..	0.3682	0.4755	0.3528	0.3206

It will be observed in Tables VI, VII and VIII that the different samples of starch decomposed by the amylase of cheese can be arranged in the following order according to the amount decomposed.

*Cheese Sample I.*

30 mins.	...	...	...	R.S. II	P.S. V	P.S. III	R.S. III
1 hr.	...	...	...	R.S. II	P.S. V	P.S. III	R.S. III
3 hrs.	...	...	...	P.S. V	R.S. II	R.S. III	P.S. III
5 "	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III
24 "	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III

*Cheese Sample II.*

30 mins.	...	...	...	P.S. V	R.S. II	P.S. III	R.S. III
1 hr.	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III
3 hrs.	...	...	...	P.S. V	R.S. II (P.S. III)		R.S. III
5 "	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III

Thus we could confirm the same facts as were found with milk that the preparation P.S. V was decomposed most easily, that the digestion of R.S. III was the worst and that the digestion of R.S. II was better than that of P.S. III.

SUMMARY.

1. The results of Spontagh, Zeitscheck, Wohlgemuth and Strich, Koning, Giffhorn, and Lane-Claypon regarding the presence of amylase in raw milk are confirmed.
2. For the quantity of soluble potato-starch decomposed by 100 cc. of raw milk, we find practically the same result as Koning and Giffhorn at half-an-hour, but more than was observed by Lane-Claypon in three hours.
3. As a result of our investigation, it appears that amylase is universally present in commercial Cheddar cheese, although the amount of this ferment varies within considerable limits.
4. Soluble potato-starch is digested more easily than potato- and rice-starch, both by milk and by the amylase of cheese.
5. Rice-starch seems to be more decomposed than potato-starch within a certain number of hours but the difference is so small as not to be distinct.
6. Soluble rice-starch is less decomposed than other starches in each experiment but it is uncertain what is responsible for this and further investigation is required.

I have to thank Prof. H. C. Sherman for helpful suggestion and interest throughout the work.

## REFERENCES.

- Béchamp (1883). *Compt. Rend.* **96**, 1508.  
Biolchini and Luzzati (1902). *Arch. Kinderheilk.* **33**, 460.  
Bouchut (1885). *Hyg. de la prem. Enfance*, 102.  
Giffhorn (1909). *Dissertation, Hannover; Rev. gen. du Lait*, 1911, **8**, No. 22.  
Grimmer (1913). *Biochem. Zeitsch.* **53**, 429.  
Hippius (1905). *Jahrb. Kinderheilk.* **61**, 365.  
Koning (1907). *Milchwirtsch. Zentr.* **3**, 41.  
Lane-Clayton (1913). *Report to the Local Govt. Board*, N.S. **76**, 52.  
Moro (1898). *Jahrb. Kinderheilk.* **47**, 342.  
— (1902). *Jahrb. Kinderheilk.* **56**, 362.  
Spolverini (1902). *Rev. Hyg. Med. Infant.* **1**, 252.  
Spontagh (1905). *Jahrb. Kinderheilk.* **62**, 715.  
van der Velde and Landtscher (1909). *Arch. Med. Infant.* **6**, 408.  
Wohlgemuth and Strich (1910). *Sitzungsber. Preuss. Akad. Wissensch.* **1**, 520.  
Zeitscheck (1904). *Arch. ges. Physiol.* **104**, 539.

## XVII. THE ANTISCORBUTIC REQUIREMENTS OF THE MONKEY.

BY ARTHUR HARDEN AND SYLVESTER SOLOMON ZILVA.

*From the Biochemical Department, Lister Institute.*

*(Received February 27th, 1920.)*

THE use of the monkey as an experimental animal in the investigation of scurvy and other deficiency diseases is becoming more and more extensive, owing chiefly to the fact that a great similarity exists between the clinical symptoms of the disease induced in these animals and the symptoms which occur in human subjects suffering from a similar disease contracted through the agency of faulty nutrition. Such experiments, however, are of a prolonged nature and several months must elapse before the results are known. This drawback coupled with the comparative scarcity and high price of the monkey makes this animal in many instances less suitable for experimental scurvy than the guinea-pig. The latter animal is plentiful in this country and results can be obtained with it within a few weeks. One can therefore consider these two animals as complementary in the technique of experimental scurvy, and it is accordingly desirable to establish some quantitative relationship as regards dose and time between them, so that results obtained with one can be translated if necessary into terms of the other.

It is now well established that guinea-pigs which are fed on a scorbutic diet of oats, bran and autoclaved milk manifest well-declared symptoms of the disease after about 15–21 days, although, as was shown by Zilva and Wells [1919], microscopical changes of the teeth may be present as early as 7–10 days after the commencement of the experiment. Our previous experiments [1919] with monkeys have shown that these animals usually manifest symptoms of scurvy after about two months. We have thus an approximate relationship as to the time taken by the two animals to develop scurvy when maintained on scorbutic diets.

It was the object of this investigation to find out the minimum dose of orange juice for monkeys. Orange juice was chosen because it is one of the most common antiscorbutics employed at present in infant feeding and on account of its sweet taste and comparatively low content in acids it can be administered without producing any digestive disturbances, both in human subjects and animals.

It is unlikely that orange juice has a constant antiscorbutic content, since this most probably varies with the variety of the fruit, time of the year and

other factors. This fact must be taken into consideration especially when, as in this case, the experiment is spread over a period of several months. It is at present impossible to standardise an antiscorbutic as regards its therapeutic potency by any chemical method, and the biological method now in vogue requires several weeks before a definite result is obtained and even then the degree of accuracy is not very great. Under these circumstances freshly expressed orange juice was considered to be the most suitable form of the antiscorbutic for our purpose. According to Chick and Rhodes [1918] the minimum dose of orange juice for a guinea-pig lies between  $1\frac{1}{2}$  cc.—3 cc. per diem. Our own experience in this laboratory confirms this observation.

Five monkeys receiving 5 cc., 2 cc., 1 cc., 0.75 cc., 0.5 cc. of orange juice respectively were utilised in this experiment. The animals received a scorbutic diet consisting of rice, wheat germ, salt mixture, and butter. The daily ration, which was qualitatively and quantitatively adequate, was made up as follows:

Rice	...	...	300 g.
Wheat germ	...	...	50
Salt mixture	...	...	2
Butter	...	...	5

The orange juice was administered daily *per os* under strictly quantitative conditions, the smaller doses being previously diluted with sweetened water. The orange juice was pressed out fresh every day and endeavours were made to obtain oranges of the same variety throughout the duration of the experiment.

Doses of orange juice of 5 cc. and 2 cc. have proved to be sufficient to ward off scurvy. The monkeys receiving these doses thrived well and gained in weight. The animals on the smaller quantities of juice developed scurvy which in some cases was severe enough to prove eventually fatal. The following are the protocols of the experiments.

Monkey No. 16. *Macacus rhesus*, male. Started on a scorbutic diet on May 13th, 1918 and received 0.5 cc. of orange juice daily. The weight of the animal, which at the commencement of the experiment was 2600 g., was maintained, apart from small fluctuations, until the end of June. After that period the animal commenced losing weight. Scorbutic symptoms became quite definite early in August, when purple and swollen gums were observed. The scorbutic condition developed gradually during the month of August. By the commencement of September the animal had lost the use of its hind limbs. At this stage it was at first intended to cure the monkey but after the administration of a single dose of antiscorbutic equivalent to about 20 cc. of lemon juice the plan was changed and the original daily dose of 0.5 cc. was resumed. The effect of the single high dose was very noticeable as the condition of the animal improved immensely. The pseudo-paralysis of the hind limbs disappeared but the gums and teeth did not clear up. The scurvy, however, soon became severe again and in spite of the continued daily dose

of 0.5 cc. of orange juice the animal declined and died on October 21st, 1918. At the post mortem examination very severe scurvy was found.

Monkey No. 24. *Macacus rhesus*, male. Experiment started July 29th, 1918. Dose 0.75 cc. of orange juice. The initial weight of this animal was 2900 g. Even in the early stages of the experiment the animal showed a slow loss in weight. This decline became very rapid at the end of October and lasted to the end of the experiment. As early as the middle of September the animal showed a tendency to bleed from the gums when handled but no more definite symptoms of scurvy were discernible at this stage. Spongy and purple gums however soon developed and by October 21st pseudo-paralysis of the hind limbs was recorded. The animal declined and died on November 22nd, 1918. At the post mortem examination severe scurvy was found.

Monkey No. 25. *Macacus rhesus*, male. Placed on scorbutic diet on August 26th, 1918. Dose 1 cc. of orange juice. Initial weight 2160 g. This monkey increased in weight until November 9th, 1918 when it reached 2600 g. After that time it commenced to lose slowly in weight. Early in December 1918 the animal developed spongy gums and its incisors became loose. At the same time it lost its liveliness and became dejected. It could however move its limbs without discomfort. As the mild scurvy could not be responsible for this condition it was concluded that the animal was suffering from some other disease. It was therefore decided to stop the experiment and the monkey was placed on a mixed diet on December 12th and a strong dose of an antiscorbutic was also administered. The scurvy cleared up but the animal died five days later. At the post mortem examination no scurvy was found and the cause of death could not be ascertained.

Monkey No. 15. *Macacus rhesus*, female. Placed on scorbutic diet February 16th, 1918. Dose 2 cc. of orange juice. This experiment lasted a year during which time the animal kept in excellent health and gained in weight regularly. The initial weight of the monkey was 3300 g. It weighed 4400 g. on February 19th, 1919 when it was chloroformed. At the post mortem examination the animal was found to be in excellent condition and no trace of scurvy was observed.

Monkey No. 14. *Macacus* (species not determined), male. Placed on scorbutic diet February 16th, 1918. Dose 5 cc. of orange juice. Initial weight 3040 g. This animal increased regularly in weight. After about four months, July 29th, 1918, the experiment was terminated as it became evident from this and the previous experiment that 5 cc. of orange juice was an ample dose. The animal weighed 3450 g. at the end of the experiment and was in good health.

Examining the above results it is seen that the daily minimum dose of fresh orange juice for the monkey lies between 1 cc.-2 cc. No delay in the onset of scurvy was discerned in the animals receiving 0.5 cc. and 0.75 cc. of the juice, compared with those receiving no antiscorbutic. On the other hand, scurvy was definitely delayed in the animal which received 1 cc. of orange

juice. This is plain both from the time of the onset of the symptoms and from the growth of the animal during the early stages of the experiment. Unfortunately this animal succumbed to some other disease and it was not possible to ascertain the extent to which the severity of the symptoms of scurvy could be modified by the daily administration of 1 cc. of fresh orange juice. The higher doses of 2 cc. or 5 cc. have proved an adequate prophylactic. A monkey therefore of 2-3 kilos is protected from scurvy by about the same quantity of orange juice as a guinea-pig weighing 300-400 g. It is interesting to note that while the minimum dose of antiscorbutic required by the two animals to protect them from scurvy is of the same order, the time taken for the development of the disease is very different, being about two months for a monkey and three weeks for a guinea-pig. This suggests that the monkey possesses a higher store of the antiscorbutic factor than the guinea-pig while their daily requirements for metabolism are equal.

## REFERENCES.

- Chick and Rhodes (1918). *Lancet*, Dec. 7th, 1918.  
Harden and Zilva (1919). *J. Path. Bac.* **12**, 246.  
Zilva and Wells (1919). *Proc. Roy. Soc. B*, **90**, 50E

# XVIII. THE PRODUCTION IN MONKEYS OF SYMPTOMS CLOSELY RESEMBLING THOSE OF PELLAGRA, BY PROLONGED FEEDING ON A DIET OF LOW PROTEIN CONTENT.

BY HARRIETTE CHICK AND ELEANOR MARGARET HUME.

*From the Department of Experimental Pathology, Lister Institute.*

*(Received March 2nd, 1920.)*

(With Plate V.)

THE dietary interpretation of the etiology of pellagra has been growing in favour and the disease seems to stand in much the same relationship with the eating of maize as beriberi stands with the eating of rice.

More than 50 years ago, Roussel [1866] pointed out that pellagra can be cured by good food and that without dietetic improvement all remedies fail.

The various theories of its causation which have been advanced are discussed by Funk (1913) who is of opinion that the facts can be best explained by that one which attributes the disease to partial starvation of some important dietary constituent.

Voegtlin [1914], although inclining to the view that pellagra is a chronic intoxication, points out that in the U.S.A. it is endemic only upon a population whose diet is mainly vegetarian and that the possibility of its causation by a vitamine deficiency, or by the defective supply of certain amino-acids in the protein ingested, must be seriously considered.

Lorentz [1914] and Willets [1915] treated advanced cases with mental symptoms by a generous diet with favourable results.

Goldberger [1916] investigated the conditions at two orphanages in Jackson, Missouri, in which cases of pellagra occurred. Some of the children had been admitted pellagrous but others became ill after prolonged residence in the orphanages. Milk and meat were sparingly used in the institutions. By greatly increasing the quantity of meat, milk and milk products, but without disturbing the other conditions, the disease disappeared and failed to recur next spring.

Goldberger, also, made observations upon eleven criminals who volunteered for the following dietetic experiment. The pellagra squad was fed upon a diet consisting of white flour, corn grits, corn meal, corn starch, polished rice, sugar, sweet potatoes, pork-fat, cabbage and turnip-tops. The only animal protein was 4 oz. of meat once a week. The calorific value of the daily diet was 2950 and the prisoners performed only light work. Nevertheless, loss of weight occurred early. After  $7\frac{1}{2}$  months cutaneous manifestations were noticed which, in the opinion of experts who were asked to see the cases,

justified a diagnosis of pellagra. These symptoms cleared up on the resumption of a normal diet. Goldberger concludes that the evidence strongly suggests that pellagra will be proved to be a deficiency disease closely related to beriberi but, whatever its nature, it is capable of correction or prevention by including in the diet suitable proportions of fresh animal protein.

Wilson [1916, 1918] had an exceptionally favourable opportunity to study the relation of diet to pellagra in the case of an outbreak of the disease amongst the population of a camp of Armenian refugees at Port Said during 1915, 1916 and 1917. The diet at first supplied was inadequate. It contained only 2200 calories and 92 % of its protein was derived from vegetable sources, of which about  $\frac{3}{4}$  was from wheat and  $\frac{1}{4}$  from maize. Wilson (using Thomas' [1909] data) calculates that the biological value of the total protein only equalled 22 g. of caseinogen.

Nearly 10 % of the inhabitants of the camp developed pellagra within a year. When, however, the diet was enriched so as to contain 3000 calories and proteins of a total biological value equal to 41 g. of caseinogen, fresh cases soon ceased to occur and the camp remained thereafter free from the disease.

Wilson concludes, from the above observations and from extensive studies of Egyptian and other dietaries, that the cause of pellagra is to be found in an inadequate supply of some one or more of the essential amino-acids and is generally occasioned by relying too exclusively upon cereals, and especially maize, for a supply of protein.

Wilson considers the danger line to be crossed when the total protein intake has a biological value of less than that of 40 g. of caseinogen.

Chittenden and Underhill [1917] produced in dogs a condition which they regard as suggestive of pellagra, by feeding for eight months upon a diet of biscuits, peas and cotton-seed oil. They referred the symptoms to a deficiency of some essential dietary constituent of unknown character. McCollum and his colleagues [1917 to 1919] made a biological analysis of pellagra-producing diets by experiments on rats. They obtained merely evidence of malnutrition and conclude therefore that pellagra is caused by an infection supervening upon lowered vitality due to faulty diet.

A further study of the relation between the incidence of pellagra and dietary was made by Goldberger, Wheeler and Sydenstricker [1918], who have compared the diets partaken of by households in South Carolina villages, in which pellagra occurred, with those of similar households free from the disease. The food consumed by the latter contained more milk, fresh meat, eggs, butter and cheese, and depended less upon cereals for proteins.

The effect of the absence of tryptophan from the protein zein, which forms nearly 50 % of the proteins of the maize endosperm, was demonstrated by Willcock and Hopkins [1907] in the case of young mice, and that of the absence of lysine from the same protein was studied by Osborne and Mendel [1914, 2; 1915]. These two obvious deficiencies, particularly that in trypto-

phan, have frequently been suggested as the cause of pellagra in human beings, the protein of whose diet has been too exclusively derived from maize endosperm. Were such an explanation correct, *i.e.* that pellagra is a lysine or tryptophan deficiency, it would also cover those cases of the disease which are reported in persons who have never touched maize, for doubtless a diet deficient in tryptophan or lysine can be attained by other means than by eating too exclusively of maize endosperm.

The present paper gives, very tentatively, an account of preliminary experiments which were planned in order to make a close study of the symptoms which might develop in monkeys fed on a diet of which the protein was deficient in tryptophan and lysine. The results obtained seemed sufficiently promising to warrant publication.

#### EXPERIMENTAL.

The diet selected had to satisfy a number of requirements. It had to be adequate from the point of view of fat and of calorific value; it had to contain enough of all the known accessory food factors and it had to be sufficiently palatable for the animals to eat it. It had also to be composed of materials which could be prepared on a large scale, as the experiments were likely to last over many months. The use of purified foodstuffs was thus excluded and the diets had to be so planned as to consist of ordinary foodstuffs or of products of manufacturing processes.

The accessory food factors were supplied throughout as follows:

Fat-soluble *A*. Butter. 10–20 g. per head daily.

Water-soluble *B*. Marmite. 2 g. per head daily.

Anti-scorbutic factor. Orange juice. 10 cc. per head daily.

The fat-soluble *A* ration also provided the fat ration. The orange juice and the marmite were always consumed separately but the butter was mixed with the other food and was not therefore always all consumed. As regards the adequacy of these rations previous experience had shown that 200 cc. daily of milk (about 7 g. of butter fat) would keep a monkey in health over long periods (six months and over) when it was the almost exclusive source of fat-soluble *A* [Barnes and Hume, 1919]. The same amount of fat derived from the same milk ration and about 5 g. of fat derived from wheat germ, of which by no means all was eaten, has been found adequate throughout our scurvy experiments on monkeys.

The antiscorbutic ration was far in excess of that needed to maintain a monkey in health; 2.5 cc. of lemon juice has been found adequate [Chick, Hume and Skelton, 1918], and lemon juice and orange juice, by experiments on guinea-pigs, have been found to have approximately equivalent values (unpublished experiments to which reference is kindly permitted by Miss A. J. Davey).

As regards the water-soluble *B* ration, the amount needed to protect a monkey is quite unknown, but it was judged that 2 g. daily of marmite should

amply suffice, as it evidently did, for the onset and control of any symptoms which occurred were entirely independent of the supply of this factor. The value of the commercial yeast preparation "marmite," for preventing the onset of polyneuritis in pigeons fed on a polished rice diet, had already been demonstrated by us [Chick and Hume, 1917].

The rest of the diet was composed of sugar, cornflour (maize starch, almost protein-free, supplied by the kindness of Messrs Brown and Polson), salt, and corn gluten; a small daily ration of apple or banana was given as a relish. The corn gluten which was almost the sole source of protein in the diet was a product of the manufacture of corn starch; it was similar to that described by Osborne and Mendel [1914, 2, p. 5] for which the following composition is given:

	%
Zein ... ..	31.25
Maize glutelin ... ..	13.75
Total protein ...	45.00

The remaining 55 % of the corn gluten is oil and carbohydrate.

The product was supplied as a dry cake by the kindness of Messrs W. Mackean of Paisley, and served the purpose well as it was not too unpalatable. No special analysis of it has been attempted, for the figures would have had no value in the interpretation of the results since it has not been attempted within wide limits to determine how much the monkeys consumed.

Table I.

*Daily ration offered but not always consumed.*

Items common to Diets I, II and III.

Food stuff	Total quantity	Protein	Carbohydrates	Fat
Butter ... ..	10-20 g.	0.1-0.2 g.	—	8.4-16.8 g.
Banana ... ..	25 g.	0.3 g.	5.5 g.	0.15 g.
or		or	or	or
Apple ... ..	25 g.	0.1 g.	3.5 g.	0.1 g.
Sugar ... ..	26 g.	—	26 g.	—
Orange juice ... ..	10 cc.	—	—	—
Cornflour biscuit ... ..	34 g.	—	34 g.	—
Marmite ... ..	2 g.	—	—	—
Totals ... ..	—	0.2-0.5 g.	63.5-65.5 g.	8.5-17.0 g.

Table II.

Variable constituents of Diets I, II and III.

	Foodstuff	Total quantity	Protein			Carbohydrate and fat
			Zein	Other protein	Total protein	
Diet I ...	Corn gluten	50 g.	15.6 g.	6.8 g.	22.4 g.	27.5 g.
	Corn starch	30	—	—	—	30
Diet II ...	Corn gluten	32	10.0	4.4	14.4	17.6
	Corn starch	45	—	—	—	45
Diet III ...	Corn gluten	17	5.3	2.3	7.6	—
	Corn starch	62	—	—	—	62

The corn gluten was fed in three different proportions, Diets I, II and III as set out in Table II. Table I shows the food common to Diets I, II and III, Table II shows the starch and protein values (calculated from the above percentage composition for corn gluten) in which Diets I, II and III differ from one another. For the total value of any of the three diets the values in Table I must be added to it and it must be remembered that these figures represent, approximately only, the amount offered to the animals; it is not to be supposed that the total ration offered was consumed for it never was. In experiments such as these the difficulty of getting the animals to consume the monotonous and rather tasteless food for a period of many months is very great; it is a difficulty which it would seem can never be completely overcome, for even if the animal eats the diet well when in health it usually begins to lose appetite when it begins to sicken. The partial starvation which results, with the consequent general lowering of the metabolism, seems to have the effect of moderating the development of the typical and florid symptoms of any deficiency disease. The distaste of a sick animal for the diet also makes it impossible to obtain that rise in weight, following a cure of the symptoms, which is desirable in order satisfactorily to clinch the cure.

Every effort in the present experiments was made to render the food palatable; part of the cornflour was cooked up with the corn gluten into a sort of blancmange and the rest of the cornflour was made into a biscuit through the kindness of Messrs Huntley and Palmer. These efforts were however only moderately successful as partial starvation certainly hastened the decline in weight, particularly in the case of two out of the three monkeys. After symptoms had been alleviated by a modification in the diet it was also found that it was not possible to do more than arrest the fall in weight; the animals did not begin to regain weight until they were put on to a normal diet; on that however they regained weight rapidly, showing that the whole of their trouble was dietary.

The experiments concern three monkeys only, Lazarus, Diana and Jonathan. They all belonged to the species *Macacus rhesus*; this species was chosen because it is one which has an almost hairless, flesh-coloured face, upon which it was hoped that any skin lesion or erythema would show up clearly; much of the rest of the body is dark bluish in colour.

Attempts were made to study the effect of direct sunlight in producing or in increasing any skin lesions that might appear; some results were obtained during the summer but the most important part of the experiment, *i.e.* that in which Diana was upon Diet III, fell in the winter months when sunshine was not available.

Of the three animals, two, Lazarus and Jonathan, lost weight on Diet I (total protein content 23 g.; total protein other than zein 7.3 g.); they were therefore retained on it throughout (Lazarus 92 days, Jonathan 195 days). The third, Diana, liked the corn gluten feed much better and almost maintained weight for 90 days on Diet I and for 26 days on Diet II (total protein

15 g.; total protein other than zein 5 g.). It was not until she received Diet III (total protein 8.2 g.; total protein other than zein 2.9 g.) with its very much lower protein value that she also began seriously to lose weight. The three diets did not differ to any extent in palatability and there seems no doubt that the only reason, at any rate at the commencement of Diet III, for the loss in weight in this case, when at last it did begin, was the lower level of protein intake on Diet III.

The two monkeys, Jonathan and Lazarus, which lost weight on Diet I form one group and the third monkey, Diana, which only began to lose weight when put on to Diet III stands apart. The two first showed only incipient symptoms, the third developed skin lesions of a striking type; the inconclusive skin symptoms of the first two were however identical with the incipient symptoms of the third. It seems probable that Jonathan and Lazarus were suffering from partial starvation, Diana on the other hand was not, at any rate in the earlier stages of the experiment.

A description of the symptoms, together with an account of attempts which were made to perform cures with tryptophan, lysine and caseinogen, will be found described in detail in the protocols; the cures were only partially successful except in the case of Diana, whose cure with caseinogen was of a startling and dramatic nature.

#### PROTOCOLS.

*Lazarus.* Diet I, Male. Initial weight 2540 g. (see weight curve, Fig. 1). This animal at once began to lose weight on Diet I and on the 51st day showed a faint erythema on the face, particularly on the bridge of the nose, after spending an hour on the roof in direct sunlight (May 15th).

The mouth at this time was rather unclean and the gums inclined to bleed; the animal was subdued and there was some intestinal upset, the abdomen being often distended with gas. It should be noted that this animal was one always rather subject to digestive disorders. The stools were loose, sometimes amounting to diarrhoea.

The erythema, which was of a papillo-macular type, continued intermittently but progressively for the whole of the rest of the experiment. It was never very marked; it left patches which remained faintly pigmented for a few days and sometimes scaled off; it was most marked on the brows, retreating back into the hair, but it was also apparent on the cheeks and on the bridge of the nose and sometimes on the neck, down on to the chest and trunk and even on the inner sides of the thighs.

The appetite was bad and the meals were only partially consumed. About the 85th day the animal was becoming very weak, all the symptoms were aggravated and the mouth was somewhat sore inside and at one corner. The abdomen was very much distended with gas.

On the 89th day the condition seemed very precarious and 0.2 g. tryptophan, *i.e.* the tryptophan from about 13.3 g. of caseinogen, taking the value 1.5 %

tryptophan in caseinogen, as given by Mathews [1916], was given by the mouth.

On the 90th day the animal was much weaker, the lips sore and the diarrhoea bad; 0.2 g. tryptophan was again given and later in the day 3-4 g. of caseinogen with brandy. No improvement took place; the animal was however probably in any case too far gone before a cure was attempted; the next day it was moribund and was chloroformed.

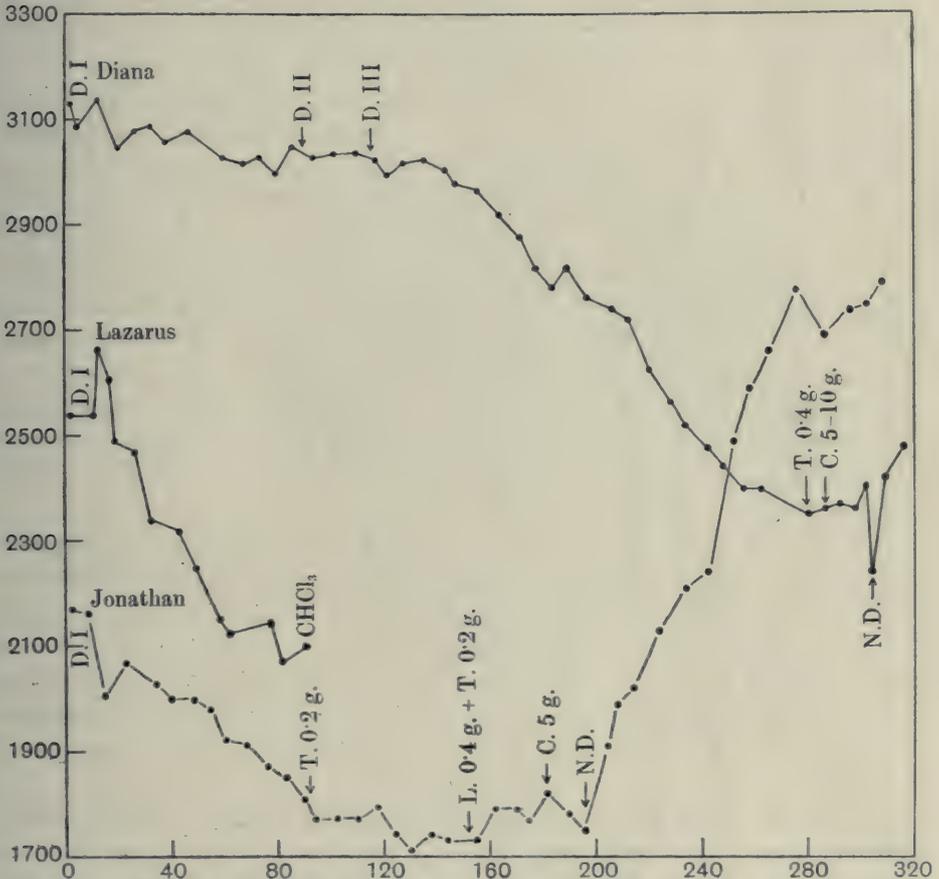


Fig. 1. Weight Chart of the three monkeys, showing dates of changes in the diet.

Ordinates. Weight in grams.

Abscissae. Days from commencement of experiment.

D. (diet), T. (tryptophan), L. (lysine), C. (caseinogen), N.D. (normal diet) indicate day on which the change was commenced.

The post mortem showed nothing remarkable; the body was much emaciated and the caecum and colon greatly distended with gas and soft faeces, but the walls of the intestine showed no special alteration.

There was no sign of tuberculosis or other intercurrent disease.

*Jonathan.* Diet I, Male. Initial weight 2170 g. (see weight curve, Fig. 1). This animal also began to lose weight from the first. On the 33rd day a dull

reddish patch began to appear about the centre of the lower lip, where the skin and mucous membrane meet, and there were also patches on the scrotum. These were at the time attributed to some skin disease, but they cleared up on a good diet and the patch on the lip closely resembled the patch on the lips developed in the case of Diana.

On the 49th day slight erythema below one eye was noticed but it did not appear to be much enhanced by exposure to sun.

The stools were generally well formed, sometimes rather soft and of a greenish brown colour; diarrhoea was never observed.

The erythema progressed and appeared on the brows and face; on the 71st day it was observed faintly on the chest and on the inside of the thighs. The appetite became bad and the intake of food much diminished, even the ration of banana was often refused. The coat became very scanty, the skin scurfy and chaffy, the gums receded from the teeth but the tongue and inside of the mouth were not sore. The legs were tremulous. The animal had now lost about 20 % of its initial weight and seemed in a very dangerous condition; a daily ration of 0.2 g. of tryptophan was therefore initiated on the 92nd day and continued without any change or addition for 61 days. The loss in weight ceased and the animal seemed brighter and better but no gain in weight took place. Erythema was still recorded from time to time and the animal continued in very poor condition; when the treatment started however there seemed no possible likelihood of life being prolonged for another 61 days if no treatment were given.

On the 153rd day of the experiment 0.4 g. of the unseparated di-amino-acids lysine, arginine and histidine (prepared from caseinogen) was administered by the mouth together with the 0.2 g. of tryptophan already being given; this treatment was persisted with for 29 days up to the 181st day of experiment but no marked change was observed. Addition of further butter and more cornflour failed to make the animal put on weight; probably it liked the diet insufficiently well to eat enough to make it put on weight in any case.

On the 181st day the amino-acids were dropped and 5 g. caseinogen daily were substituted and this treatment was continued for 14 days; still the weight did not rise and on the 190th day it was put on to normal diet of rice, wheat germ, autoclaved milk and cabbage. The weight rose at once by leaps and bounds until it was far above that at the start of the experiment. In 34 days on normal diet the monkey had regained its weight at the start of the experiment and in another 40 days it had increased 500 g. beyond that. Its coat now became thick and glossy; normal change of dentition set in, all trace of skin lesions vanished and the animal, from being depressed and sedentary, became happy and playful. There could be no possible doubt that the whole of its disorder had been dietary.

Although the symptoms in this case were insufficiently marked to be able to say whether a real cure took place or not, it is to be noted that in the





first 92 days of the experiment it lost 20 % of weight and was judged to have reached a precarious condition in which death could not be far off if no change in treatment were instituted. The additions to the diet which are detailed above were then made, starting when the animal's weight was 1750 g.; these additions were continued in all over a period of 103 days, yet no further loss in weight took place after they were instituted and when at the end of the 103 days the animal was put on to normal diet its weight was the same, *i.e.* 1750 g.

*Diana.* Diets I, II and III. Female. Initial weight 3130 g. (see weight curve, Fig. 1). This animal very nearly maintained weight for 90 days on Diet I, during which period she was occasionally exposed to the sun and very faint erythema of the face was sometimes observed.

On the 91st day the protein and starch were readjusted to the proportions of Diet II which was continued for 26 days; weight was still practically maintained and erythema was not observed but some scurfiness of the skin was present.

On the 117th day Diet III (total protein 8.2 g.; total protein other than zein 2.9 g.) was started and a marked loss of weight set in. Faint erythema with scurfiness of the skin was observed which was intermittent but also progressive.

Menstruation, which had been regular previous to the experiment, ceased and was never observed even on Diet I. The stools were fairly firm and greenish brown in colour.

On the 218th day, the face and brow ridges were distinctly marked with patchy erythema and there was oedema of the eyelids. The spots increased rapidly in brightness, particularly on the bridge of the nose, cheeks, brows and upper lip. A reddish patch of dermatitis began to develop at each angle of the mouth where skin and mucous membrane meet.

Local oedema continued to increase and appeared about the upper lip and face generally. The butter ration was increased and the oedema disappeared for a time, only to reappear later with much greater severity.

All the symptoms continued to progress; the whole skin became very scurfy; the face was often brilliantly red; the mouth showed rough red patches of dermatitis at the angles; these patches however only involved the very edge of the mucous membrane and the actual interior of the mouth appeared normal throughout. The skin of the face evidently itched for the monkey was seen to rub it and scabs were produced in this way. Strong illumination, even from an electric light, brought up the erythema and caused her to hide her face.

On the 253rd day of experiment (137th day of Diet III) the sketch (Plate V) for which we are indebted to Miss M. Rhodes was made.

On the 279th day of experiment (163rd day of Diet III) the animal's condition was deplorable; the oedema which was strictly confined to the area of erythema, *i.e.* on the face, was so severe that in the morning and evening when

the head was held down in sleep she could scarcely open her eyes; during the day, when the head was held up, the oedema tended to run down under the chin. The animal was very emaciated, having lost nearly 25 % of weight; the face was erythematous all over; the patches of dermatitis at the corners of the mouth were large and very red, the skin was scurfy, the hair almost all gone, the tail was unhealthy looking with patches of dermatitis. On this day 0.2 g. of tryptophan was given by the mouth and by the next day the oedema was distinctly less. The next day 0.4 g. of tryptophan was given and the same dose continued daily for six days; the oedema continued somewhat less but it had always been intermittent; the erythema did not decrease and the animal's condition appeared to be very precarious. Fearing intercurrent disease, 5 g. caseinogen was given which was replaced after one day by the commercial preparation plasmon, this being simply caseinogen in a more palatable form, since much difficulty was experienced in administering the caseinogen.

On the third day from the beginning of the caseinogen treatment, the oedema was almost completely gone, the erythema was reduced, with the nose and eyelids desquamating healthily. The backs of the fingers, parts of the feet and tail also desquamated extensively, showing that some superficial dermatitis must have been present though not easily observable on the dark skin of those parts. Some slight gain in weight took place and the angles of the mouth began to heal; these patches of dermatitis were so severe that, although they began to improve at once, 20 days of the plasmon treatment elapsed before they were fully healed. After 12 days the plasmon had been increased until sometimes as much as 10 g. were taken, but no marked increase in weight ensued. The lesions however completely healed; although the nose remained for long afterwards of a rather purplish red, the oedema had completely disappeared and the whole skin of the face, body, tail, hands and feet was clean. The appetite, however, continued very bad, the animal seemed weak, the weight dropped suddenly and it was decided on the 307th day of experiment (191st day of Diet III), 28 days after the attempts at cure started, to put her on to normal diet, as it was deemed impossible to secure a rise in weight while the same monotonous food was continued. The animal fell upon the food ravenously and a rise in weight at once commenced, strength began to come back and at the time of writing the animal bids fair to show the same return to condition as was shown by Jonathan in similar circumstances [see *Postscriptum*]. Considerable importance is attached to this return to form as it excludes all possibility of tubercular or other infection having played any part in the decline.

## SUMMARY AND CONCLUSIONS.

The foregoing experiments show that three monkeys fed on, or consuming, a low protein diet, whose proteins are at the same time of low biological value, lose weight rather slowly, two possibly from partial starvation through distaste for the diet and only showing mild skin lesions, while the third, which ate well of the diet, at first, showed florid skin lesions and co-extensive localised oedema. The development of these lesions took place after variable, rather lengthy periods.

Attempts to cure with tryptophan failed in one case which was probably too far gone when the cure was attempted, staved off death for many weeks in one case and slightly modified the severity of symptoms in one case.

An attempt to cure with a mixture of lysine, arginine and histidine seemed to have little added beneficial effect in the one case in which it was tried.

Addition of 5-10 g. caseinogen failed in the one very ill case, gave little added benefit in the second case but brought about a dramatic cure in the case where the skin lesions were most severe.

Owing to the small number of animals under consideration, it is not permissible to draw too definite a conclusion; the skin symptoms produced did however closely resemble those of pellagra and there can be no doubt that they were of dietary origin. The skin lesions were bilaterally symmetrical and appeared to be heightened by direct sunlight as in pellagra. They were the only symptom present in all three cases. Diarrhoea and flatulence only occurred in one case out of three, in an animal normally liable to digestive upset. In human pellagra diarrhoea is by no means universal. Nervous symptoms, such as occur in advanced cases of pellagra, were not observed.

As regards the localised oedema which developed in the case of the monkey Diana, which was co-extensive with the dermatitis, this is not uncommon in pellagrins when the dermatitis is acute. The association of general oedema and pellagra has been described by Bigland [1920] among Turkish prisoners of war in Egypt. General oedema has, however, been widespread amongst starving populations during and since the war, but whether it be occasioned by one or many dietetic deficiencies is at present uncertain. A single experiment by Harden and Zilva [1919], in which it followed prolonged feeding of a monkey upon a diet devoid of fat-soluble *A* but otherwise adequate, is an indication.

In the case of our experiments, the diet offered to the animals was deficient only in protein of good biological value but the exact amount of food consumed could not be determined, the quantity uneaten being often considerable, as their appetites became capricious. As to how far the pellagra-like symptoms and wasting were due to an inadequate supply of tryptophan, of lysine or of both, or of some other unappreciated constituent of the protein, the experiments do not supply an answer.

A portion of the expenses of the investigation was defrayed by a grant from the Medical Research Council, to whom our thanks are due.

*Postscriptum.*

On the 324th day, that is 17 days after the return to normal diet and at a time when the monkey appeared to be rapidly recovering weight and strength, it was found early one morning in convulsions. These recurred at frequent intervals during the day.

Three grains of chloral hydrate were administered and the animal slept, but on waking a further series of epileptic fits succeeded one another at shorter and shorter intervals.

The spasms were entirely bilateral, at first clonic and involving the flexors and terminating with tonic contractions in which the extensors overpowered. Next day the animal was very exhausted, refused food and the intervals between the fits became reduced to a few minutes.

As by evening there seemed little chance of it surviving the night, it was killed by chloroform. At the autopsy there was total absence of fat and the hair had to a large extent disappeared from the body, but the organs all appeared perfectly healthy. There was no abnormality discovered by the naked eye in the nervous system. Portions were taken of various parts for microscopical examination and will be reported upon later by Miss Tozer.

## REFERENCES.

- Barnes and Hume (1919). *Biochem. J.* **13**, 306.  
 Bigland (1920). *Lancet*, **i**, 243.  
 Chick and Hume (1917). *Proc. Roy. Soc. B*, **90**, 44.  
 Chick, Hume and Skelton (1918). *Lancet*, **i**, 1.  
 Chittenden and Underhill (1917). *Amer. J. Physiol.* **64**, 13.  
 Goldberger (1916). *J. Amer. Med. Assoc.* **66**, 471.  
 Goldberger, Wheeler and Sydenstricker (1918). *J. Amer. Med. Assoc.* **71**, 944.  
 Harden and Zilva (1919). *Lancet*, **ii**, 780.  
 Lorentz (1914). *Pub. Health Reports (U.S.A.)*, Sept. 11.  
 McCollum and Simmonds (1917, 1). *J. Biol. Chem.* **32**, 29.  
 — (1917, 2). *J. Biol. Chem.* **32**, 181.  
 — (1917, 3). *J. Biol. Chem.* **32**, 347.  
 — (1918). *J. Biol. Chem.* **33**, 303.  
 McCollum and Parsons (1918). *J. Biol. Chem.* **33**, 411.  
 — (1919). *J. Biol. Chem.* **33**, 113.  
 Mathews (1916). *Physiological Chemistry*.  
 Osborne and Mendel (1914, 1). *J. Biol. Chem.* **17**, 325.  
 — (1914, 2). *J. Biol. Chem.* **18**, 1.  
 — (1915). *J. Biol. Chem.* **20**, 351.  
 Roussel (1866). *Traité de la pellagra*. Paris, 529.  
 Thomas, K. (1909). *Arch. Physiol.* 219.  
 Voegtlin (1914). *J. Amer. Med. Assoc.* **63**, 1094.  
 Willcock and Hopkins (1907). *J. Physiol.* **35**, 88.  
 Willets (1915). *South. Med. Journ.* **8**, 1044.  
 Wilson (1916). Appendix to Report No. 2, On a pellagra epidemic at Armenian refugees camp, Port Said, issued by the Public Health Dept., Egypt.  
 — (1918). Report of a committee appointed by the Dir. Med. Serv., Egypt. Exped. Force, regarding the prevalence of pellagra amongst Turkish prisoners of war, Cairo, 20. (Reprinted in *J. Roy. Army Med. Corps*, **33**, 426, 508; **34**, 70.)

# XIX. ON THE SWELLING OF GELATIN IN HYDROCHLORIC ACID AND CAUSTIC SODA.

BY DOROTHY JORDAN LLOYD.

*Report to the Medical Research Committee.  
From the Biochemical Laboratory, Cambridge.*

*(Received February 19th, 1920.)*

## CONTENTS

	PAGE
I. Introductory . . . . .	147
II. Materials and methods . . . . .	148
III. The swelling of gelatin in water, hydrochloric acid and caustic soda . . . . .	148
IV. The state of gelatin dissolved by acid or alkali in the cold . . . . .	153
V. The basic and acidic properties of gelatin . . . . .	155
VI. The behaviour of swollen gels in an atmosphere of saturated water vapour . . . . .	156
VII. Theory of gel structure deduced from a study of gelatin swelling . . . . .	162
VIII. Appendix. On the molecular weight of gelatin . . . . .	166
IX. Summary . . . . .	168
X. Bibliography . . . . .	169

## I. INTRODUCTORY.

IN 1900 Hardy [1900] put forward evidence to show that gelatin gels are physically heterogeneous, *i.e.* 2-phase systems, and stated in addition that one phase was a solid solution of water in gelatin, the other a liquid solution of gelatin in water. The solid phase (unless the concentration of gelatin were very great) formed an open framework throughout the system.

Procter [1914] discarded the 2-phase theory of gel structure as gratuitous, merely postulating a "molecular network" throughout the jelly. He considered that the equilibrium in a gelatin-acid-water system was due to the chemical combination of gelatin and acid to form dissociated salts, and that the osmotic pressure of these salts determined the volume of the jelly. Procter's theory will be discussed in detail later; at this juncture it may be summarised as a theory which postulates physical homogeneity, but chemical heterogeneity.

In the following pages it is hoped to establish the thesis that both physical and chemical heterogeneity are essential to the gel state, and to show that the volume occupied by a gelatin gel is determined by the resultant of two opposing sets of forces localised respectively in the two phases of the jelly. The experimental evidence will now be considered.

## II. MATERIALS AND METHODS.

Coignet's "Gold Label" gelatin was used for all experiments. An analysis of the sample used showed that it contained 20 % of water, which was removable by drying for 6-8 hours in a hot air oven at 110°. Actually, therefore, there was probably a slightly higher percentage of water, but a correction for 20 % of water only has been made in the initial weights of leaf gelatin taken for the experiments. The gelatin had an ash content of 1.0 %. This consisted mainly of calcium sulphate, with traces of phosphates and chlorides. A small fraction of the base present was combined with the gelatin.

The distilled water used was always of recent manufacture; it was made in an ordinary laboratory still and had a reaction of about  $P_H = 5.5$ . After boiling and cooling the reaction moved to  $P_H = 7.0$ . The acidity therefore was due to dissolved carbon dioxide. Solutions of the gelatin in boiled distilled water gave a reaction  $P_H = 5.25$ , at concentrations of 0.2 and 0.5 %. Since the iso-electric point of gelatin is at  $P_H = 4.6$ , this shows that the gelatin contained a small quantity of base.

In the experiments recorded below discs of leaf gelatin weighing from 0.05 to 0.08 g. were used in most cases. These were placed in Gooch crucibles where they remained at all stages of the experiment. In experiments on equilibria in liquids, both crucible and gel were dried rapidly with filter paper before each weighing. The gels were weighed in their crucibles, but a cover was always placed on the crucible during weighing to check loss of weight by evaporation. The same applies to weighings made during experiments on equilibria in saturated vapour. In drying the gels care was taken to avoid mechanical pressure. The smoothness of the experimental curves appears to justify the simplicity of the method.

The volume of liquid used was in most experiments 150 cc. The experimental liquids were placed in glass bottles with wide necks and ground-in stoppers. The crucibles were easily suspended in these by a harness of fine thread. For experiments conducted in vapour, a glass desiccator was used with distilled water at the bottom. This was partially evacuated, but the air pressure in the chamber does not appear to have an appreciable influence on the shape of the experimental curves.

In all experiments, apparatus in use was kept submerged in a water-bath at 20°. The variation of temperature was  $\pm 0.25^\circ$ .

In all experiments a few drops of toluene were added to the system to prevent the development of bacteria and moulds.

## III. THE SWELLING OF GELATIN IN WATER, HYDROCHLORIC ACID AND CAUSTIC SODA.

The swelling of gelatin in water and the influence of acid or alkali in increasing both the rate and degree of such swelling has been described many times by many writers. The rate at which the dry gelatin absorbs water,

and the final amount taken up, depend on a variety of factors among which may be mentioned: hydrogen-ion concentration [Chiari, 1911; Procter, 1914; Loeb, 1918]; temperature [Procter and Burton, 1916]; the ratio of the area of surface of the gelatin to its volume [Spiro, 1910; Fischer, 1910; Hofmeister, 1890; Pauli, 1897]; the ratio of the mass of the gelatin in the system to the mass of dissolved electrolyte (see below), the elastic modulus of gelatin [Procter and Wilson, 1916], and finally, since in every case of absorption of

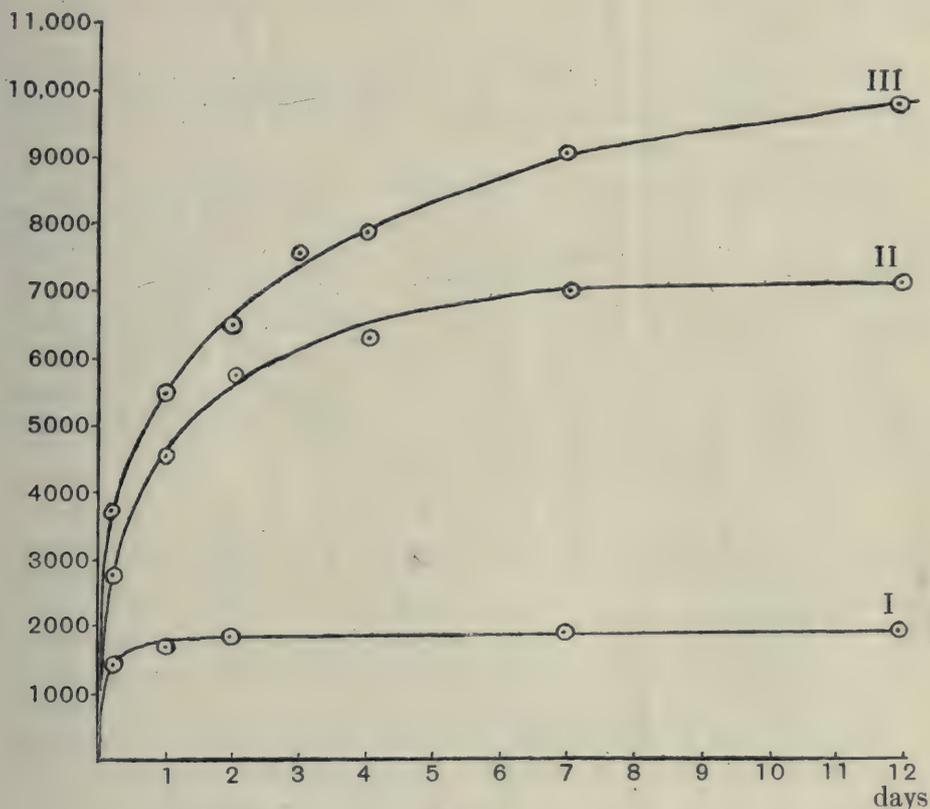


Fig. 1.

Abscissa = time.

Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

water by dry leaf gelatin the initial stage must consist in the restoration of a preformed structure, a factor must be introduced involving the conditions under which the gel was originally formed [see Hardy, 1900]. This last factor is usually unknown. The importance of the mass relations in the system gelatin-hydrochloric-acid-water (other variables being kept constant) is illustrated by the curves shown in Fig. 1. These curves illustrate the progress of swelling in three systems where the ratios  $\frac{\text{dry weight of gelatin}}{\text{volume of } 0.001N \text{ HCl}}$  were 0.8/100, 0.8/1000, 0.8/10,000 respectively.

The actual experimental figures for the three systems are:

	I	II	III
Weight of leaf gelatin containing 20 % moisture =	0.820 g.	0.1443 g.	0.0148 g.
Corrected weight of gelatin . . . . . =	0.656 g.	0.1154 g.	0.0118 g.
Volume of <i>N</i> /1000 HCl added . . . . . =	82 cc.	144 cc.	148 cc.
Weight of swollen jelly at equilibrium . . . =	12.04 g.	8.21 g.	—
Weight at equilibrium expressed as percentage of the weight of dry gelatin } =	1840	7100	—
Initial reaction of acid, $P_H$ . . . . . =	3.0	3.0	3.0
Final reaction of acid, $P_H$ . . . . . =	4.4	3.57	(3.25)

The intermediate weights of the gel system expressed as percentages of the dry weight of gelatin, and the time relations, are shown in Fig. 1. System III ultimately went into solution.

The influence of the reaction, or hydrogen ion concentration, on both rate and degree of swelling is shown by the curves in Figs. 2 and 3. The problem of the influence of concentration of acid on the degree of swelling has been studied very thoroughly by Procter. It is to be noticed that Procter used 1 g. of moist gelatin in 100 cc. of experimental fluid. In the experiments shown below the ratio of dry gelatin to fluid varies from 1/2200 to 1/3750. The importance of keeping this factor constant unfortunately had not been fully realised at the time the experiment was made. The curves therefore carry a small relative error due to this cause.

	I	II	III	IV	V	VI
Concentration of HCl. Volume in } all cases = 150 cc.	0.001 <i>N</i>	0.005 <i>N</i>	0.01 <i>N</i>	0.05 <i>N</i>	0.1 <i>N</i>	0.5 <i>N</i>
Weight of leaf gelatin, g.	0.0640	0.0500	0.0644	0.0852	0.0838	0.0781
Weight of gelatin corrected for } water content, g.	0.0512	0.0400	0.0515	0.0682	0.0670	0.0625
Ratio, weight of gelatin/acid volume	1/2930	1/3750	1/2950	1/2200	1/2240	1/2400

The progress of swelling in the six systems can be followed in the curves in Fig. 2. In systems IV, V, and VI the gelatin dissolved in the acid in the course of a few days. System III was unfortunately attacked by mould and the end of the curve was therefore lost, but from other experiments it is known that 0.01*N* hydrochloric acid also dissolves gelatin in the proportions given. It will be shown later that this is a true case of solution and that it does not involve any break-down of the gelatin molecule.

Curves I and II appear to reach an equilibrium state. The daily increase of weight becomes imperceptible, but even after two or three months the swollen jellies still show a slow, steady increase in weight. This type of behaviour has already been recorded by Masson [1905], studying the absorption of water by silk fibres. It may therefore be characteristic of all proteins. It suggests the possibility that both gelatin and silk fibre would ultimately dissolve in water or dilute acid, given a sufficient quantity of solvent and sufficient length of time.

It is interesting to compare curve I, Fig. 2 with curve II, Fig. 1. The very different level reached in the two cases is probably due to the difference in thickness of the two sheets of gelatin from which the discs were cut in the two different experiments. This factor, *i.e.* thickness of the plate, appears in the equations of both Hofmeister [1890] and Pauli [1897] for the swelling of gelatin in water.

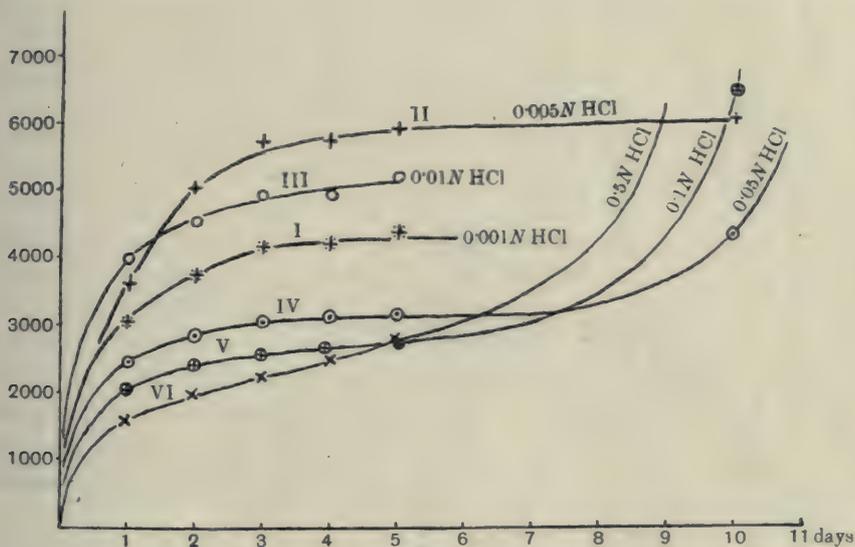


Fig. 2.  
Abscissa = time.  
Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

In Fig. 3 are given the time-weight curves of the alkaline series. The isoelectric point of gelatin is at  $P_H = 4.6$ . All media having a value for  $P_H$  greater than 4.6 are therefore "alkaline" to gelatin. For this reason the curve for distilled water has been placed in the same series as the curves for caustic soda. The experimental quantities used in the experiment were:

	I	II	III	IV	V	VI	VII
Concentration of NaOH, Volume in all cases, 150 cc.	0.5N	0.1N	0.05N	0.01N	0.005N	0.001N	Water
Weight of leaf gelatin, g.	0.070	0.0627	0.0659	0.0595	0.0592	0.0586	0.0801
Weight of gelatin corrected for water content, g.	0.056	0.0502	0.0527	0.0476	0.0474	0.0469	0.0641
Ratio, weight of gelatin/alkali volume	1/2680	1/2980	1/2850	1/3150	1/3160	1/3200	1/2350

The same sheet of gelatin was used for cutting the discs used in all the experiments charted in Figs. 2 and 3. The variation between the two series due to varying thickness of the gelatin has therefore been eliminated. The two series differ in two striking ways; the first is the much greater rapidity with which the gelatin is dissolved in the more strongly alkaline solutions

than in acids of equal normality, the second is the much greater degree of swelling attained by the gels in the acid systems. The last point is well shown in Fig. 4.

In Fig. 4 the curve weight of gelatin gel against reaction of fluid has been plotted. The time has been fixed arbitrarily at 48 hours. The similarity of this curve to the corresponding curve for skeletal muscle [Jordan Lloyd, 1916] is very striking.

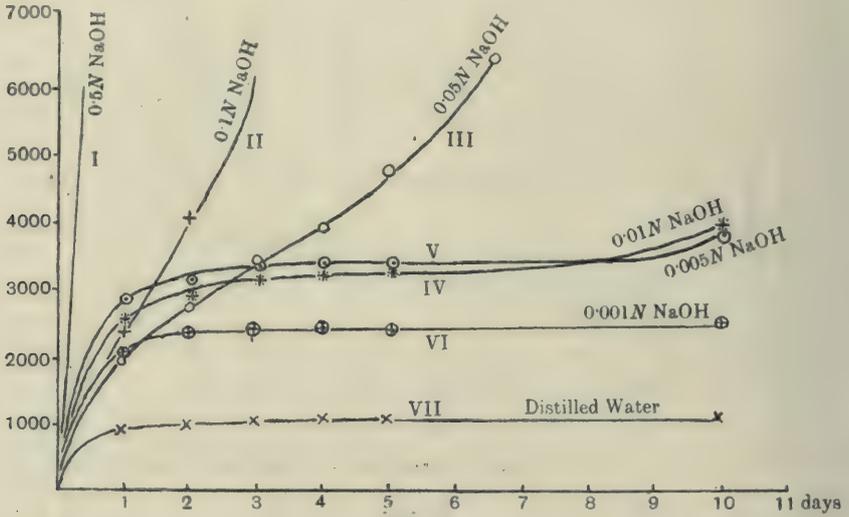


Fig. 3.  
Abscissa = time.  
Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

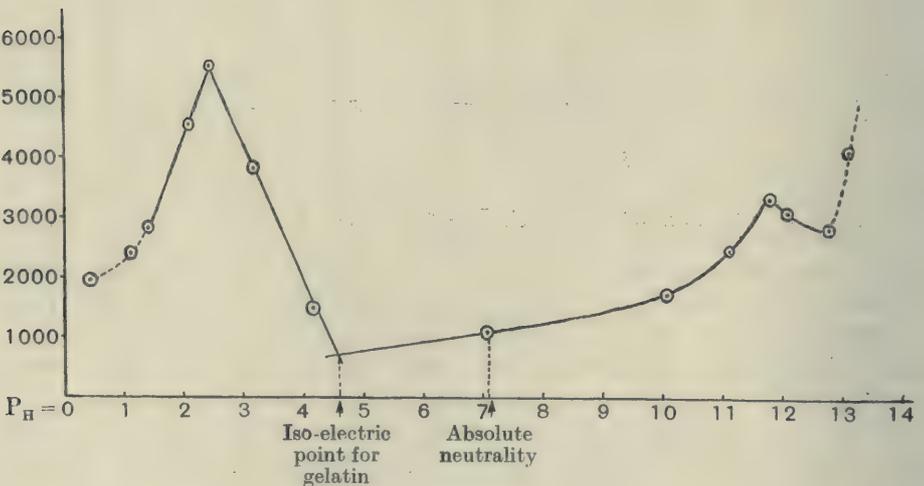


Fig. 4.  
Abscissa = hydrogen-ion concentration.  
Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

## •IV. THE STATE OF GELATIN DISSOLVED BY ACID OR ALKALI IN THE COLD.

If solutions of gelatin in hydrochloric acid or in caustic soda which have been made by allowing the gelatin to stand in contact with these fluids in the cold are examined *at the moment of solution*, there is no chemical evidence that there has been any break-down of the protein molecule.

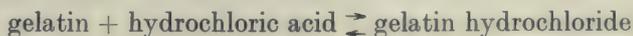
Consider first the case of gelatin dissolved in dilute hydrochloric acid. The following experiment may be cited: 2 g. of gelatin were placed in a flask containing 200 cc. *N*/10 hydrochloric acid. The gelatin was completely dissolved after six days at 20°, 20 cc. *N* NaOH were added to the solution, which was tested and found neutral to litmus, and 220 cc. saturated ammonium sulphate. A white flocculent precipitate was formed. This was filtered off. The filtrate was tested with the biuret test and shown to be free from protein. The precipitate was insoluble in cold water, and was washed several times. It was found to be rapidly soluble in hot water. The precipitate was completely dissolved in 2 cc. of hot water and the solution set to a gel on cooling. A control experiment made by dissolving 2 g. of gelatin in 220 cc. of water with 1.12 g. of sodium chloride behaved in a similar manner.

Gelatin therefore can be recovered from an acid solution.

A similar solution of gelatin (*i.e.* 2 g. in 200 cc. *N*/10 hydrochloric acid) was poured into 3 litres of 96 % alcohol. A white gelatinous precipitate formed which settled slowly to the bottom of the flask. This was filtered off and was washed with absolute alcohol. On cold water being added to the precipitate it swelled rapidly to a clear, glassy, but very soft gel. This was melted and was found to have an acid reaction. The volume was brought to 2 cc. and the solution set to a gel on cooling.

Solutions of gelatin in acid (made in the cold) were compared against control solutions in water for the presence of free ammonia and free amino-groups. Nessler's method was used for the detection of ammonium groups, and Sørensen's formol titration method for the estimation of amino-groups. No difference could be detected between the acid solution and the water solution, *i.e.* there was no evidence of break-down of the gelatin molecule at the moment of solution. If the two solutions are left standing for a day at room temperature, it can be shown that in the acid solution a very slow hydrolysis is taking place.

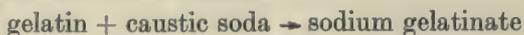
These facts accord fully with Procter's theory that gelatin forms salts with hydrochloric acid. They also show that the change



is reversible.

The case of solution in alkali is more complicated. Though the gelatin goes rapidly into solution without molecular break-down, there is evidence that the formation of the solution is accompanied by some internal structural

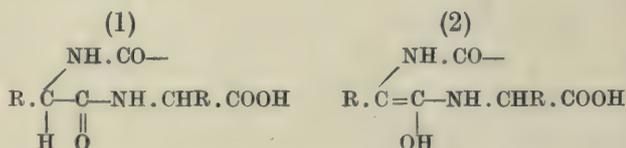
change in the protein molecule since gelatin is not readily recovered from a caustic soda solution, *i.e.* the change



is not reversible. Brailsford Robertson [1918] has suggested that acids and bases attach themselves to protein molecules at the  $-\text{COHN}-$  linkage. He points out that this linkage may exist as an enol-linkage  $-\overset{\text{C}}{\underset{\text{OH}}{=}}\text{N}-$  or as a

keto-linkage  $-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{H}}{\underset{\text{O}}{\text{N}}}-$ , and favours the enol-form since it offers a point of

attachment to both acids and bases. A more probable explanation seems to be that under the action of acids gelatin goes to the keto-form, and under the action of bases to the enol-form. This would conform with the observation that the free acid from sodium gelatinate differs in properties from the free base of gelatin hydrochloride. It can also be harmonised with Dakin's theory [1913] that the non-terminal groups in proteins go from the keto-form (1) to the enol-form (2)



with loss of optical activity under the action of bases at low temperatures.

The experimental evidence that gelatin dissolves in caustic soda with change of properties, but without molecular break-down, is given below.

0.2 g. gelatin was placed in a flask with 200 cc. *N*/10 NaOH. The gelatin was completely dissolved after two days at 20°. 20 cc. hydrochloric acid were added to the solution, which was tested and found neutral to litmus, and 220 cc. saturated ammonium sulphate. A white flocculent precipitate formed immediately and was filtered off. The filtrate was tested with the biuret test and found free from protein. The precipitate dissolved completely and rapidly in a few cc. of cold water. The solution was reduced to a volume of 2 cc. (= 10% concentration of gelatin) but it did not gelatinise on cooling.

A similar solution (*i.e.* 0.2 g. of gelatin in 200 cc. *N*/10 NaOH) was poured into 3 litres of 96% alcohol. A white gelatinous precipitate was formed which settled slowly. This was filtered off and washed with alcohol. It was strongly alkaline. Enough *N* HCl was added to the gelatinous, slimy mass to make it neutral to litmus. 2 cc. of hot water were added; the precipitate dissolved completely forming a turbid solution but this did not set to a gel on cooling.

Two solutions of gelatin were made:

(A) by dissolving 0.2 g. of gelatin in 200 cc. *N*/10 NaOH in the cold and neutralising with *N* HCl.

(B) by dissolving 0.2 g. of gelatin and 1.12 g. of sodium chloride in 200 cc. water by means of heating.

These two solutions contained identical concentrations of gelatin, sodium ions and chlorine ions, and had the same reaction (neutral to litmus). They were dialysed against distilled water for a week to reduce the concentration of sodium chloride. They were then evaporated to small volume, 2 cc.: B set to a firm jelly on cooling; A remained fluid. The jelly B was melted and the two solutions A and B were half saturated with ammonium sulphate. A dense white precipitate formed in both tubes, and was filtered off. The filtrates were free from protein. Precipitate A was soluble in cold water, precipitate B was insoluble in cold water but dissolved readily on warming.

Gelatin therefore cannot be recovered unchanged from alkaline solution.

Solutions of gelatin in caustic soda, made in the cold, were compared against control solutions in water for the presence of free ammonia and free amino-groups. No difference could be detected between the alkaline solution and the control, *i.e.* there was no evidence of break-down of the protein molecule at the moment of solution. It is important that the solutions should be examined immediately after solution, as the alkaline solution shows evidence of hydrolysis after two or three hours' standing at room temperature. The fresh gelatin-alkaline solutions were examined for their rotatory power. No change in optical activity of the gelatin was detected, and indeed from Dakin's experiments would hardly be expected.

#### V. THE BASIC AND ACIDIC PROPERTIES OF GELATIN.

The observation that gelatin solutions can dissolve considerably greater quantities of lime than can pure water is recorded by Heintz [1853], and the solubility of gelatin in acid or alkali in the cold by Kuhne [1888]. The first definite description of these compounds of gelatin with acids and bases respectively, as gelatin salts, appears to be in a paper by Nasse [1889] published in 1889. Pauli [1912, 1913] further postulated that gelatin salts were ionised in aqueous solution. A considerable amount of work has since been done on the subject and the present position may be summarised briefly as follows: gelatin can combine chemically with both acids and bases, the amount of acid or base combined with unit weight of gelatin being a function of the hydrogen-ion concentration. This is well shown in both Procter [1914] and Loeb's [1919, 1] recent experimental figures. This inter-relation of combined acid and reaction has been interpreted as an adsorption phenomenon, but it can equally well be deduced from the theory that gelatin is a multi-basic acid or a multi-acid base, which dissociates in stages according to the reaction in a way strictly analogous to the dissociation of phosphoric acid. Procter considers that gelatin acts as a di-acid base and has a molecular weight of 839. Consideration of the chemical evidence (see Appendix) shows that the molecular weight of gelatin must be of the order 10,000. A few rough experiments made by means of indicators during the course of this work have indicated that gelatin when acting as a base is not completely neutralised by hydrochloric acid until the reaction is at  $P_H < 2.5$ . At this reaction, and taking

10,000 as the molecular weight, the basicity is 8. Conversely in an alkaline system, the acid valencies of gelatin are not yet satisfied at  $P_H = 13$ , and the acidity at this reaction appears to be 28. This figure however is not reliable. Guttenberg [1896] and Berrar [1912] using different quantitative methods both reach the figure for gelatin, 10 atoms of nitrogen correspond to 1 molecule of acid. As Berrar states that the binding power of gelatin for acid is unaffected by excess of acid, this value may be taken to be a maximum. Assuming 133 nitrogen atoms in the gelatin molecule (see Appendix) the basicity of gelatin becomes 13.

The chemistry of the gelatin salts is at present under investigation, and it is hoped shortly to publish more satisfactory evidence of their constitution. The theory that gelatin forms ionisable salts which are more soluble in water than either the free acid or the free base is in accordance with all the evidence at present available and has been dealt with briefly here, as it is an essential part of the theory of gel structure which is discussed in Section VII of this paper. The further question of the nature of the ions to which these gelatin salts give rise in solution is also discussed.

#### VI. THE BEHAVIOUR OF SWOLLEN GELS IN AN ATMOSPHERE OF SATURATED WATER VAPOUR.

Masson [1905] has recorded for silk fibres that the maximum degree of swelling in liquid water or in saturated water vapour is identical, and that the rates of swelling in the two cases are very nearly so. Schroeder [1903] has stated that this is not the case with gelatin. He gives figures showing that dry gelatin in contact with water vapour will increase its weight up to about 140 % of the dry weight; removed to liquid water further increase up to 1200 % will occur. Conversely gelatin in equilibrium with, or even absorbing water from, liquid water will give up water if transferred to an atmosphere of saturated vapour. Schroeder's experiments were made under apparently controlled conditions, but his controls were not very rigid. Wolf and Buchner [1915] re-investigated the question of the equilibrium of gels in liquid and gaseous water and found that by conducting their experiments in a very reliable thermostat, and using vessels silvered on their internal surfaces, it was possible to transfer gelatin in equilibrium with liquid water into the saturated vapour phase without subsequent loss of weight occurring. With even the slightest temperature variation in the apparatus, loss of water from the swollen jelly promptly occurred. This loss however occurred equally from small basins of pure water suspended in the chamber, and Wolf and Buchner regard it as a distillation on to the walls of the containing vessel under a temperature gradient. It appears therefore to have been satisfactorily demonstrated that the equilibrium of gelatin gels in water and water vapour does not form an exception to the second law of thermodynamics as has been claimed. But in spite of the undoubted truth of Wolf and Buchner's results, and the disproof of Schroeder's phenomenon *per se*, it is hoped to demonstrate

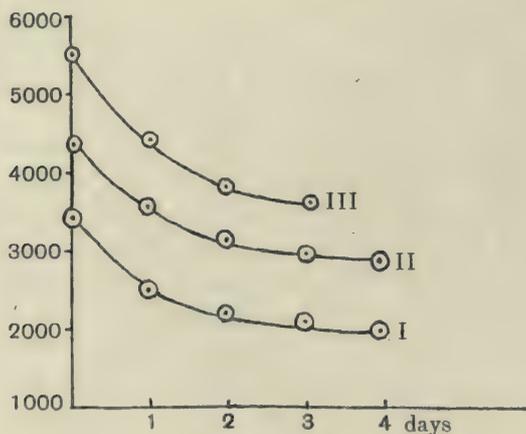
that in systems consisting of acid or alkaline gelatin two separate equilibria do actually occur in the fluid and gaseous media, but that the existence of these two points follows naturally from Donnan's membrane potential theory [1911] and does not therefore form an exception to the second law of thermodynamics.

If gels which are swelling in dilute hydrochloric acid, or in caustic soda, are lifted out of the liquid into a vapour phase, they immediately contract and express fluid. The expulsion of water is frequently so great and so rapid that after three or four minutes in air, drops of liquid can be seen gathering at points all over the surface of the jelly even though the latter be kept in the horizontal plane. In the course of a few hours these beads of water may run together and flow away from the jelly, causing loss of weight, and if the gels are kept in an atmosphere of saturated vapour over a plane surface of water and at a steady temperature, further marked loss will continue to occur for three or four days, doubtless owing to the raised vapour pressure at the curved surface of the fluid drops. It is particularly wished to emphasise that the water does not merely drip away from the lowest edge of a suspended jelly, but is squeezed out at definite *loci* scattered evenly all over the free surface of the gel. This observation confirms the existence of definite pores in a jelly surface, which was postulated both by Bütschli [1896, 1898] and v. Bemmelen [1898]. It may be noticed that Hardy's figures of coagulated colloidal solutions all show an orientation of the solid particles at the surface, which would lead to the formation of pores. This phenomenon of recoil appears to be a good demonstration of the existence of elastic forces within the gel, and it will be shown below that these elastic forces must be localised in the solid framework. The amount of water squeezed out by a gel in this way is independent of the total amount of water in the gel. This is shown by the following experiment: discs of leaf gelatin were soaked in  $N/100$  NaOH for 1, 2 and 5 days respectively, the swollen jellies were transferred to the same moist chamber, and weighed every 24 hours.

	I	II	III
Weight of moist gelatin, g. . . . .	= 0.0655	0.0590	0.0610
„ „ gelatin corrected for 20 % moisture, g. . . . .	= 0.0524	0.0472	0.0488
Volume of $N/100$ NaOH added . . . . .	= 150 cc.	140 cc.	140 cc.
Days in $N/100$ NaOH . . . . .	= 1	2	5
Weight on removing from NaOH, g. . . . .	= 1.8515	2.1410	2.754
Ditto calculated as percentage of dry weight of gelatin =	3530	4420	5630
Weight after 24 hours in moist chamber calculated			
as % of dry weight . . . . .	= 2520	3590	4440
Do. after 48 hrs . . . . .	= 2270	3160	3840
Do. „ 3 days . . . . .	= 2180	3000	3550
Do. „ 4 days . . . . .	= 1900	2900	—

These figures have been plotted and are shown on the curves in Fig. 5. It will be seen that the three curves are parallel. If the shrunken gels are again immersed in fluid the reaction is reversed, water being again absorbed.

Though the amount of water in a gel does not determine the amount lost in an atmosphere of saturated water vapour, the reaction of the fluid in which the initial swelling of the dry leaf gelatin has taken place is a very potent factor. By varying the reaction it is possible to determine at will whether a gel shall lose weight on being transferred to a vapour phase, gain weight, or remain at a steady equilibrium. Curves for acid gels of varying reaction are shown in Fig. 6, and for alkaline gels in Fig. 7. In these curves, the abscissa is time, and the ordinate the gain or loss in weight which occurs after transferring to the vapour phase. This is expressed in percentages of the dry weight of gelatin. Notice that in all cases the weight at transference is placed at the origin. In both figures a control curve has been plotted. This shows the change in weight of a disc of moist filter paper kept in the same apparatus. Under ideal conditions this should of course run parallel to the  $x$ -axis. It will be seen that in reality it falls, slowly and steadily.



Abscissa = time.

Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

The initial weights of the gelatin discs and their weights after soaking for 48 hours in the experimental fluid are given below. The various weight changes in the saturated vapour phase can be read from the curves. 150 cc. of acid or alkali were used in all the experiments. The acid figures (see Fig. 6) are given first.

Concentration of hydrochloric acid, 150 cc. taken	$N/2$	$N/10$	$N/20$	$N/200$	$N/1000$	$N/10,000$	$N/100,000$
Weight of gelatin disc, g.	0.0676	0.0676	0.0870	0.0695	0.0604	0.0640	0.0650
Do. corrected for 20% water content, g.	0.0541	0.0541	0.0696	0.0558	0.0484	0.0512	0.0520
Weight after 48 hrs. in acid, g.	0.864	1.031	2.0268	2.169	1.430	0.922	0.788
Do. expressed as percentage of dry weight of gelatin	1590	1910	2920	3900	2960	1800	1510

The experimental quantities in the alkaline series (Fig. 7) were:

Concentration of NaOH, 150 cc. taken	N/10	N/20	N/100	N/200	N/1000	N/10,000	N/100,000	Dis- wat
Weight of gelatin, g.	0.0622	0.0621	0.0620	0.0637	0.0581	0.0636	0.0622	0.062
Do. corrected for 20% water content, g.	0.0498	0.0497	0.0496	0.0510	0.0465	0.0509	0.0498	0.055
Weight after 48 hours in NaOH, g.	1.617	1.135	1.206	1.297	0.835	0.501	0.495	0.551
Do. expressed as percentage of dry weight of gelatin	3250	2280	2440	2545	1790	990	996	990

It can be seen from Figs. 6 and 7 how very powerfully the reaction of the fluid influences the behaviour of the gel in the saturated vapour. Though the water loss is considerable in some of the acid systems, it is even exceeded in the alkali systems. In the experiment with N/200 HCl (Fig. 6) 100 units

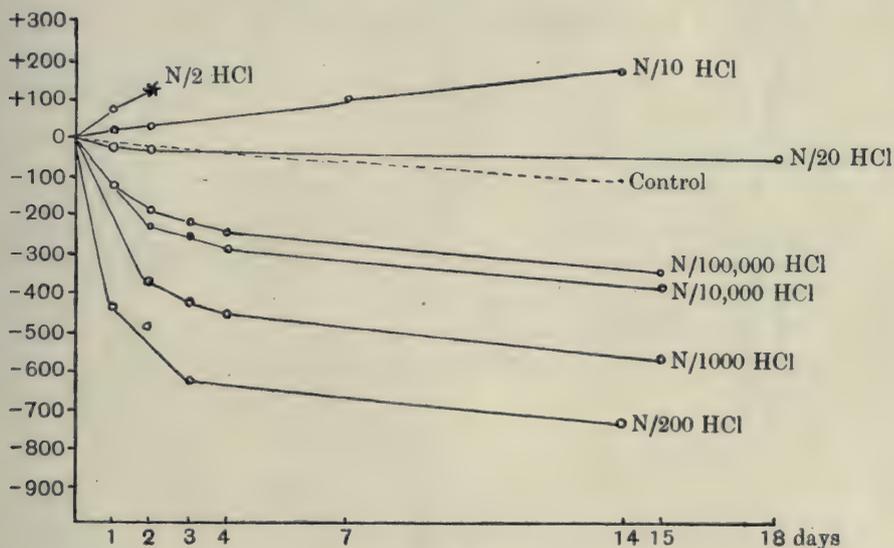


Fig. 6.

Abscissa = time.

Ordinate = change of weight in saturated vapour calculated as a percentage of the dry weight of gelatin.

of gelatin were holding at transference 3900 units of water; 650 of these, equalling one-sixth of the water content of the jelly, were lost in three days. In the alkaline series the greatest loss again occurs at the concentration N/200. In the experiment shown in Fig. 7, 100 units of gelatin were holding 3500 units of water; 1700 units of water, equalling nearly a half of the total water content, were expressed in three days. After three or four days the weight curves all run parallel to the filter paper control. Further loss of weight may therefore be attributed to experimental error. It is interesting that the reaction causing maximum swelling in the liquid systems should coincide with the reaction allowing maximum recoil in the vapour phase. It should be noted that the fluid expressed by the gels is not pure water, but contains gelatin

in solution. From acid gels the fluid has an acid reaction; from alkaline gels an alkaline. The reaction of the expressed fluid is not the same as the reaction of the external medium, but lies nearer to the neutral point. This fluid is the "internal phase" of the gel.

Gels which have been swollen in hydrochloric acid or caustic soda of normality  $N/20$  or greater all show further absorption of water on being transferred to the saturated vapour. (In the  $N/20$  NaOH curve, shown in Fig. 7, there is loss of water followed by absorption.) In some cases the gels

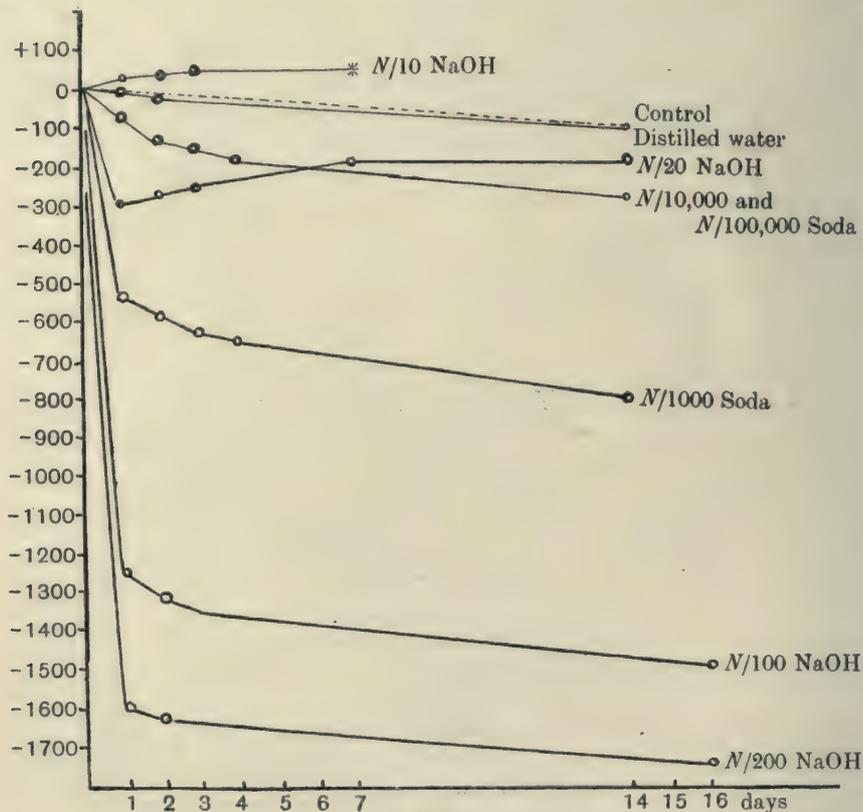


Fig. 7.

Abscissa = time.

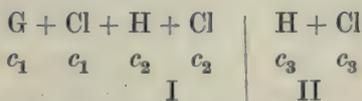
Ordinate = change of weight in saturated water vapour calculated as percentage of dry weight of gelatin.

ultimately liquefy. These curves are starred on Figs. 6 and 7. It can be shown here, as in solution in strong acid or caustic soda, that the gelatin has not been hydrolysed but has only gone into solution. It can be recovered from the acid solutions, but not from the alkaline ones.

The behaviour of the swollen gels in a vapour phase seems to be most easily interpreted on the theory that it is determined by a solid framework in the gel, which possesses elastic properties. The stretching force in the gel may be taken to be the osmotic pressure of the gelatin salts in solution. The

forces of recoil appear to conform to Hooke's law, stress  $\propto$  strain, *i.e.* they become greater the more swollen the jelly. Osmotic forces and elastic counterforces will therefore increase in relation to each other, and in the experiments shown in Figs. 6 and 7 it will be seen that with increasing concentration of acid or alkali—up to a reaction corresponding to a concentration  $N/200$  for the strong acid hydrochloric, and the strong base caustic soda—that the greater the swelling in the fluid, the greater the recoil in the vapour. Notice that the acid and alkaline systems, though similar, are not symmetrical: the greatest swelling is reached in acid solutions, the greatest recoil in the alkaline ones. As acid and base become more and more concentrated, the gelatin passes to a greater and greater extent from the solid external phase to the fluid internal phase. This gradual corrosion of the solid framework is accompanied by a decrease of elastic properties, until finally they vanish, the framework ceases to exist and the whole system becomes a hydrosol.

The recoil of swollen jellies on transference to a system of saturated water vapour has been quoted as an exception to the second law of thermodynamics. It can however be anticipated from Donnan's membrane theory, which is based on an application of the second law. Donnan has shown that if a system containing in solution an ionisable crystalloid and an ionisable colloid, which dissociates giving one colloidal ion, be separated into two phases by a membrane impermeable to the colloidal ion, then the crystalloid will distribute itself unequally across the membrane, the phase of greater concentration of the crystalloid being the phase free from colloid. Consider the system gelatin jelly in equilibrium with a hydrochloric acid solution. This can be represented as:



where  $c_2$  and  $c_3$  represent the concentrations of hydrochloric acid in the jelly and liquid phases respectively, and where  $c_1$  represents the concentration of the gelatin salt. Both salt and acid are assumed to be completely dissociated. Hydrolysis of the gelatin salt will be suppressed in the presence of free acid. If  $c_1, c_2, c_3$  represent ionic concentrations, then  $c_3$  must always be greater than  $c_2$  owing to the electrostatic repulsion between non-diffusible G, the gelatin ion, and diffusible H. But now consider what must happen if the acid solution in phase II be replaced by saturated water vapour. This system is unstable since the electrostatic forces between G and H are no longer balanced. A certain fraction of the hydrogen ions from I, with their accompanying chlorine ions, will therefore be transferred across the membrane, and for this to be possible water in the liquid form must be transferred too. Notice that the greater the ratio  $c_1/c_2$ , the greater will be the fraction of  $c_2$  which has to move from I to II in order to adjust the equilibrium, and the greater therefore the loss of water from the swollen gel. Although the weakening of the osmotic forces brought about in this way within the jelly will partially account for

the diminished volume, it is important to notice that the escape of large quantities of liquid water only occurs in gels in which it may be presumed that the elastic framework is still intact. The water is therefore partly expressed under the action of the recoil of the gel.

#### VII. THEORY OF GEL STRUCTURE DEDUCED FROM A STUDY OF GELATIN SWELLING.

Though the gel state is easily recognised in the laboratory, by its appearance and properties, the molecular mechanism which underlies these has been very variously pictured. Three types of mechanical models have been postulated, all differing in their physical conceptions, and all offering evidence in their own support. These are:

- (1) The model of a gel as a two-phase solid-liquid system.
- (2) The model of a gel as a two-phase liquid-liquid system.
- (3) The model of a gel as a one-phase (presumably fluid) system.

In the last model a molecular network is generally thrown through and round the system, in order to cope with the anomaly, that though there is only one phase and that fluid, yet it has lost the property of flowing! The theory that gels in equilibrium with fluids are one-phase structures has been strongly advocated by Procter. It is also the theory finally accepted by Brailsford Robertson after a general survey of the literature of the subject. The one-phase theory of gel structure has been held by Pauli for gelatin, by Hayes [1914] for pecten, etc. The chief line of argument in its favour used by its supporters is that it is sufficient to explain the various properties of gels, and that the appearance of a second phase at setting is an unnecessary assumption.

The two-phase liquid-liquid theory of gels was postulated by Ostwald [1905, 1909]. It is very easy to show that oil and water, for example, can be made, in certain proportions, to form stiff emulsions that will not flow in a test-tube. These emulsions, however, have not really the solid properties of gels. They cannot, for instance, be handled like blocks of gelatin jelly, retaining their shape the while. Hatschek [1916] has shown by mathematical reasoning that the liquid-liquid theory is untenable.

The two-phase solid-liquid theory of gel structure appears in several forms. Hardy, basing his belief on microscopical evidence, states that in most cases gelation consists of the breaking up of the sol into an open solid framework, with an interstitial fluid phase. The surface of the jelly on this theory would appear as a solid membrane perforated by numerous pores. This structure has also been postulated by van Bemmelen and by Andersen [1914] for gels of silica, and is accepted by Freundlich [1909]. In this theory, which is the one accepted by the writer, both solid and liquid phases are continuous.

Another form of the solid-liquid theory of gels is that of Bradford [1918]. Bradford's conception of the gel state is that the solid phase is discontinuous, and he compares a gel in structure to a pile of shot. He states that gels cannot have a rigid framework since they flow slowly and take on the form

of the containing vessel. This observation has not been confirmed during the course of the present work, in fact it has constantly been noticed that thin flat discs of gelatin maintain a rigid attitude when immersed in fluid, and keep perfectly flat for months, even though not lying in a horizontal plane, and unsupported except at the upper and lower edges. Gelatin discs which have been placed in a suspended crucible in order to swell in an experimental fluid will, if they grow sufficiently large, lift themselves right out of the crucible, rather than accommodate themselves to its limitations by bending. Throughout the course of this work, both in liquid and gaseous media, if flowing was observed in a jelly, it was always a sign that liquefaction of the gel was immanent. The theory of the discontinuity of the solid phase of gels appears to be upheld by Arisz [1914].

The process of separation of the solid phase from the liquid has been compared to the process of crystallisation, from the early days of the study of colloids. Both Frankenheim [1835] and von Nägeli [1858] state that the solid part of a gel is crystalline, and the same theory is held by von Weimarn [1908], Levites [1908] and Bradford. Hardy has called the external phase a solid solution, and has shown that it contains water, the amount of the latter depending on the rate of cooling. If the water can be regarded as present in the solid phase as water of crystallisation, and if the existence of several hydrates of gelatin is postulated, then the variation of water content with rate of cooling remains in harmony with the crystalline theory of the solid phase. Moreover, if the external phase is solid then there must be a definite amount of adsorbed water at the solid-liquid interface [see Parks, 1902, 1903]. Any variation in the interfacial area, such as is brought about by variations in the cooling rate, will therefore again affect the water content of the solid phase. Osborne [1918] has recently examined the problem as to whether the solid phase of a gel is crystalline or amorphous. His results are not very conclusive.

Although it is obvious that both the "liquid-liquid" theory and the "pile of shot" theory of gel structure would lead to the formation of highly viscous fluids which would closely simulate gels, yet the theory that gels possess a continuous solid framework appears to explain most satisfactorily the elastic properties dealt with in Section VI of this paper. This continuous framework forms a *locus* for the contracting forces which have been demonstrated to exist in the gels.

There remains to be considered the liquid phase of the gel. Hardy showed this to be a solution of gelatin in water, and Procter brought forward very satisfactory evidence that in a gelatin-hydrochloric acid system the gelatin is present as a soluble salt. Procter's theory of gelatin swelling, though now well known, is summarised here for the sake of clearness. Gelatin is an amphoteric substance which possesses the power of combining with acids to form di-acid, ionisable salts. These dissociate to form one diffusible ion, the anion, and one non-diffusible ion, the gelatin cation. At equilibrium in

a system consisting of gelatin jelly plus acid external fluid, the ionic products on each side of the jelly-water interface are equal, but the sums of the diffusible ions in the two systems are unequal, owing to the unbalanced diffusible anions of the jelly salt which are retained in the jelly phase. This is of course an application to a special case of Donnan's general theory of membrane equilibria [1911]. This inequality of diffusible ions in the two systems causes an osmotic pressure in the jelly, which is greater than the osmotic pressure in the external fluid by a quantity which Procter calls  $e$ . This osmotic excess  $e$  is the force which causes the jelly to swell, but there must be some other force opposing  $e$ , since otherwise the jelly would swell to infinity. The origin of this opposing force is not very clear in Procter's paper, where it is assumed that the jelly only forms a single phase, but if a continuous solid framework is accepted as an integral part of the gel structure, then the counter-force,  $-e$ , is readily located in the elastic properties of this framework. If  $e$  becomes sufficiently great, the framework is stretched beyond its breaking point,  $-e$  vanishes, and  $e$  does cause the jelly to swell to infinity, in other words to dissolve in the external fluid. The volume occupied by a gel in an acid solution therefore is that due to the resultant of two sets of forces, osmotic and elastic, located respectively in the two phases of the gel. Loeb [1919, 2] has considered it due to the resultant of two sets of forces, osmotic and electric. The obvious importance of the electric forces is included in Donnan's original theory. One common misinterpretation of Donnan's theory might be mentioned here. It has been held that Donnan's "membrane potential" implies that there must be a charge at the surface of the membrane, and various workers, including Ehrenberg [1913], have endeavoured in vain to demonstrate this. An examination of Donnan's theory, however, shows that the membrane potential is due to an electric repulsion between the non-diffusible ions and the diffusible ions of the same sign. This leads to a condition of electric strain across the membrane, but not to polarisation in the ordinary sense of the term. Procter claims to have detected a potential of a few millivolts at a gelatin-water interface. The theory of acid-gelatin equilibrium can also be applied to alkaline-gelatin equilibrium, though the qualification must be added that the former equilibrium is reversible, the latter is not. This distinction is probably one of considerable significance in biological problems, especially in view of the fact that the muscle colloids have been shown in freshly excised muscles to be a protein-acid system [Jordan Lloyd, 1916]. It should be noticed before leaving the subject of the gelatin salts that Procter considers that the jelly cation of gelatin hydrochloride is non-diffusible and contributes nothing to the osmotic pressure. But the fact that the internal phase of swollen jellies can be shown to contain gelatin hydrochloride as a non-gelling compound, shows that the colloidal ion must be diffusible and must therefore exert an osmotic pressure. Brailsford Robertson believes that protein salts ionise across the middle of the protein molecule, giving two colloidal ions. If this view were correct, the distribution of free hydrochloric acid across

a jelly water interface would result in equal concentration on both sides. But in Procter's experimental results, the concentration of the acid in the external medium is always greater, at equilibrium, than in the jelly itself, a result which follows naturally if gelatin hydrochloride gives rise to one colloidal and one crystalloidal ion.

A gelatin gel may therefore be taken to consist of two phases, solid and liquid, and two chemical states of gelatin, *viz.* gelatin *per se* and gelatin in the form of soluble salts. Such gels therefore are three component systems, the components being water, gelatin, and an acid (or base).

It is necessary to consider the evidence that there must be some third component—an electrolyte—in a gel system. The most convincing work on the subject is that of Jordis [1902] on colloidal silicic acid. Jordis shows that the nearer silicic acid comes to chemical purity the more insoluble it becomes, and that ultimately when chemically pure it is practically insoluble in hot or cold water, and cannot be made to pass into the colloidal state. This only occurs in the presence of traces of acid, alkali, or organic groups. Loeb [1918] has shown recently for gelatin that at the iso-electric point it is an inert substance, insoluble in cold water, which moreover releases neither hydrogen nor hydroxyl ions, *i.e.* it has all the characteristics of an insoluble solid precipitate. Consider the case of a hot solution of gelatin cooling at a reaction  $P_H = 4.6$ . The iso-electric gelatin will be precipitated at numerous crystallisation centres, the solid drops will run together to form a framework, but since there can be no osmotic forces in the system (the only available source being the + and - ions of the dilute acid, to which the pores of the framework may safely be presumed to be freely permeable), the framework will contract under the action of its own surface forces, and the internal phase will be squeezed out. In short the system will show minimal swelling. That minimal swelling does coincide with the iso-electric point has been shown by Chiari [1911]. The properties of iso-electric gelatin have not been studied to any extent, probably owing to the extreme difficulty of keeping the system at the iso-electric point, and free from electrolytes. The purest water obtainable is still an electrolyte, and is alkaline to gelatin, and therefore will react with it to form salts. Much work, especially by Dheré [1913], Dheré and Gorgolewski [1910], Nasse [1889], Mörner [1889], etc., has been devoted to preparing ash-free gelatin, and studying its properties. Ash-free gelatin, however, is not necessarily iso-electric, and the fact that Dheré states ash-free gelatin to be electro-negative is sufficient to show that he was dealing with an alkaline system, and not with pure iso-electric gelatin. It is therefore justifiable in view of the work of Loeb, Chiari and others, to deduce that the gel state as a two-phase system cannot exist as a stable system at the iso-electric point.

It is now necessary to consider the fate of the system in strongly acid and strongly alkaline media, respectively. It has been shown during the course of this work that in the presence of sufficient acid or base the hydrogel

becomes a hydrosol. This process is reversible in an acid system, irreversible in an alkaline one. Gelatin, therefore, cannot form gels even in concentrated solutions, if it is present entirely as a gelatin salt. The process of gelation is therefore pictured as follows: gelation will only occur on the cooling of a sol which contains in solution iso-electric gelatin, and gelatin salts in equilibrium with free electrolytes. As the sol is cooled the insoluble iso-electric gelatin is precipitated in a state of suspended crystallisation and forms a solid framework throughout the system. The more soluble gelatin salts remain in solution, and by their osmotic pressure keep the framework extended. Gels therefore are two-phase systems, the solid phase consisting of iso-electric gelatin, the liquid of gelatin in the salt form.

This paper forms a preliminary part of an investigation undertaken on behalf of the Medical Research Committee on the equilibrium of the tissues and body fluids. I have to thank Professor Hopkins for much encouragement during the course of the work.

## APPENDIX.

### ON THE MOLECULAR WEIGHT OF GELATIN.

The molecular weight of gelatin determined by physical methods has been estimated at values ranging from 800 to 31,000. The following estimations are quoted:

	Mol. wt. of gelatin
Schutzenberger and Bourgeois [1876]. . . . .	1836
Paal [1892] . . . . .	900
Procter [1914] . . . . .	839
Berrar [1912] . . . . .	823
Biltz, Bugge and Mehler [1916] . . . . .	5500 to 31,000 for different varieties

Considerations based upon the most accurate estimations of individual amino-acids lead to a figure for the reacting weight of the order of 10,000. The value of the evidence which is given below should be duly weighed. The evidence of the distribution of nitrogen in the gelatin molecule is taken from van Slyke's [1912] estimations.

	% of Total nitrogen
{ Ammonia nitrogen . . . . .	2.25
{ Melanine     " . . . . .	0.07
{ Cystine       " . . . . .	0.00
{ Arginine     " . . . . .	14.7 =
{ Histidine   " . . . . .	4.48
{ Lysine       " . . . . .	6.32
Mono-amino " . . . . .	56.3
Non-amino   " = proline + oxyproline	14.9
	99.02

In order to reduce these percentage figures to others which will show the relations in terms of nitrogen atoms, it is necessary to multiply throughout

by some factor arbitrarily determined. Now the histidine molecule contains three atoms of nitrogen, the arginine four, and the lysine two. The atomic ratios therefore must be such that the histidine nitrogen atoms form some multiple of 3, the arginine of 4, and the lysine of 2. Histidine, being present in very small quantity, may be taken as a convenient basis of calculation. If 1 histidine grouping is present in the gelatin molecule then there must be 3 histidine nitrogens. The percentage histidine value 4.48 can be reduced to 3 by multiplying it by 0.665. If, however, 0.665 is taken as the common factor, both ammonia and arginine yield figures corresponding to fractional (half) molecules. If 2 histidine groupings are assumed, the factor becomes 1.33. Multiplying throughout by 1.33 we obtain:

Ammonia nitrogen	...	...	...	...	2.9 = 1 × 3 approximately	
Arginine	„	...	...	...	19.7 = 4 × 5	„
Histidine	„	...	...	...	6.0 = 3 × 2	„
Lysine	„	...	...	...	8.4 = 2 × 4	„
Mono-amino	„	...	...	...	75.2 = 1 × 76	„
Proline + oxyproline nitrogen	...	...	...	...	20.0 = 1 × 20	„
Total	...	...	...	...	132.2 = 133	„

These figures are sufficiently satisfactory to justify the assumption that 1 gelatin molecule contains  $133x$  nitrogen atoms distributed amongst:  $3x$  ammonia (amide) groupings,  $5x$  arginine,  $2x$  histidine,  $4x$  lysine,  $76x$  mono-amino, and  $20x$  proline + oxyproline groupings. In the absence of further evidence,  $x$  may be taken as unity. Two of the figures call for special comment—the lysine value, 8.4 instead of 8.0 is too high, but since the lysine is estimated by difference in van Slyke's calculation, it will carry as a positive error the negative error from both histidine and arginine. The mono-amino figure, 75.2 has been approximated to 76 instead of 75, in order to bring the total from the various groupings to the necessary value of 133.

Nitrogen may be taken to form 18.0% of the total weight of dry gelatin. The following analyses of purified gelatin are quoted:

Paal [1892]	...	...	...	...	N = 18.12% (ash content = 0.07%)
Sadikoff [1903]	...	...	...	...	N = 17.47 Kjeldahl's method
„ [1903]	...	...	...	...	N = 18.18 Dumas' method
Schutzenberger and Bourgeois [1876]	...	...	...	...	N = 18.3
Mulder [1843]	...	...	...	...	N = 18.3
v. Name [1897]	...	...	...	...	N = 17.81
Chittenden and Solley [1891]	...	...	...	...	N = 18.0

Mean = 18.0% error = ± 2%

If 133 atoms of nitrogen form 18.0% of the weight of the gelatin molecule, then the lowest weight of gelatin which can act as a chemical individual must be  $\frac{133 \times 14 \times 100}{18.0} = 10,344$ , or approximately 10,300. The error in the mean of the analyses given above falls within ± 2%; the error in van Slyke's analyses is of the order ± 1%; the total error in the computed value is therefore of the order ± 3%.

## SUMMARY.

1. Gelatin is an amphoteric substance which can exist as the free base (or acid), the so-called neutral gelatin, or as a salt in combination with an acid or a base. The molecular weight of gelatin is about 10,000, or some multiple of 10,000.

2. Pure neutral gelatin is only stable at the iso-electric point ( $P_H = 4.6$ ); it is slightly soluble in hot water, but insoluble in cold water; but it has the power of combining with water (? as hydrates, or as solid solutions).

3. At  $P_H < 4.6$  gelatin acts as a base and combines with hydrochloric acid to form soluble, ionisable salts—hydrochlorides. Gelatin is a multi-acid base, and combines with increasing quantities of acid as the reaction of the fluid becomes more and more acid. At about  $P_H \approx 2.5$  gelatin exists mainly in the salt form.

The reaction: gelatin + hydrochloric acid  $\rightleftharpoons$  gelatin hydrochloride is reversible.

4. At  $P_H > 4.6$  gelatin acts as an acid and combines with soda to form soluble, ionisable salts—so-called gelatinates. Gelatin is a multibasic acid and combines with increasing quantities of base as the reaction of the fluid becomes more alkaline. At  $P_H \approx 13$  gelatin exists mainly in the salt form.

Gelatin acts more readily as an acid than as a base; its dissociation constant for  $H'$  is greater than for  $OH'$ , and its valency towards bases is greater than towards acids.

The reaction: gelatin + soda  $\rightarrow$  sodium gelatinate is not reversible.

5. The formation of keto salts in acids and enol salts in alkalies is postulated, ordinary gelatin being taken as a keto compound.

6. The figures given above apply to a system at  $20^\circ$ .

7. Gels are two-phase structures, which can only be formed in the first place from a fluid system (*i.e.* by cooling). They consist (1) of a solid framework of precipitated neutral gelatin, with which is combined a certain amount of water; (2) of an interstitial fluid which is a solution of gelatin in the salt form (either acid or basic).

8. The volume of any system in the gel condition is determined by the equilibrium between two opposing sets of forces. These are:

(1) The elastic forces of the solid framework which tend to make the volume contract.

(2) The osmotic forces of the ionised gelatin salts dissolved in the interstitial fluids which tend to make the volume expand.

9. Neutral gelatin at the iso-electric point cannot make a stable gel, since the constituent of only one phase is present. The gel formed on cooling will be free to contract till it is solid throughout, *i.e.* it will no longer be a gel.

Gelatin in the salt form cannot make a gel, since the constituent of only one phase is present. Gelatin in this form will form only sols with water.

10. The proportion of neutral gelatin to gelatin salts in any system is determined by the reaction of the system.

11. The volume occupied by a gelatin gel swelling in a fluid medium depends on: (1) the ratio of the mass of the dissolved electrolyte to the mass of the gelatin; (2) the geometrical form of the gel; (3) the hydrogen ion concentration of the medium, etc. Gels which are swelling in a weakly acid (or alkaline) medium lose water on being transferred to an atmosphere of saturated vapour. The amount of water lost by the gel is a function of the reaction of the previous external fluid. This water loss is shown to follow from the elastic properties of the gel, and to be in accordance with Donnan's membrane theory.

12. Van Bemmelen's theory that the surface of a gel is to be regarded as a solid membrane, perforated by pores, is confirmed by observation.

#### BIBLIOGRAPHY.

- Andersen (1914). *Zeitsch. physikal. Chem.* **88**, 191.  
Arisz (1914). *Kolloidchem. Beihefte*, **7**, 1.  
van Bemmelen (1898). *Zeitsch. anorg. Chem.* **18**, 20.  
Berrar (1912). *Biochem. Zeitsch.* **47**, 189.  
Biltz, Bugge and Mehler (1916). *Zeitsch. physikal. Chem.* **91**, 705.  
Bradford (1918). *Biochem. J.* **12**, 382.  
Bütschli (1896). *Ueber den Bau quellbarer Körper*, p. 19, Göttingen.  
— (1898). *Untersuchungen über Strukturen*, Leipzig.  
Chiari (1911). *Biochem. Zeitsch.* **38**, 167.  
Chittenden and Solley (1891). *J. Physiol.* **12**, 33.  
Dakin (1913). *J. Biol. Chem.* **13**, 357.  
Dheré (1913). *J. Physiol. Path. Gén.* **13**, 158, 167.  
Dheré and Gorgolewski (1910). *Compt. Rend.* **150**, 934. *J. Physiol. Path. Gén.* **12**, 646.  
Donnan (1911). *Zeitsch. Electrochem.* **17**, 572.  
Ehrenberg (1913). *Biochem. Zeitsch.* **53**, 356.  
Fischer (1910). *Das Oedem*, Dresden.  
Frankenheim (1835). *Die Lehre von der Kohäsion*, Breslau.  
Freundlich (1909). *Kapillarchemie*, Leipzig.  
Guttenberg (1896). *Münch. med. Wochensch.* **147**.  
Hardy (1900). *Roy. Soc. Proc.* **66**, 95.  
Hatschek (1916). *Trans. Farad. Soc.* **12**, 17.  
Hayes (1914). *Biochem. J.* **8**, 553.  
Heintz (1853). *Lehrbuch der Zoochemie*.  
Hofmeister (1890). *Arch. exp. Path. Pharm.* **27**, 395.  
Jordan Lloyd (1916). *Proc. Roy. Soc.* **89 B**, 277.  
Jordis (1902). *Zeitsch. Electrochem.* **8**, 677.  
Kuhne (1888). *Lehrbuch der physiologischen Chemie*.

- Levites (1908). *Kolloid. Zeitsch.* **2**, 161, 273.  
Loeb (1918). *J. Gen. Physiol.* **1**, 41.  
— (1919, 1). *J. Gen. Physiol.* **1**, 379, 483.  
— (1919, 2). *J. Gen. Physiol.* **1**, 717.  
Masson (1905). *Proc. Roy. Soc.* **74**, 230  
Mörner (1889). *Zeitsch. physiol. Chem.* **28**, 471.  
Mulder (1843). *Annalen*, **45**, 63.  
v. Nägeli (1858). *Pflanzenphysiologischen Untersuchungen*, Zürich.  
van Name (1897). *J. Exp. Med.* **2**, 117.  
Nasse (1889). *Jahresber. Thier-Chem.* **19**, 29.  
Osborne (1918). *Proc. Roy. Soc. Vic.* **30**, N.S. 153.  
Ostwald (1905). *Pflüger's Arch.* **108**, 581.  
— (1909). *Grundriss der Kolloidchemie*, Dresden.  
Paal (1892). *Ber.* **25**, 1202.  
Parks (1902). *Phil. Mag.* (6), **4**, No. 2, 240.  
— (1903). *Phil. Mag.* (6), **5**, No. 1, 517.  
Pauli (1897). *Pflüger's Arch.* **67**, 219.  
— (1912). *Fortschr. naturwiss. Forschung*, **4**, 223.  
— (1913). *Koll. Zeitsch.* **12**, 222.  
Procter (1914). *J. Chem. Soc.* **105**, 313.  
— and Burton (1916). *J. Soc. Chem. Ind.* **35**, 404.  
— and Wilson (1916). *J. Chem. Soc.* **109**, 307.  
Robertson (1918). *Physical Chemistry of the Proteins*, New York.  
Sadikoff (1903). *Zeitsch. physiol. Chem.* **37**, 397.  
Schroeder (1903). *Zeitsch. physikal. Chem.* **45**, 109.  
Schützenberger and Bourgeois (1876). *Jahresbericht Thier-Chem.* **30**.  
Spiro (1910). *Van Bemmelen Festschrift*, 261. Quoted from Mahly's *Jahresbericht*, **40**, 123.  
van Slyke (1912). *J. Biol. Chem.* **10**, 15.  
v. Weimarn (1908). *Kolloid. Zeitsch.* **2**, 76, 230, 275, 301, 326.  
Wolf and Buchner (1915). *Zeitsch. physikal. Chem.* **89**, 271.

## XX. THE ANTI-SCORBUTIC PROPERTIES OF CONCENTRATED FRUIT JUICES. PART III.

BY ARTHUR HARDEN AND ROBERT ROBISON.

*From the Biochemical Department, Lister Institute.*

*(Received March 2nd, 1920.)*

IN a previous paper [1919] a method was described by which orange juice could be reduced, by evaporation at low temperatures, to a crisp, dry solid, retaining in a considerable degree the anti-scorbutic properties of the raw juice. This residue, which was very hygroscopic, was stored in a desiccator over sulphuric acid, at room temperature. Tests on guinea-pigs were carried out when the material was from four to seven months old and showed that a daily ration of 0.5 g., equivalent to about 4.5 cc. of the raw juice, afforded complete protection from scurvy, but the minimum daily amount sufficient to afford such protection was not determined. A quantity of the dried juice was set aside for further tests after a longer interval of time, and these tests have now been carried out. The material formed part of the original quantity prepared in June 1917 and since then had been stored in a desiccator at room temperature. The tests were carried out during April-June 1919 so that the average age of the dried juice was nearly two years. Unfortunately the amount available did not permit of an extended series of tests in order to determine the minimum dose and it was thought advisable to repeat the somewhat high daily ration (0.5 g.) used in the previous tests rather than risk more inconclusive results by feeding single animals on varying smaller amounts. The results of these tests are shown below. In these and other experiments described in this paper the animals employed were guinea-pigs and the basal diet consisted of oats and bran *ad lib.* with 60 cc. daily of milk autoclaved at 120° for one hour.

Table I.

No. of animal	Diet	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result
136	Basal + 0.5 g. dried orange juice daily from the 13th day	326	Weight increased steadily throughout the experiment (maximum weight 519 g. on 75th day). No symptoms of scurvy were observed	77	503	Animal in good health; killed on 77th day; no signs of scurvy
137	Basal + 0.5 g. dried orange juice daily from the 13th day	307	Weight increased steadily throughout the experiment (maximum weight 436 g. on 75th day). No symptoms of scurvy were observed	77	422	Animal in good health; killed on 77th day; no signs of scurvy

It will be seen that the daily ration of dried orange juice was not given until the 13th day of the experiment. This was on account of the limited amount of the material available, but the advisability of the procedure is questionable. The animal's reserve stock of the anti-scorbutic accessory factor must have been very much reduced and this may perhaps account for the somewhat low rate of growth shown by guinea-pig 137.

Microscopical examinations of the ribs of both animals were very kindly carried out by Miss Tozer to whom we are indebted for the following reports:

*Guinea-pig 136*:—"The two ribs examined are practically normal, a slight shortening of the trabeculae being the only abnormality."

*Guinea-pig 137*:—"The two ribs examined are normal."

In these experiments, therefore, complete protection from scurvy was afforded by a daily ration of 0.5 g. of dried orange juice, after this had been kept during nearly two years in a dry atmosphere at room temperature. This quantity was equivalent to about 4.5 cc. of the raw juice.

From the work of Chick and Rhodes [1918] and Davey [quoted by Delf, 1920] it would appear that the minimum daily ration of raw orange juice sufficient to protect a guinea-pig completely from scurvy is about 1.5 cc., though it seems probable that this figure may vary to some extent with the character and age of the fruit. Hence the experiments described above do not exclude the possibility of considerable loss of the anti-scorbutic factor either during the process of drying or during storage. It can only be stated that if such loss took place it probably did not exceed 66 % of the amount originally present in the raw juice. It seemed advisable, therefore, to repeat these experiments on a larger scale in order to decide whether any loss of the anti-scorbutic factor actually occurs. Accordingly a considerable quantity of dried orange juice was prepared during November and December 1919. The oranges were fine new season's fruit and yielded a high proportion of juice which was, however, very tart. It was clarified by centrifuging and then passing through filter paper in a Buchner funnel. Five litres of juice containing 10.0 % of total solids were evaporated in quantities of 500-600 cc. at a time, the temperature of the bath being maintained at 50°-52° while the receiver was surrounded by ice. About four hours were required to evaporate this quantity of juice to a thick syrup, the final drying of which was completed by the method previously described. One portion of the dry product has been stored in a screw-top glass bottle, and kept at a uniform temperature of 26°. It is intended to test this portion after a lapse of one year. With another portion animal experiments were at once commenced, two guinea-pigs receiving 0.15 g., two receiving 0.3 g. and two receiving 0.45 g. daily, equivalent to 1.5, 3.0 and 4.5 cc. of raw juice respectively. On account of the tartness of the juice a little cane sugar was added to the ration so that the animals should take it readily. As will be seen from the tables below, all six animals were satisfactorily protected from scurvy during the 85 days that the experiment lasted and we may therefore conclude that no appreciable loss of the anti-

scorbutic factor occurs when orange juice is evaporated to dryness under the given conditions.

Table II.

No. of animal	Diet	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result
156	Basal + 0.15 g. dried orange juice daily from 1st day	283	Weight increased throughout experiment. Maximum weight 550 g. on the 83rd day. No symptoms of scurvy were observed	85	538	Animal in good health; killed on the 85th day; no signs of scurvy
157	Basal + 0.15 g. dried orange juice daily from 1st day	282	Weight increased throughout experiment. Maximum weight 490 g. No symptoms of scurvy were observed	85	490	Animal in good health; killed on the 85th day; no signs of scurvy
158	Basal + 0.3 g. dried orange juice daily from 1st day	283	Weight increased throughout experiment. Maximum weight 573 g. on the 75th day. No symptoms of scurvy were observed	85	555	Animal in good health; killed on the 85th day; no signs of scurvy
159	Basal + 0.3 g. dried orange juice daily from 1st day	332	Weight increased throughout experiment. Maximum weight 627 g. on the 75th day. No symptoms of scurvy were observed	85	613	Animal killed on 85th day. Post mortem examination revealed acute congestion of the left lung but there were no signs of scurvy
160	Basal + 0.45 g. dried orange juice daily from 1st day	283	Weight increased throughout experiment. Maximum weight 557 g. on the 75th day. No symptoms of scurvy were observed	85	525	Animal in good health; killed on the 85th day; no signs of scurvy
161	Basal + 0.45 g. dried orange juice daily from 1st day	300	Weight increased throughout experiment. Maximum weight 506 g. on the 82nd day. No symptoms of scurvy were observed	85	505	Animal in good health; killed on the 85th day; no signs of scurvy

[NOTE:—On the 75th day the animals were moved into another building. As a result of this change five of the animals suffered a temporary fall in weight from which some had not completely recovered when killed on the 85th day.]

That considerable loss of anti-scorbutic potency may occur when other methods of evaporation are employed is shown by the experiments of Givens and McClugage [1919] in which orange juice in shallow dishes was dried by a current of air heated to 55°–60°, the duration of drying varying from 36 to 63 hours. A daily ration of the dried product equivalent to 12.5 cc. of the raw juice was required in order that guinea-pigs might be completely protected from scurvy

whereas on the equivalent of 6.25 cc. the animals developed scurvy in less than 20 days. The loss by this method must therefore have amounted to more than 76 %.

It had been our intention to extend this investigation to the various industrial processes for the rapid evaporation of liquids, particularly those used in the preparation of milk powders, in the hope that one of these processes or some practicable modification might yield from orange juice a product of high anti-scorbutic potency, that could be stored for considerable periods under varying conditions of climate, without serious loss of the accessory factor. Preliminary experiments had been commenced with the co-operation of the Kestner Evaporator Co. Ltd., to whose managing director Mr J. A. Reavell and chemist Mr Nicol we are greatly indebted for much valuable assistance. In July 1919 Givens and McClugage published an account of some successful experiments with dried orange juice prepared by the Merrell-Soule patented process for making milk-powder. The orange juice, mixed with corn-syrup, was forced as a fine spray into a chamber where it met a current of air heated to 75°-80°. The drying was almost instantaneous and the product, when tested on guinea-pigs, was found to afford complete protection from scurvy. The daily dose given to the animals was 1 g. equivalent to 3 cc. of raw juice, which was taken, from the earlier experiments of Chick and Rhodes [1918], as the minimum daily dose of fresh orange juice sufficient to afford complete protection. Givens and McClugage state that they intend to report on the activity of this product after a long period of storage and it is to be hoped that smaller doses will also be tested.

In view of the satisfactory results quoted above it appeared to us unnecessary to continue our attempts, for there would seem to be little doubt that a highly active dried orange juice can be readily prepared on a commercial scale, and from our own experiments recorded in this paper it seems very probable that such a product will retain its potency after prolonged storage under suitable conditions. Orange juice in fact would appear to be particularly suited for the preparation of a dried anti-scorbutic, as it has been shown by Delf [1920] to lose very little of its potency, even when heated at 100° for an hour. The peculiar value of such a substance would lie in its adaptability for infant feeding and for the use of expeditions of long duration, where fresh fruit and vegetables are unobtainable and transport must be reduced to a minimum. The investigation of the effects of storage at tropical temperatures is therefore advisable.

A brief account of the preliminary experiments on large scale evaporation mentioned above may here be given.

(A) The juice of 360 oranges (18.5 litres) was strained through muslin and concentrated in the "Kestner Evaporator," under 25 inches of vacuum and a steam pressure of 10-15 lbs. The time taken for the passage of any portion of the juice through the evaporator was less than one minute and the temperature of the escaping liquid was below 60° (usually below 50°). Owing

to the juice being insufficiently clarified its passage through the inlet valve was somewhat erratic and the consequent evaporation less than the maximum obtainable under the stated conditions of temperature and pressure. It was consequently necessary to pass the juice three times through the evaporator in order to obtain the desired degree of concentration. The product was a viscous syrup containing 68 % of total solids as against 11.6 % in the original juice. A portion of this syrup was tested on guinea-pigs (see Table III, A), and during the course of the experiments was kept in a loosely-corked bottle at room temperature. Two guinea-pigs, each receiving a daily ration of 0.14 g., equivalent to 0.8 cc. of raw juice, developed symptoms of scurvy in 26 days and died in 42 and 39 days respectively. Two guinea-pigs received a daily ration of 0.27 g., equivalent to 1.6 cc. of raw juice. Unfortunately the condition of both animals was unsatisfactory from the first and there was no appreciable gain in weight even during the initial 14 days. In one animal scurvy symptoms were observed after 36 days and the weight commenced to fall slowly. No such symptoms were observed in the other animal though its general condition was not good and its weight declined after the 52nd day. Both animals were killed on the 73rd day and signs of scurvy were found in each case. Two animals received a daily ration of 0.67 g., equivalent to 4.0 cc. of raw juice; one of these was completely protected from scurvy during 93 days but in the other scurvy symptoms were observed after 77 days. From these results it might appear that a loss of anti-scorbutic potency, amounting to more than 63 %, had occurred during concentration of the orange juice. It is very possible, however, that the actual loss due to the process of concentration was considerably less than this, but that further loss occurred during storage in the course of the animal tests, and some evidence in favour of this view can be derived from a comparison of the results obtained with the different doses and with the corresponding amounts of the dried product after further treatment (see below).

(B) The bulk of the concentrated syrup described above was submitted to further concentration in a small vacuum pan fitted with a mechanical stirrer and scraper. A vacuum of 24–25 inches was maintained but the evaporation was very slow and it was found necessary to raise the temperature of the outer bath to 80° for 2½ hours. Even then the result was unsatisfactory, the product being a viscous mass, very difficult to remove from the pan. On cooling it set to a dark coloured, toffee-like solid containing 7.5 % of water.

The final drying of orange juice by this method was obviously impracticable but the product still retained its anti-scorbutic potency in greater degree than might have been expected after such drastic treatment (see Table III, B). Two guinea-pigs, each receiving 0.1 g. daily, equivalent to 0.8 cc. of raw juice, developed symptoms of scurvy in 26 and 31 days and died in 39 and 44 days respectively. A daily ration of 0.2 g. also afforded only partial protection, but two guinea-pigs each receiving 0.5 g. daily, equivalent to 4.0 cc. of

Table III.

A (Concentrated orange juice : total solids = 68%)							E (Dried orange juice : total solids = 92.5%)						
No. of animal	Daily ration of "A"	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result	No. of animal	Daily ration of "B"	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result
138	0.14 g. <sup>1</sup> (0.8 cc.)	364	Maximum weight 368 g. Scurvy symptoms observed on the 26th day	42	217	Death from scurvy on the 42nd day	144	0.1 g. (0.8 cc.)	312	Maximum weight 356 g. Scurvy symptoms observed on the 26th day	39	207	Death from scurvy on the 39th day
139	0.14 g. (0.8 cc.)	332	Maximum weight 367 g. Scurvy symptoms observed on the 26th day	39	232	Death from scurvy on the 39th day	145	0.1 g. (0.8 cc.)	312	Maximum weight 323 g. Scurvy symptoms observed on the 12th day	44	193	Death from scurvy on the 44th day
140	0.27 g. (1.6 cc.)	399	Maximum weight 420 g. Scurvy symptoms observed on the 36th day	73	301	Animal killed on the 73rd day: severe scurvy	146	0.2 g. (1.6 cc.)	339	Maximum weight 375 g. Scurvy symptoms observed on the 19th day	52	233	Death from scurvy on the 52nd day
141	0.27 g. (1.6 cc.)	318	Weight remained constant during 52 days and then fell slowly. No symptoms of scurvy observed but general condition bad	73	241	Animal killed on the 73rd day: signs of mild scurvy	147	0.2 g. (1.6 cc.)	287	Maximum weight 338 g. Scurvy symptoms observed on the 33rd day	73	266	Animal killed on the 73rd day: severe scurvy
142	0.67 g. (4.0 cc.)	349	Maximum weight 442 g. on the 80th day. No symptoms of scurvy observed	93	440	Animal killed on the 93rd day: no signs of scurvy	148	0.5 g. (4.0 cc.)	317	Maximum weight 423 g. on the 50th day. No symptoms of scurvy observed	93	405	Animal killed on the 93rd day: no signs of scurvy
143	0.67 g. (4.0 cc.)	285	Maximum weight 448 g. Scurvy symptoms observed on the 77th day	93	341	Animal killed on the 93rd day: scurvy not very severe	149	0.5 g. (4.0 cc.)	290	Maximum weight 431 g. Scurvy symptoms observed on the 78th day. No symptoms of scurvy observed but the animal did not appear in good health. From the 94th day it was given a highly anti-scorbutic diet but its condition did not improve, and it was killed on the 115th day, its weight being 402 g.	93	425	Animal killed on the 115th day. No signs of scurvy were found but there was acute congestion of both lungs

<sup>1</sup> The figures in brackets give the equivalent amount of raw orange juice.

raw juice, did not show any symptoms of scurvy during 93 days, though their general condition was not entirely satisfactory and the normal rate of growth was not maintained throughout the whole period.

#### SUMMARY.

Orange juice can be reduced by evaporation under suitable conditions to a dry residue without suffering appreciable loss of the anti-scorbutic accessory factor, and this residue still retains a considerable degree of potency after storage during two years in a dry atmosphere at ordinary temperatures.

The preparation on a commercial scale of such a dried orange juice appears to be quite practicable, and should prove of considerable value where an anti-scorbutic material is required in a highly concentrated and stable form.

#### REFERENCES.

- Chick and Rhodes (1918). *Lancet*, ii, 774.  
Delf (1920). *Biochem. J.* **14**, 211.  
Givens and McClugage (1919). *Amer. J. Diseases of Children* (July), **18**, 30.  
Harden and Robison (1919). *J. Roy. Army Med. Corps* (Jan.), 48.

# XXI. OBSERVATIONS ON ANTHOCYANINS.

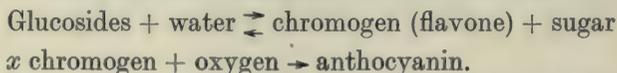
## I. THE ANTHOCYANINS OF THE YOUNG LEAVES OF THE GRAPE VINE.

BY OTTO ROSENHEIM.

*From the Physiological Laboratory, King's College, London.*

*(Received March 2nd, 1920.)*

THESE investigations were undertaken with the object of obtaining experimental evidence of a biochemical nature with regard to the formation of anthocyanin pigments by the plant. Until recently two working hypotheses had been advanced in this connection. They both owe their origin to the views of Palladin [1909], who looked upon anthocyanin as a respiratory pigment. Miss Wheldale [1911, 1916] suggested that Palladin's respiratory prochromogens were flavone (or xanthone) derivatives, and expressed their change into anthocyanins in general terms as follows:



The first reaction was assumed to be controlled by a glucosidase, and the second by an oxidase. Keeble, Armstrong and Jones [1913] proposed a very similar hypothesis and assumed a preliminary reduction (not necessarily of an enzymatic nature), followed by an oxidation due to the interaction of a peroxidase and an organic peroxide. Both these theories have this in common; they require the presence of oxidases and they assume oxidation as the essential condition for anthocyanin formation. The evidence for the simultaneous occurrence in plants of oxidases and anthocyanins was, however, by no means satisfactory [see Atkins, 1915], and necessitated the further assumption of "inhibitors" of oxidases, if the theory was to be retained. The two theories became finally untenable when it was shown by Combes [1913] and confirmed by Everest [1914], by Willstätter and Mallison [1914], and by Shibata [1919] that the conversion of flavonol derivatives into anthocyanins is not an oxidative process at all, but is due to reduction.

As any hypothesis on the biochemical anthocyanin formation must be based on the chemistry of these substances, a short résumé of our present knowledge of the anthocyanin chemistry is necessary here. A detailed account, based on the work of Willstätter and his co-workers, is given by Perkin and Everest [1918].

It has now been established that the anthocyanin pigments of flowers, fruits and leaves are glucosides and they are the first representatives of a

new class of non-nitrogenous vegetable bases [Willstätter and Everest, 1913; Willstätter, 1914]. They are derived from a reduced  $\beta$ -phenyl- $\gamma$ -pyrone (*i.e.* 2-phenyl-pyrylium) nucleus (see Formula III). The characteristic of this nucleus is a quadrivalent oxygen atom, which conveys basic properties to its derivatives and enables them to form salts with mineral and organic acids. The methods for the isolation of anthocyanins are based mainly on the formation of these well-crystallised oxonium salts. The differences in constitution of the various anthocyanins are due (1) to the number, nature and position of the carbohydrate radicles attached to the nucleus, (2) to the number and position of phenolic OH groups introduced, whilst (3) methylation of one or more of the OH groups gives rise to further variation. One and the same anthocyanin may occur in three different modifications in the plant, and these modifications may artificially be produced from the isolated crystallised pigment, *viz.* (1) the oxonium salts with mineral or organic acids are red, (2) the phenolic alkali salts are blue and (3) the neutral form, the free oxonium base, is violet. According to Shibata organo-metallic complex compounds of calcium and magnesium are also important factors in the production of flower colour. A fourth colourless modification will be discussed later.

On heating with 20 % hydrochloric acid the anthocyanins are hydrolysed into carbohydrates (glucose, galactose or rhamnose) and their chromogenic components, for which the general name of *anthocyanidins* was introduced by Willstätter and Everest. A very simple reaction makes it possible to decide whether the glucoside, anthocyanin, or the sugar-free pigment, anthocyanidin, occurs in any plant. The reaction depends on the difference which these pigments show in their distribution between amyl alcohol and aqueous acid. The diglucoside anthocyanins are insoluble in amyl alcohol, and are therefore quantitatively retained as oxonium salts in the aqueous layer on shaking their faintly acid solution with amyl alcohol. The sugar-free anthocyanidins on the other hand are soluble in amyl alcohol and are quantitatively removed from their aqueous solution by shaking with amyl alcohol. There are a few exceptions to this general rule, *e.g.* some monoglucosides and rhamnoglucosides are partially soluble in amyl alcohol, but they can be quantitatively removed again by shaking the coloured amyl alcohol solution with fresh aqueous acid.

By means of this reaction it has been established by Willstätter and Everest that all the anthocyanins of flowers, fruits and leaves occur as glucosides, and only in one single instance, the fruit of the grape vine, has it been found that a variable but small percentage of free anthocyanidin is present in the ripe fruit [Willstätter and Zollinger, 1915, 1916].

It was this exception which led me in the first instance, apart from other considerations, to select the grape vine as a suitable subject in which to investigate the relationship of the pigments of the leaves to those of the fruit. The fact that one of the constituents of the pigment exists in the free condition in the fruit in measurable amounts, suggested that such an investigation might throw some light on the mechanism of the synthesis by which

the living plant builds up its anthocyanins. Further, in plants bearing fruits rich in anthocyanin, such as the grape vine, the chances of following the gradual pigment formation during the slow ripening process seemed to be more hopeful than in the case of the flowering plants, in which the pigments are rapidly formed and the pigment carriers themselves are relatively short-lived.

The material for this study was obtained from two established vines, growing in the open, covering several square yards of wall space on my house and producing yearly a large crop of fully matured grapes. On examining the red pigment of the young leaves of these vines, I found the rather remarkable fact that the whole of it was soluble in amyl alcohol and consisted of an anthocyanidin, most probably oenidin, which occurs in combination with glucose as the chief pigment of the purple grape. The pigment itself was obtained in well-formed crystals by a relatively simple method. It was further found that the leaves contained, in an amount equal to that of the preformed red pigment, a colourless modification of the pigment for which the term *leuco-anthocyanin* is proposed. This is convertible into the anthocyanidin by means of hydrochloric acid. The occurrence of anthocyanidin in young leaves appears to be limited to the species *Vitis vinifera*, and presents a Mendelian character of possible value in genetic investigations.

#### EXPERIMENTAL.

*The red pigment of the young leaves of Vitis vinifera.* The young red leaves of two varieties ("Black Hamburg" and "Esperione") were used. They were at first examined separately, but as no qualitative or quantitative difference with regard to their pigments could be detected, the leaves of both varieties were used indiscriminately. The young leaves are produced during the whole of the growing season, and the nature and distribution of their pigments is practically the same in spring and in autumn. Identical results were obtained with the freshly gathered leaves and with air dried material. As the latter has the advantage of smaller bulk and makes its investigation independent of the season, a large quantity of the leaves was collected and dried at ordinary temperature in air. The powdered material was kept for examination in a desiccator over sulphuric acid.

(a) *Oenidin.* In some preliminary experiments 5 g. of the fresh leaves (or 1 g. of the dry powder) were ground up finely with 15 g. of clean sand and treated with 40 cc. of 1 % hydrochloric or sulphuric acid. The extract filtered easily through a folded filter and gave a bright wine red filtrate. Of this 10 cc. were shaken gently with an equal volume of amyl alcohol in a separating funnel. A large amount of the pigment present passed into the amyl alcoholic layer and a second extraction removed practically the whole of the pigment. The extracted fluid has a slightly yellowish tint, which appears to be largely due to the presence of flavone derivatives (see later).

The amyl alcohol solution of the pigment is of a fine bluish-red colour and none of the pigment is removed by shaking it with fresh dilute acid (absence of mono- and rhamno-glucosides). On shaking with a dilute solution of sodium acetate the tint becomes more purple, but the pigment remains in the amyl alcoholic layer. On the other hand, dilute sodium carbonate solution removes the whole of the pigment from amyl alcohol, its alkaline solution turning rapidly brown and yellow. On neutralisation with hydrochloric acid in the early stages, the soda solution again turns red and gives up its pigment once more to amyl alcohol. These reactions clearly characterise the pigment as an anthocyanidin.

*Preparation.* The pigment crystallises remarkably easily, and extracts of leaves made with 1 % hydrochloric acid at a temperature of about 30° gradually deposit the pigment as a microcrystalline sediment on standing in an open flask for some days. The yield of the crude pigment from two extracts is about 2.5 % of the dry material or 0.5 % of the fresh leaves. It is contaminated by a relatively small amount of colourless substances, from which it can easily be freed by repeated recrystallisation from 3 % hydrochloric acid. The pigment is insoluble in this solvent in the cold, but dissolves easily on slight warming. On allowing its solution to cool gradually, the dark red pigment crystallises out in advance of the colourless admixtures. It is filtered under suction, washed with cold 3 % hydrochloric acid and dried in a desiccator over soda lime.

The pigment appears to be pure and consists of uniform crystals when recrystallised several times in the above way. It may be further purified by making use of its solubility in amyl alcohol and by converting it into its well-crystallised picrate. For this purpose the hydrochloride of the pigment is dissolved in slightly warmed 1 % hydrochloric acid and shaken out with amyl alcohol. From its amyl alcoholic solution it is precipitated by adding 2½ volumes of light petrol (B.P. 40°–60°). A bright carmine red watery layer settles out, from which the hydrochloride soon begins to crystallise. The crystals are redissolved by slightly warming the separated watery layer and removing at the same time the dissolved petrol by a current of air. A saturated solution of picric acid in water is added. On cooling, the picrate crystallises in fine deep red prisms, from which the hydrochloride may be easily obtained by the general method described by Willstätter.

This method does not yield the whole of the pigment present in the young leaves, but its simplicity and the relative purity of the product obtained in the first instance make it on the whole preferable to the method subsequently worked out. The latter need therefore only be described in its outlines. It was found that whilst butyl alcohol is an excellent solvent for anthocyanins [see Rosenheim, 1920] it does not dissolve any red pigment from the dry leaf powder in the absence of mineral acid. It removes, however, the whole of the yellow and green pigments (flavones, chlorophyll, etc.). The dry powder is

therefore extracted at ordinary temperature<sup>1</sup> with butyl alcohol until the solvent remains colourless. Extraction is continued with moist butyl alcohol containing 3–5 % hydrochloric acid. The dark red extract is filtered through a layer of sand on a Buchner funnel, and the pigment precipitated with a mixture of ether and light petrol. It is taken up again in amyl alcohol, reprecipitated by light petrol and finally recrystallised several times from warm 3 % hydrochloric acid.

*Properties of the crystallised pigment.* The hydrochloride of the pigment is obtained on recrystallisation from 3 % hydrochloric acid in well-formed uniform needles, often arranged in rosettes (see Fig. 1). In transmitted light

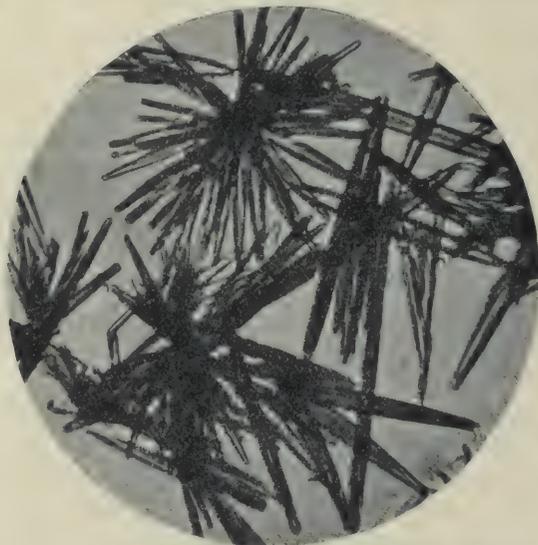


Fig. 1. Oenidin hydrochloride, the red pigment of young vine leaves.

the crystals, seen singly, are transparent with a brownish red tint; where the crystals are superimposed or cross each other their colour is carmine red. They show a red bronze appearance in reflected light, and are anisotropic under the polarisation microscope. Macroscopically they form a reddish brown non-hygroscopic powder. The purest product so far obtained melts on rapid heating at 200°–202° (uncorr.); on slow heating between 195°–197° (uncorr.). The melting point may possibly be raised on further purification. The crystals dissolve with a brownish red colour in warm water, but the solution becomes rapidly colourless on dilution with water, owing to isomerisation into the pseudo-base. This isomerisation also takes place quickly when the alcoholic solution is diluted with hot water, without the intermediate precipitation of the violet colour base, as in the case of cyanidin. The colour is immediately restored by hydrochloric acid. The salt is readily

<sup>1</sup> Extraction in a Soxhlet apparatus is not to be recommended since the pigment appears to become partially isomerised at the high temperature obtaining during the extraction.

soluble in methyl, ethyl, amyl and butyl alcohol, giving fine violet red solutions. The hydrochloride is easily soluble in 0.1 % hydrochloric acid, very easily if warmed; in cold 1 % hydrochloric acid it is only soluble with difficulty and still more so in 3 % hydrochloric acid, from which it may be recrystallised by allowing its hot solution to cool. The salt is also soluble, especially on warming, in dilute sulphuric acid, even up to 5 % sulphuric acid. With picric acid it forms a characteristic picrate, crystallising in deep carmine red prisms. Ferric chloride gives no colour reaction, and an excess quickly destroys the pigment.

The solutions of the pigment show a single dark band in the green, when examined spectroscopically. The following measurements were taken, when a solution of 20 mg. in 10 cc. butyl alcohol was examined:

5 mm. layer: 530—570; blue absorbed from 420 onwards.

10 mm. layer: 515—585;    "   "   "    430   "

The substance is laevorotatory. The observations were made in white light, and as monochromatic red light was not available no measurements were recorded.

In all its properties and reactions the crystallised pigment isolated from young vine leaves agrees with oenidin, the colour component of the pigment of the purple grape. According to Willstätter and Zollinger, oenidin represents a dimethyl ether of delphinidin, the exact positions of the methyl groups in the molecule being still doubtful. Theoretically, eleven dimethyl ethers are possible, of which only two are known (oenidin and malvidin). It will be necessary therefore to determine not only the number of methoxy groups, but also the products of alkaline decomposition in order to settle the identity of the pigment. Material is being collected for this purpose.

(b) *Leuco-anthocyanin*. The presence of a colourless modification of the pigment was first observed during the course of some experiments in which the quantitative distribution of the pigments contained in an acid extract of leaves was investigated. For this purpose 1 g. of the dry leaf powder was extracted with 30 cc. of 1 % hydrochloric acid. 10 cc. of the extract were shaken with an equal volume of amyl alcohol (saturated with 1 % hydrochloric acid) which removed 70 % of the red pigment. Two more extracts with amyl alcohol were made, the third showing only a faint pink tint. The completely extracted residual fluid was compared in a Hellige-Autenrieth colorimeter with the original extract, and the result showed that more than 99 % of the pigment had been removed by amyl alcohol. A more exact estimation of the small fraction left in the solution could not be made, owing to the slightly yellowish tint of the fluid. An attempt was therefore made to determine the minute residual anthocyanin fraction indirectly, by converting it into free anthocyanidin through hydrolysis with hydrochloric acid. On boiling the practically colourless solution with 20 % hydrochloric acid, the surprising fact was noticed that the solution assumed immediately a deep

wine red tint, of apparently the same colour intensity as the original extract. The pigment formed was easily soluble in amyl alcohol with a violet red colour, which could be matched with the original extract in the colorimeter. Its strength was determined and found to be exactly the same as that of the original pigment present.

The experiment was repeated in a modified form with another sample of leaves. In this case the extraction was made with acid alcohol in order to exclude the possibility of the formation of any colourless pseudo-base during the extraction, which was rapidly carried out. From the acid-alcoholic extract the pigment was precipitated by a mixture of ether and light petrol and dissolved subsequently in 0.5 % hydrochloric acid. The solution was divided into two equal parts, of which (1) was hydrolysed with hydrochloric acid and the whole of the oenidin present transferred into amyl alcohol (= total anthocyanidin). From (2) the free oenidin was removed by means of amyl alcohol (saturated with 0.5 % hydrochloric acid) and the colourless residual fluid hydrolysed with 20 % hydrochloric acid (= combined anthocyanidin). This was subsequently extracted with the same volume of amyl alcohol as (1) and the two extracts compared in the colorimeter. The following results were obtained:

- |                            |        |
|----------------------------|--------|
| (1) Total anthocyanidin    | = 100. |
| (2) Combined anthocyanidin | = 52.  |
| (3) Free anthocyanidin     | = 48.  |

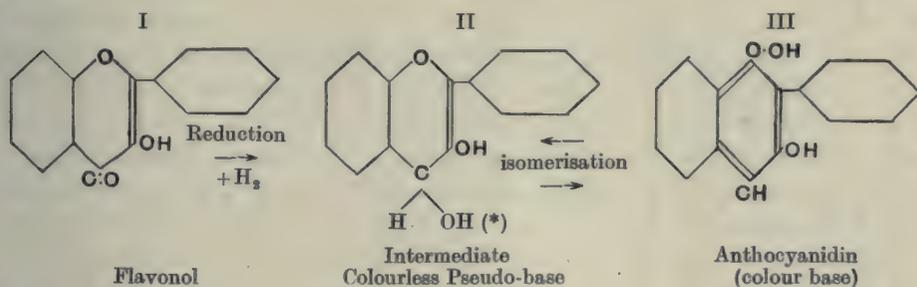
It will be seen that this result agrees well with the previous one and shows that the amount of anthocyanidin present in the colourless modification is about equal to that of free anthocyanidin. This result was confirmed by several other experiments in which various modifications of the procedure were adopted.

The pigment possessed all the characteristics of an anthocyanidin; it remained in the amyl alcoholic layer on shaking with fresh dilute acid, assumed a bluish-violet tint on shaking with dilute sodium acetate solution, and descended quantitatively into dilute sodium carbonate. On rapid acidification of the latter the colour changed into red and the pigment was again given up to amyl alcohol.

On investigating the conditions under which the change from the colourless stage to the pigment takes place, it was found to be independent of the presence of oxygen, for the identical colour intensity is developed with equal rapidity whether the process is carried out in a current of carbonic acid or in air. The chromogenic substance is not extracted by ethyl acetate, after neutralisation of its solution with sodium bicarbonate, thus showing that it does not belong to the rather indefinite group of substances called catechol- or phloba-tannins. These two negative observations are mentioned, since they disprove an assumption, current in the botanical literature of the subject,

according to which the pigments of the grape are formed by oxidation of a colourless oenotannin<sup>1</sup>.

In the light of the recent work on the constitution of the anthocyanins, the following suggestion is advanced, as affording an easy and natural explanation of the observed facts. According to Willstätter and his co-workers, the oxonium salts of all anthocyanins and anthocyanidins are hydrolytically dissociated in dilute solutions, giving rise to the formation of the free oxonium base (III) which more or less easily isomerises into the colourless pseudo-base (II). This isomerisation takes place owing to the migration of the basic hydroxyl group from oxygen to carbon. The sugar-free pseudo-bases have been obtained crystallised. They are again transformed into the coloured oxonium salts by acids. The process is therefore a reversible one and according to my observations it is the reverse of this reaction which plays an important rôle in the synthesis of anthocyanins by the plant. It is suggested that the colourless pseudo-bases are the primary products formed by the plant during the reduction of flavonols to anthocyanidins. A consideration of the formulæ given below demands, indeed, their formation as a theoretical necessity.



This reduction has been successfully carried out in the laboratory by Willstätter and Mallison [1914] and, since I have now shown that the free anthocyanidin occurs in young vine leaves, it is not surprising to find also the intermediary product. In the young leaf, however, the pseudo-base does not occur in the free state, but in combination with either a carbohydrate or possibly another complex. For this combination the general name *leucoanthocyanin* is proposed. If we assume that the carbohydrate (or similar complex) is attached to the pseudo-base by means of the hydroxyl group marked \* in II, one can easily understand that the change from the colourless modification into the basic pigment cannot take place until the carbohydrate radicle is split off. This hydrolysis is rapidly effected by strong acids, but only very slowly if at all by 1 % hydrochloric acid. In the presence of the strong

<sup>1</sup> On the other hand, however, I have obtained certain experimental evidence which points to a relationship of a different character between anthocyanins and phlobatannins and which suggests the existence of reduced flavonol rings in the latter substances. Additional support for this suggestion is afforded by the correlation in the distribution of tannins, flavonols and anthocyanins, and by the fact that they all three furnish identical cleavage products on alkaline decomposition (phloroglucinol and various hydroxy-phenolic acids).

acid, the liberated pseudo-base immediately isomerises into anthocyanidin. An attempt to effect hydrolysis by means of emulsin has so far not yielded any decisive results. The chromogenic substance is not extracted by ether even after saturation of its solution with ammonium sulphate. As the free pseudo-bases are soluble in ether, under these conditions, their absence is proved, and this fact also shows incidentally that the colourless modification is not artificially produced, during the preparation of the extract, by the isomerisation of preformed anthocyanidin.

I have so far been unable to isolate the leuco-anthocyanin itself from the young leaves of the grape vine. I found, however, that the unripe berries of the purple grape and the ripe berries of white grapes contain leuco-anthocyanin in much larger amounts and from this source it may be easily obtained, so far only in an amorphous condition. (Further details will be given in a future communication.)

It is interesting to note that Willstätter and Nolan [1915] recorded an unexplained observation in their work on the pigment of *Rosa gallica*, which points to the existence of leuco-anthocyanins in this flower. They noticed that an acid methyl alcoholic extract of the flower showed, on standing, the remarkable phenomenon of gradually increasing its colour to twice its original intensity. They were unable to explain this phenomenon as being due to the occurrence of the pseudo-base of cyanin, since this rapidly changes into the coloured salt under these conditions. It was also not due to oxidation since it occurred equally in the absence of oxygen. They assume the presence of an unknown anthocyanin, the colourless isomer of which is slowly changed into the coloured salt.

*Oeninidin as a Mendelian Factor.* The occurrence of free anthocyanidin as the only red pigment of young leaves in *Vitis vinifera* naturally suggested an investigation of the pigments of young leaves of other plants, in order to find out whether this occurrence represents a general stage in the formation of anthocyanins by the plant organism. For this purpose I examined the pigmented leaves of 85 different species of various families, the enumeration of which would occupy too much space. For a large selection of suitable material I am indebted to the authorities of the Royal Botanic Gardens at Kew, and especially to Mr Dallimore, who also kindly supplied the botanical names. In most cases the young as well as the autumnal leaves were examined. The fresh material was ground with clean sand and extracted with 1 % sulphuric acid. A clear filtrate was obtained in most cases, 5 cc. of which was shaken out with amyl alcohol. The result showed that in all the varieties of *Vitis vinifera* examined, the pigment of the young leaves was soluble in amyl alcohol, whilst all the other plants contained only anthocyanins, insoluble in amyl alcohol, in both their young and autumnal leaves. In a few cases (several species of *Euonymus* and *Geranium*) the amyl alcohol extract was slightly pigmented. The pigment, however, was easily removed by fresh dilute acid, thus proving the absence of anthocyanidins.

It would appear therefore that the occurrence of free anthocyanidins is limited to *Vitis vinifera*, and it became of interest to examine other species of the *Vitis* family. As is well known, *Vitis vinifera* is the only European species of this family, whilst at least 18 species are known in America and about eight in Asia. I was able to examine six Asiatic species (*V. pulchra*, *Thomsonii*, *Wilsonii*, *amurensis*, *Baileyana* and *Coignetiae*) and three American species (*V. arborea*, *aestivalis* and *Bourquiniana*). They all gave negative results with the single exception of *Vitis Bourquiniana*. In this case the pigment of the young leaves was to a large extent soluble in amyl alcohol and consisted of anthocyanidin.

This result is rather suggestive in so far as the origin of *V. Bourquiniana* and its recognition as a species has given rise to a great deal of discussion in viticultural journals<sup>1</sup>. According to the standard work on American grapes by Hedrick [1907], the name *V. aestivalis Bourquiniana* was given by Munson, who ranks a group of several similar varieties as a species, in honour of the Bourquin family of Savannah, whose ancestors were supposed to have brought them from France to America over 150 years ago. Munson's derivation of the origin of this vine has not been accepted by either French or American botanists, and the general opinion appears to be that *V. Bourquiniana* is a hybrid between *V. aestivalis* and some form of *V. vinifera*.

This conclusion seems to receive an experimental confirmation by the above described reaction, which clearly demonstrates the influence of *V. vinifera* on the nature of the pigment of the supposed hybrid. If further investigation of other species of *Vitis*, which I hope to carry out, should confirm the observation that the occurrence of free anthocyanidin is limited to *Vitis vinifera*, this biochemical test might prove useful for investigations of genetic problems.

#### SUMMARY.

(1) The red pigment of the young leaves of the grape vine has been isolated in a crystalline form. It is most probably identical with oenidin, the non-glucosidic component of the pigment of the purple grape. This is the first known, and so far the only instance, in which the red pigment of leaves consists of free anthocyanidin.

(2) The occurrence of a colourless modification of the pigment has been demonstrated, for which the general name *leuco-anthocyanin* is proposed. It is present in combination possibly with a carbohydrate or other complex, and is converted into anthocyanidin by strong acids.

(3) The European species *Vitis vinifera* appears to be the only representative of the family *Vitis*, which is characterised by the production of free anthocyanidin, and the bearing of this observation on genetic problems is discussed.

<sup>1</sup> I am greatly indebted to Mr E. A. Bunyard, of Maidstone, for kindly referring me to the extensive literature of this subject.

## REFERENCES.

- Atkins (1915). *Proc. Roy. Soc. Dublin*, **14**, 317.  
Combes (1913). *Compt. rend.* **157**, 1002, 1454.  
Everest (1914). *Proc. Roy. Soc.* **87 B**, 444.  
Hedrick (1907). *The Grapes of New York, Rep. N. Y. Agric. Stat.*  
Keeble, Armstrong and Jones (1913). *Proc. Roy. Soc.* **86 B**, 308.  
Palladin (1909). *Biochem. Zeitsch.* **18**, 151.  
Perkin and Everest (1918). *The Natural Organic Colouring Matters* (London).  
Rosenheim (1920). *Biochem. J.* **14**, 73.  
Shibata, K., Shibata, Y., and Kasiwagi (1919). *J. Amer. Chem. Soc.* **41**, 208.  
Wheldon (1911). *J. Genetics*, **1**, 133.  
—— (1916). *The Anthocyanin Pigments of Plants* (Cambridge).  
Willstätter (1914). *Ber.* **47**, 2831.  
—— and Everest (1913). *Annalen*, **401**, 189.  
—— and Mallison (1914). *Sitz. Ber. Preuss. Ak. Wiss.* **29**, 769.  
—— and Nolan (1915). *Annalen*, **408**, 1.  
—— and Zollinger (1915). *Annalen*, **408**, 83.  
—— — (1916). *Annalen*, **412**, 195.

## XXII. THE ACTION OF THROMBIN UPON FIBRINOGEN.

BY JOHN OGLETHORPE WAKELIN BARRATT,

*Beit Memorial Research Fellow.*

*From the Lister Institute.*

*(Received February 27th, 1920.)*

### METHOD.

THE solutions of fibrinogen, thrombin and sodium chloride employed in these experiments contained about 0.5 % of tri-sodium citrate.

As source of fibrinogen human plasma was ordinarily used, being prepared by mixing 12 cc. of blood, immediately after withdrawal from a vein of the forearm, with 1 cc. of 0.85 % sodium chloride solution to which 0.13 g. sodium citrate had been added. After centrifugalisation the plasma was pipetted off and stored at 0°. In a few experiments fibrinogen was precipitated by pouring human plasma into twenty volumes of distilled water through which a stream of carbon dioxide had been passed, the precipitate being separated by centrifugalisation and then redissolved in citrated 0.85 % sodium chloride solution.

As source of thrombin freshly prepared whipped blood, to which 1 % of sodium citrate had been added as soon as separation of fibrin was completed, was used in a few experiments. As, however, the range of concentration of thrombin obtainable in this way was small, in most experiments the thrombin employed consisted of the venom of *Echis carinatus*<sup>1</sup> dissolved in 0.85 % sodium chloride solution, to which 0.5 % of sodium citrate had been added [Barratt, 1915]; the limit of utility of this form of thrombin was about 1 in 30,000,000 and the highest concentration used rarely exceeded 1 in 3000; it was completely destroyed by heating to 75° for ten minutes.

Dark background illumination was obtained by means of a paraboloid condenser with central stop, an arc lamp being used as source of light.

Coagulation was usually studied at room temperature, the mixture of fibrinogen and thrombin, which formed the subject of observation, being placed upon a glass slide, within an area ringed with vaseline, and closed by a coverslip: the thickness of the layer of fluid examined was usually about 50 $\mu$ . Occasionally coagulation was carried out in small glass tubes.

<sup>1</sup> For a supply of this I am indebted to the kindness of Dr C. J. Martin, Director of the Lister Institute.

## FIBRIL FORMATION.

When a liquid containing fibrinogen is mixed with a solution of thrombin in the presence of sodium citrate or oxalate coagulation occurs, due to the formation of fibrin fibrils. If the production of such fibrils occurs with sufficient slowness the course of the process can readily be studied<sup>1</sup>.

Fig. 1 represents the appearance, under a dark background illumination, of a mixture of (1) citrated human blood (containing fibrinogen, but no thrombin), and (2) whipped human blood<sup>2</sup> (containing thrombin, but no fibrinogen): at the end of three hours a fine fibril of fibrin, about  $0.17\mu$  thick, previously invisible or on the threshold of visibility, was observed

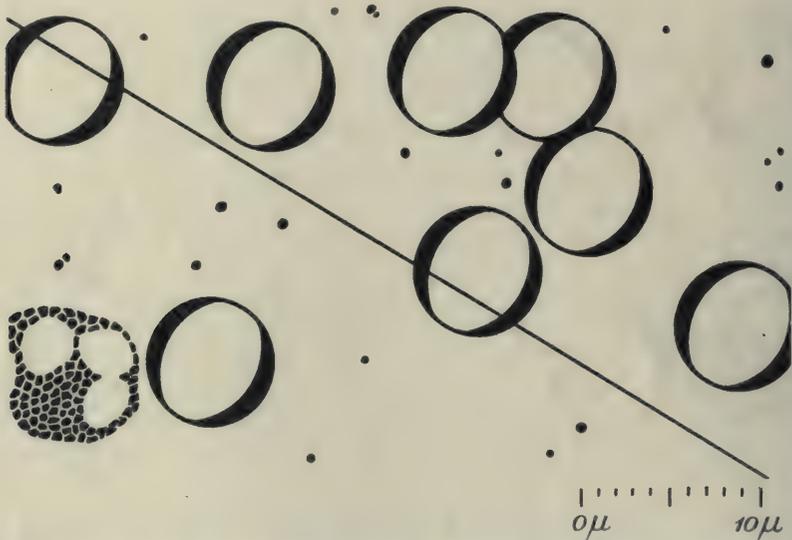


Fig. 1. Early stage of coagulation of a mixture of citrated human blood and whipped human blood (subsequently citrated). Film preparation three hours old. A single fibril is seen. In addition to red cells highly refractile dancing particles and a polymorphonuclear leucocyte are seen.  $\times 2300$ .

lying across the field. At the end of a further period of twenty hours, this fibril was found to have increased considerably in thickness, while a second delicate fibril, about  $0.2\mu$  thick and  $25\mu$  long, had appeared (Fig. 2): the length of the large fibril was found on measurement to be  $0.342$  mm. Fibrils vary in length from  $20\mu$  or less to  $500\mu$  or more; their apparent diameter ranges from about  $0.17\mu$  to  $1.1\mu$ ; the smallest fibrils do not present any

<sup>1</sup> Fibril formation has been investigated ultramicroscopically by several observers, two views having been advanced as to the nature of the process occurring. According to Mayer [1907] and Cesana [1908] coagulation consists in the appearance of very fine granules, which increasing in size arrange themselves in rows, forming threads, subsequently becoming numerous and thus giving rise to a network. Stübel [1914] compares coagulation to a crystallisation process and Howell [1914, 1916] states that fibrin is deposited not as a network, but as separate needles or crystals, which are massed to form a close meshwork.

<sup>2</sup> Citrated after separation of fibrin.

obvious structure, but the largest exhibit an irregular imperfectly reticular aspect (Fig. 3 c), due to refractile material, which is in more or less marked contrast to the rest of the substance of the fibril, the pattern thus produced sometimes extending along the whole of the fibril, sometimes being observed in short lengths of fibril, the intervening portion being apparently homogeneous. No further increase in size or number of fibrils could be recognised subsequently in the microscopic field shown in Fig. 2.

Two processes are therefore concerned in the formation of fibrin fibrils: (1) production of primitive fibrils; and (2) subsequent growth of these in presence of fibrinogen. The number of the finest fibrils actually seen depends

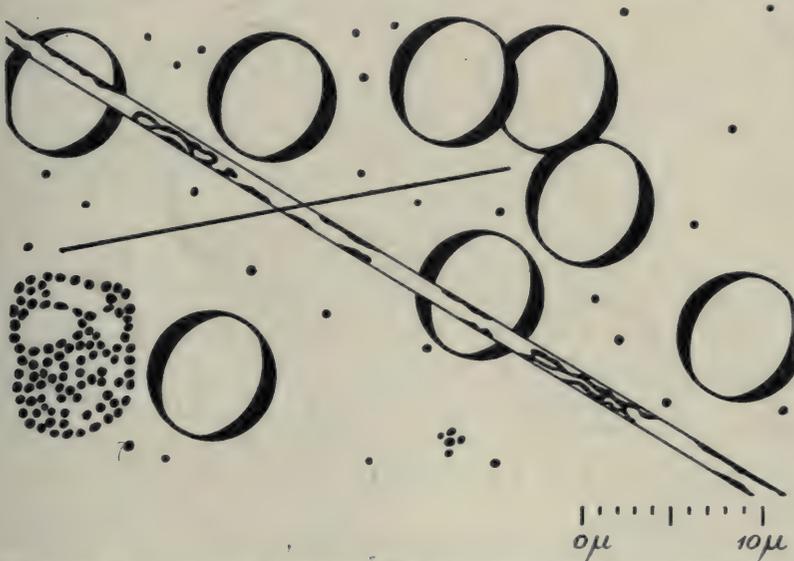


Fig. 2. Same as Fig. 1, twenty hours later. The fibril seen in the former figure has increased considerably in size and a second fine fibril has appeared. The leucocyte has become swollen and its granules, which were fixed in the preceding figure, are now in oscillatory motion.  $\times 2300$ .

upon the intensity of the illumination employed: with a poor arc light only a few fine fibrils are seen; the more intense the arc, the larger the number of exceedingly fine fibrils which can be rendered visible. The possibility must therefore be borne in mind that in mixtures of fibrinogen and thrombin excessively small fibrils may be already in existence, which cannot be rendered visible until their thickness has been increased by further addition of fibrin. It will be shown later that the exact moment at which fibrils are first formed can be studied only by indirect methods (p. 201). On the other hand the time of first appearance of visible fibrils can be determined by direct observation: to this method of investigation most of the results obtained in the present work are due.

In order to determine the relative amounts of fibrinogen and thrombin taking part in coagulation the following experiment was made.

*Exp. A.* Equal volumes of citrated human blood plasma (fibrinogen solution) and citrated 1 in 8,000,000 venom (thrombin) solution were mixed together. Coagulation occurred at room temperature (18°). At the end of sixteen hours a portion of serum expressed from the clot was mixed with an equal volume of venom solution (1 in 100,000) and allowed to evaporate at room temperature to about one-tenth of its bulk: no further coagulation occurred.

The amount of fibrinogen contained in the plasma employed was determined by precipitation with twenty volumes of distilled water through which a stream of carbon dioxide had been passed, the precipitate being then dried to constant weight at 120°. The plasma contained 0.78 % of fibrinogen<sup>1</sup>.

In the above experiment the weight of fibrinogen employed was more than 60,000 times as great as that of the venom added. It follows therefore that fibrin arises by transformation of fibrinogen: thrombin merely acts as a determinant, that is to say catalytically<sup>2</sup>.

Fibrils of fibrin are frequently attached to each other and may coalesce over part of their length, but no branching occurs (*cp.* Figs. 1 to 3). When present in sufficiently scanty numbers they are observed to be quite separate and to be unconnected with any of the suspended particles present, so that their characters can be readily studied, especially if the conditions of experiment are such as to lead to the production of fibrils of relatively large size: they are then seen to be attached to the surfaces of glass bounding the layer of liquid in which they have been formed.

Whatever may be the ultimate mode of production of these fibrils it is obvious that they form separate entities and that they are therefore particulate in origin, their appearance being determined by the addition of thrombin and the material of which they are constructed being provided by fibrinogen. Such particles of origin appear to be amicroscopic, for none of the particles seen in plasma or solution of fibrinogen can be observed to disappear or diminish in number after fibril formation is completed, and again the number of particles of origin, estimated from the number of fibrils developing, is very considerably in excess of the number of particles ordinarily observable in fibrinogen solution by ultramicroscopic methods. Presumably these particles of origin of the fibrils may be contained either (1) in the venom solution or (2) in the fibrinogen solution, employed in experiments on fibril formation. As a preliminary step in the investigation of this point the appearance presented at the junction of a solution of fibrinogen with a liquid containing thrombin was next studied.

<sup>1</sup> *Cp.* note, p. 197.

<sup>2</sup> Howell [1910] concludes that thrombin probably does not act upon fibrinogen after the manner of an enzyme. The solution of thrombin employed by this observer could, however, be heated to the boiling-point without completely losing its coagulative action on fibrinogen.

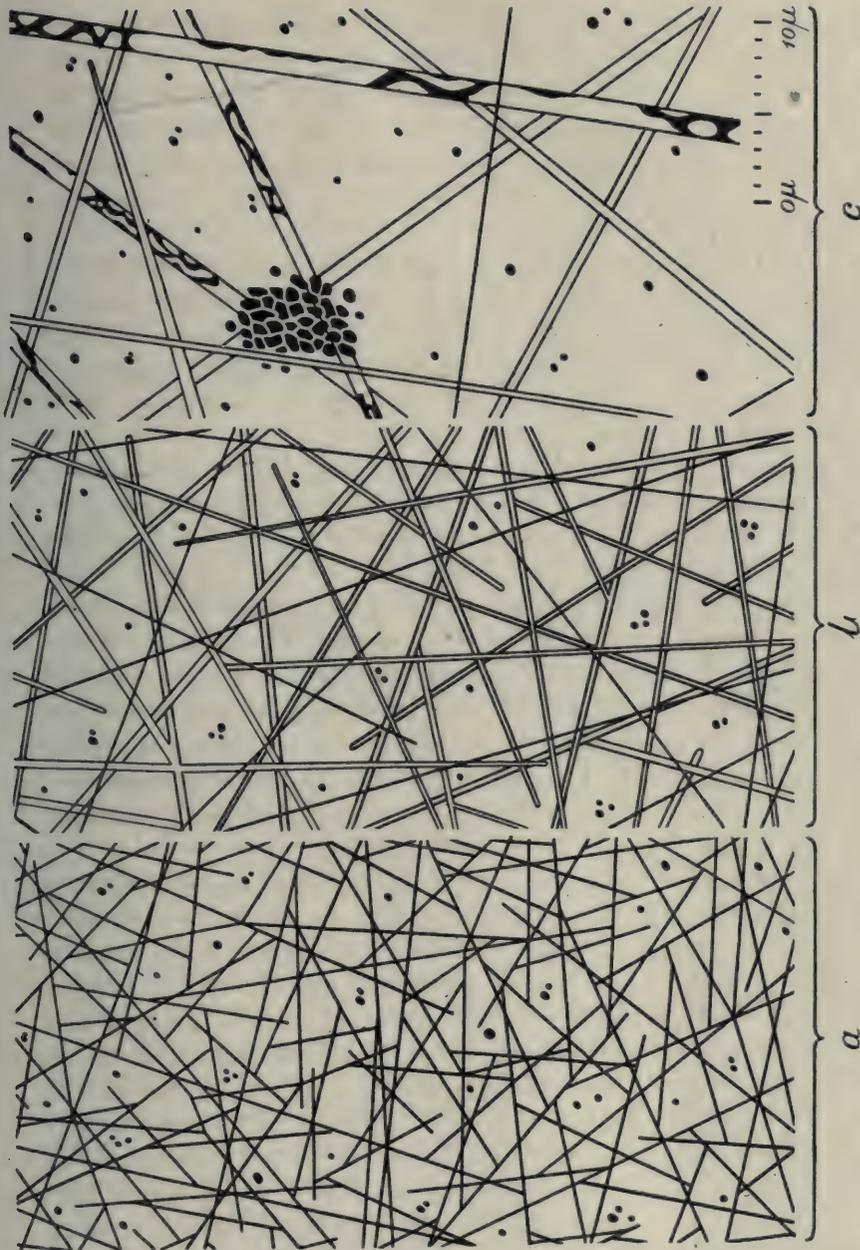


Fig. 3. Fibril formation at junction of droplets of (1) citrated human blood plasma and (2) citrated solution of venom of *Echis carinatus* (1 in 8,000,000). Film preparation six days old. *a* is taken at the venom side of the junction of liquids; *b* at the centre; and *c* at the plasma side. In *a* the fibrils are fine and numerous; in *b* most of the fibrils are larger, though less numerous; in *c* the fibrils are still further reduced in number, a few of relatively considerable size exhibiting a reticular pattern. Highly refractile dancing particles are seen among the fibrils: in *c* a fixed collection of particles attached to fibrils is observed.  $\times 2500$ .

*Exp. B.* A film preparation was made by placing droplets of human blood plasma and of a 1 in 8,000,000 solution of venom of *Echis carinatus* (both liquids being citrated) respectively near each other on a glass slide, within an area ringed with vaseline. A coverslip was then applied and lightly pressed down, so as to allow the droplets to come in contact at their opposed edges with little or no mixing of the liquids, evaporation being prevented by the ring of vaseline. The appearance of the junction of liquids at the end of six

days is shown in Fig. 3. At the side of the junction near the venom solution (*a*) very fine, exceedingly numerous fibrils are seen; in the middle (*b*) larger, less numerous fibrils; at the side next the fibrinogen solution (*c*) only relatively few fibrils, mostly long, of large diameter, and exhibiting an irregular pattern are observed.

Fibrin fibrils it may be observed are usually found to be unequal not only in length but also in thickness. After the lapse of a fortnight or three weeks, when they appear to have reached their maximum thickness, it may be observed that, mixed with the largest fibrils, medium-sized and fine fibrils are also seen (Fig. 3 *c*); when the fibrils do not exceed a medium value fine fibrils may be seen as well (Fig. 3 *b*); but when liminal visibility of fibrils has been reached differences of thickness can no longer be recognised.

It will be noted that those fibrils which are situated near the venom solution are contained in a fluid which is relatively rich in venom and correspondingly poor in fibrinogen, while at the opposite aspect of the junction of liquids the fibrils are present in a liquid in which the concentration of fibrinogen reaches its maximum, the venom being exceedingly dilute.

In order to observe more fully the effect of dilution of fibrinogen and venom respectively the series of experiments in Tables I and II were carried out. In these experiments an attempt has been made to give the actual number of fibrils observed in the field of the microscope: the higher counts are only approximate, owing to the large number and fineness of the fibrils present; the lower counts are more reliable owing to the smaller number and larger size of the fibrils. The enumeration of fibrils present in the field of the microscope is attended with difficulty if the fibrils are very numerous or are exceedingly fine. In the former case (*cp.* Fig. 3 *a*) delimitation of fibrils cannot be effected: it is a matter of uncertainty whether any given length of fibril should be regarded as a separate fibril or is merely the continuation of an adjacent fibril. Again, when fine fibrils are being examined, it is difficult to avoid overlooking some of the finest, which are indeed barely visible even with the maximum intensity of illumination available. It is obvious, therefore, that, if the fibrils under observation are very numerous or are exceedingly fine, enumeration is merely approximate. On the other hand if the conditions of experiment are such that the fibrils are few in number and if, in addition, they are relatively thick, counting ceases to be difficult and accuracy of enumeration is attainable. In Table I the effect of an 81-fold dilution of venom is exhibited. It will be seen that, within the range of concentration employed, the number of fibrils visible appears to be roughly proportional to the amount of venom present. This relation, it may be observed, does not continue to hold if the range of concentration of venom is made considerably more extensive: under these circumstances a point is reached at which no further increase occurs, but an actual diminution in the number of fibrils recognisable may be noted (*cp.* p. 209). With the highest venom concentration employed

in Table I, fibril formation is not only abundant but also rapid, occurring within a few minutes, while with the lowest concentration its course is very slow, fibrils not appearing until after the lapse of many hours: in the former case only a limited increase in thickness of the fibrils subsequently occurs; in the latter the amount of fibrinogen available is, owing to the smaller number of fibrils, relatively considerable and in consequence marked though slow growth takes place, which may not reach its maximum for several days.

Table I.

Equal parts of citrated human plasma (undiluted) and of citrated venom solution, the latter in varying dilutions, were placed on a glass slide and, immediately after mixing, a coverslip applied, the thickness of fluid examined being 0.05 mm.

	Venom	Fibrils per field	Appearance of fibrils
in	300,000	1730	Very fine, retracted
„	900,000	640	Fine
„	2,700,000	120	Medium
„	8,100,000	50	Moderately coarse
„	24,300,000	20	Coarse

In Table II the effect of dilution of plasma is exhibited: it is seen that, with diluted plasma, the number of fibrils diminishes, but the diminution is no longer accompanied by an increase in thickness, as in the preceding experiments, the fibrils remaining fine. With the higher dilutions no fibrils can be recognised. Fibrils make their appearance in diluted plasma more slowly than in undiluted plasma.

Table II.

Equal parts of citrated human plasma, in varying dilutions, and of citrated venom, diluted 1 in 8,100,000, were mixed together as in the preceding Table.

Plasma	Fibrils per field	Appearance of fibrils
100 %	43	Moderately coarse
33	7	Fine
11	—	No fibrils visible
3.7	—	„ „
1.2	—	„ „

Returning once more to the appearances presented by fibrin fibrils at the junction of two liquids containing respectively plasma and venom (*Exp. B*, p. 193, and Fig. 3 *a, b, c*), we are now, by the aid of the data obtained from the above dilution experiments, in a position to state that at the venom side of the junction of liquids (where venom is relatively abundant and fibrinogen dilute) the numerous fine short fibrils observed have been produced rapidly, while at the fibrinogen side of the junction (where plasma is almost undiluted and venom is highly dilute) the long, relatively coarse fibrils, which are seen in comparatively small numbers, have been produced with considerable slowness.

This mode of formation of fibrin fibrils in the presence of varying concentrations of venom indicates the source of the particles of origin of the fibrils.

These particles of origin, which, it may be surmised, are contained either in the thrombin solution or in the fibrinogen solution, employed for fibril production, may conceivably consist of either thrombin or fibrinogen. If the particles of origin consist of thrombin the effect of dilution of the thrombin solution, the concentration of fibrinogen remaining unchanged, would be to diminish the number of fibrils without affecting their time of formation. Such, however, is not the case: when the concentration of thrombin is lowered fibrils make their appearance (in diminished numbers) after a lengthened interval, the duration of which is dependent upon the degree of dilution of thrombin. The hypothesis that the particles of origin of fibrin fibrils consist of thrombin is therefore untenable. If, on the other hand, the particles of origin consist of fibrinogen the effect of dilution of thrombin, acting catalytically, would be exhibited by delay of formation of fibrils, and if the particles of origin were unequal (for example, in consequence of variability in size, as is not inherently improbable) in respect of their response to thrombin, the fibrils appearing would be reduced in number: since both of these effects are actually observed it is seen that the latter hypothesis, namely that the particles of origin of fibrin fibrils consist of fibrinogen, is tenable in so far as it is consistent not only with the delay in appearance of the fibrils but also with the diminution of their number<sup>1</sup>. Further experimental proof of the validity of this hypothesis will now be given.

#### FIBRINOGEN SOLUTION.

Since growth of fibrils can be observed in a fibrinogen solution the further assumption must be made that, while part of the fibrinogen is in suspension, another part is in solution: in other words the liquid containing fibrinogen must be diphasic, forming a hydrosol, the disperse phase furnishing the particles of origin of the primitive fibrils, while the continuous phase, by affording a further supply of fibrinogen to the primitive fibrils, enables an increase of their diameter to occur, both particulate and added fibrinogen alike changing under the influence of thrombin into fibrin.

A point has now been reached at which it is possible to put hypothesis to the test of experiment. If fibrinogen solution forms a two-phase system the volume of the concentrated phase which, it has just been inferred, provides the particles of origin of fibrils will diminish on dilution, that of the dilute phase undergoing a corresponding increase, until ultimately a dilution is reached, below which the concentration of the dilute phase, previously maintained unchanged, will now begin to diminish with further addition of diluting fluid.

In order to test this conclusion an attempt was made to determine the concentration of the continuous phase present in varying dilutions of plasma.

<sup>1</sup> The effect of dilution of fibrinogen solution upon the thickness of fibrils is readily explicable. The latter in low concentrations of fibrinogen can receive only a small supply of this substance and in consequence remain exceedingly small.

To this end the experiments on fibril formation, shown in Table III, in which the time of first appearance of fibrils in a series of mixtures of fibrinogen and venom is observed, were made. This method of enquiry is based upon the assumption that, since one of the two substances interacting in the production of fibrils, namely venom, is employed in the same concentration in all experiments, fibril formation will therefore proceed at a monomolecular reaction rate according to the equation

$$-lC = kt + a,$$

where  $C$  = concentration of continuous phase,  $t$  = time of first appearance of fibrils, and  $k$  and  $a$  are constants. If this assumption is verified it would become possible to determine the degree of dilution of plasma below which diminution of concentration of the continuous phase commences, and to ascertain the relative concentration of continuous phase corresponding to any given dilution of plasma. The value of the constant  $k$  is independent of that of  $a$ : if the temperature of experiment is maintained invariable its value is dependent upon the concentration of thrombin employed. If the curve is made to commence at the axis of ordinates so that the vertical part corresponds with this axis, then  $-a$  represents the natural logarithm of the lowest concentration of plasma at which the continuous phase is saturated.

Table III.

Citrated human plasma, in varying dilutions, was mixed with citrated venom, 1 in 30,000, in the proportion of eight parts of the former to one part of the latter, and the time at which fibrin fibrils were first observed noted.

Exp.	Plasma	Fibrils first seen at end of:
1	100 %	5 minutes
2	90 "	6 "
3	80 "	5 "
4	70 "	7 "
5	60 "	12 "
6	50 "	50 "
7	40 "	113 "
8	30 "	175 "
9	20 "	262 "
10	10 "	No fibrils observed

The observations when plotted with dilution of plasma as ordinates and time of first appearance of fibrils as abscissae were found to describe a curve (Fig. 4 *a*) so long as the concentration of plasma employed did not exceed 61 % (undiluted plasma<sup>1</sup> being represented by 100): above this point the curve was replaced by a vertical line. The curve corresponds to the concentration of the continuous phase, which reaches its maximum at a dilution of

<sup>1</sup> The precipitate (dried to constant weight at 120°) obtained from plasma by adding twenty volumes of distilled water, through which a stream of carbon dioxide had been passed, amounted to 0.8 % of the plasma employed. This precipitate is known to contain, in addition to fibrinogen, prothrombin and thrombokinase. It is not at present possible to obtain fibrinogen in even an approximately pure condition.

plasma represented by 61 %: at this point the conversion of the disperse phase into the continuous phase is apparently complete. Below a dilution of 61 % the concentration of the continuous phase becomes lowered when more fluid

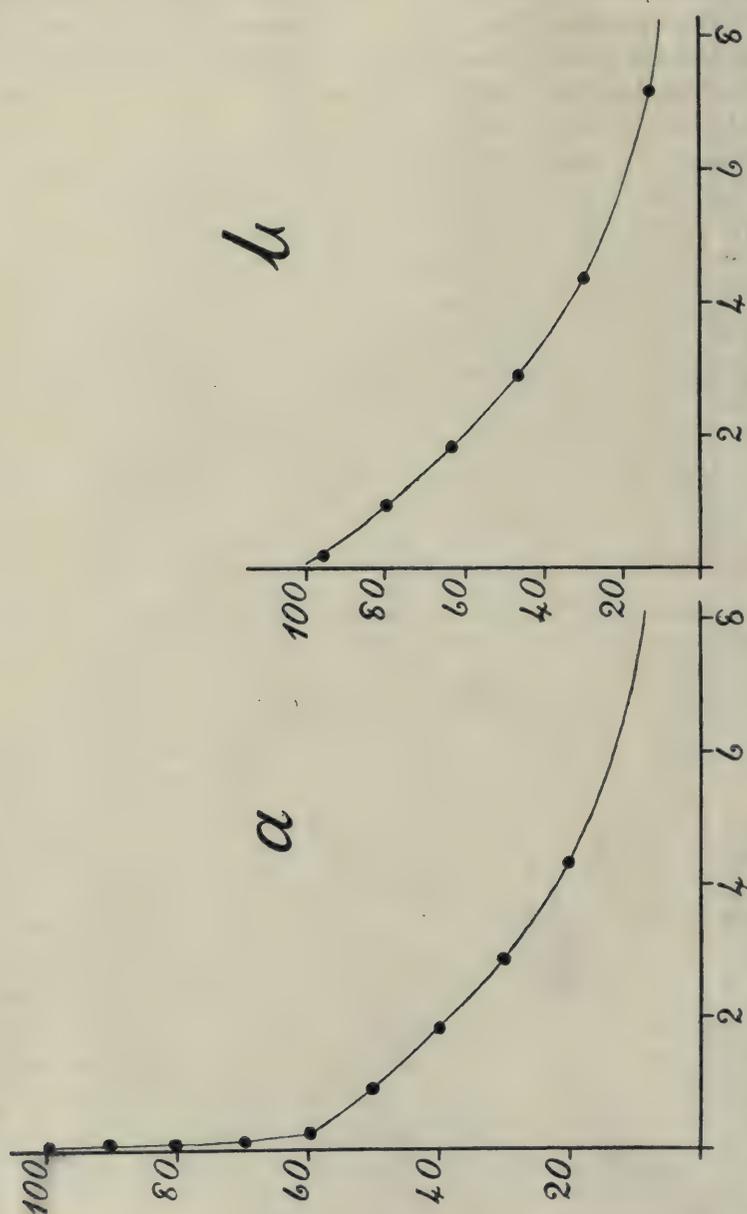


Fig. 4 a and b. Graphic representation of experiments in Table III. Ordinates in Fig. 4 a indicate the total concentration of fibrinogen contained in the plasma employed, that of undiluted plasma being represented by 100; ordinates in Fig. 4 b give the concentration of fibrinogen in the continuous phase, that corresponding to 61 % in Fig. 4 a being here represented by 100. Abscissae represent time, in hours, at which fibrin fibrils were first observed. The curves are constructed from values of  $k = 0.0042$  and  $a = -4.623$ . Exps. 1 to 4 are not indicated in Fig. 4 b.

is added; above this dilution no further change in the concentration of the continuous phase occurs, saturation having been attained. In Fig. 4 b, the same curve is again given the ordinates now indicating the concentration of the continuous phase, saturation of which (corresponding to 61 % of plasma

in Fig. 4 a) is represented by 100. If the values of the constants, determined from Exps. 5 to 10 (Table III), are taken at

$$a = -4.623, \quad k = 0.0042,$$

the calculated reaction times, given in Table IV, exhibit little divergence from those actually observed. From these experiments it may therefore be concluded that fibrinogen solution is diphasic. That the individual particles of the disperse phase have not, however, completely disappeared when the transition point of the curve is reached, is shown by the circumstance that fibril formation does not cease at this point. Nevertheless the sharpness of the transition point and the fair correspondence of calculated and observed times appear to indicate that the amount of fibrinogen still remaining, with increasing dilution of plasma, in the particles of the disperse phase after the maximum concentration of the continuous phase has been passed, is relatively insignificant, and thus the assumption, made in Table IV, col. 2, that the concentration of the latter phase now becomes proportional to the further dilution appears to be at any rate approximately correct. The significance of the absence of complete disappearance of the particles of the disperse phase, at the transition point, will be referred to later (p. 208).

Table IV.

Calculated time of appearance of fibrin fibrils in the experiments recorded in Table III.

Exp.	Plasma	Fibrinogen in continuous phase <sup>1</sup>	Reaction calculated	Time observed
1	100 %	100 %	5.5 min.	5 min.
2	90 "	100 "	5.5 "	6 "
3	80 "	100 "	5.5 "	5 "
4	70 "	100 "	5.5 "	7 "
5	60 "	98.5 "	10 "	12 "
6	50 "	82.0 "	53 "	50 "
7	40 "	65.6 "	107 "	113 "
8	30 "	49.2 "	178 "	175 "
9	20 "	32.8 "	271 "	262 "
10	10 "	16.4 "	437 "	No fibrils observed

If the dilution of plasma is considerable no visible fibrils are obtainable on adding thrombin: in Table II, for example, this was found to be the case with a dilution of 11 % of plasma. When, however, a portion of this highly diluted plasma was placed on a microscope slide and allowed to evaporate at room temperature to about one-sixth of its bulk, and then a small amount of venom (1 in 30,000) added, a striking result was obtained: at the end of fifteen minutes abundant fibril formation was found to have occurred; in a control experiment, performed at the same time, in which care was taken to prevent evaporation, no fibrils appeared.

It is thus evident that the simple addition of thrombin as a test for fibrinogen under all circumstances is not admissible. If the continuous phase is sufficiently dilute (*cp.* p. 203), no coagulation is recognisable, no fibrils

<sup>1</sup> The maximum concentration, which is present in 61 % dilution of plasma, being represented by 100.

are observable: by evaporation, however, the original concentration of fibrinogen may be regained and the former coagulability reproduced.

Incidentally the ease with which a highly diluted continuous phase can be concentrated, with reappearance of visible fibrils, in experiments made with small quantities of diluted plasma, placed on a glass slide, emphasises the necessity of very carefully avoiding evaporation in such experiments when determinations of the time of first appearance of fibrils are being made.

#### MECHANISM OF FORMATION OF FIBRIN FIBRILS.

With a view of throwing further light upon the processes concerned in coagulation, the following hypothesis as to the mechanism of production of fibrin fibrils may now be advanced.

In attempting to explain the mode of occurrence of this phenomenon in a liquid containing fibrinogen and thrombin I picture to myself fibrils originating from spherical particles, exhibiting Brownian movement like the particles ordinarily seen in plasma, but presumably of considerably smaller dimensions, which under the influence of thrombin have become semi-solid, viscid and sticky. One of these oscillating particles comes, let us suppose, into contact with the surface of the glass slide, upon which the mixture of fibrinogen and venom solutions, used for experiment, has been placed. Recoil then occurs, the particle changing its direction, continuing its oscillatory movement, and ultimately, after an exceedingly zig-zag course, colliding a second time with, let us imagine, the glass slip covering the fluid in which it is contained. But inasmuch as the particle is viscid and sticky it became adherent to the glass slide at the first impact and then, upon recoil, became drawn out, so that when the second collision with the cover-slip occurred a primitive fibril had thus been formed, which now became adherent by its previously free end (containing the remainder of the particle of origin) to the cover-slip, the fibril being thus attached at each extremity to a glass surface. By the continued action of thrombin the further change of the primitive fibril, thus formed, into fibrin is completed: the fibril becomes solid and, at the same time undergoing contraction, becomes also tense. Further addition of fibrinogen, from the continuous phase, to the primitive fibril next occurs, the new supply becoming in its turn, under the influence of thrombin, converted into solid fibrin. In this way a relatively thick fibril, visible under ultramicroscopic illumination, is ultimately produced. Adhesion of the primitive fibrils is not limited to glass surfaces. Attachment to other fibrils is common, especially when the latter are abundant, and adhesion to the particles seen singly or in groups in fibrinogen solution is also frequently observed<sup>1</sup>.

<sup>1</sup> The latter in many cases seems, however, to be a late event, accompanying and partly dependent upon gradual subsidence of the particles under the force of gravity. Characteristic rosette figures, consisting of fibrils radiating from a group of granules, may thus be formed.

Both particles of origin and primitive fibrils appear to be amicroscopic (*cp.* p. 205).

## FURTHER CHARACTERS OF FIBRINOGEN.

In relation to fibril formation the processes occurring prior to the first appearance of visible fibrils appear to be, as already indicated, three in number: (1) change, under the influence of thrombin, of the particle of origin, consisting of fibrinogen in a concentrated condition, presumably forming a liquid droplet, into a viscid, sticky, semi-solid mass; (2) conversion of the latter, by virtue of its Brownian movement, leading to contact with neighbouring surfaces, into a primitive fibril stretching between two surfaces to which its ends adhere, and changing under the further action of thrombin into fibrin; (3) growth of the fibril by deposition of fibrinogen from the continuous phase, the fibrinogen so deposited being changed in its turn, by the thrombin present, into fibrin. In the series of experiments, made to determine the time of first appearance of visible fibrils, given in Table III and Fig. 4, the period occupied by the first two processes must be alike in all experiments, whatever the dilution of fibrinogen may be, since the initial concentration of thrombin is the same throughout the series. This period was estimated for the dilution of plasma corresponding to *Exp. 5*, Table III, in the following way. As soon as the mixture of fibrinogen and thrombin had been prepared it was stirred continuously for five minutes, portions being removed for examination at the end of one, two, three, four and five minutes respectively, and the approximate number of visible fibrils appearing in a fixed volume of the sample determined. Such primitive fibrils as were formed before taking the sample would have been already removed by stirring; the visible fibrils observed in the samples must, therefore, indicate primitive fibrils formed after removal and would correspond to particles of origin which had not been converted into primitive fibrils at the time of removal of the sample. The observations made are given below:

Time	Fibrils		
	observed	calculated	%
0 min.	—	732	100
0.07 "	—	659	90
1 "	135	147	20
2 "	33	30	4
3 "	5.5	5.9	0.8
4 "	in exceedingly scanty numbers	1	0.2
5 "	"	0.2	0.03

These experiments, which are represented graphically in Fig. 5, indicate that the change of particles of origin into primitive fibrils proceeds at a monomolecular reaction rate, the constants of the reaction being  $k = 1.6$  (time being reckoned in minutes) and  $a = -6.58$ : the calculated number of fibrils, given in the third column (and in percentage form in the fourth), agree with the observed values within the range of experimental error. The time occupied in the formation of primitive fibrils appears to be small: according to the above

calculation four-fifths of the maximum number of fibrils obtainable are formed within one minute of mixing fibrinogen and thrombin, and one-tenth within the first four seconds (0.07 min.)<sup>1</sup>. On the other hand it will be noted that in the experiments recorded in Table III, in which the same concentration of thrombin was used as in the observations given above, the minimum time of first appearance of visible fibrils was five minutes. Since the fibrils first appearing form less than one-tenth of those ultimately seen it is clear that the reaction actually measured in the experiments recorded in Table III is essentially the process represented above by (3), namely growth of primitive fibrils, the processes (1) and (2) together occupying a negligible fraction of the period of first appearance of visible fibrils.

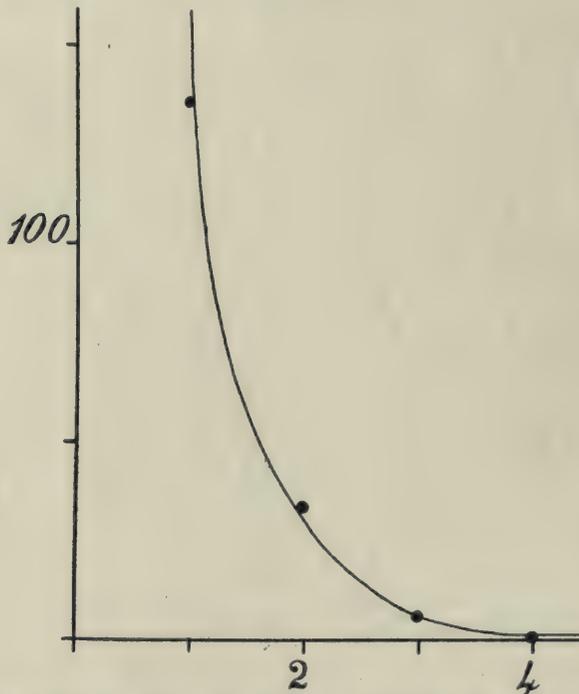


Fig. 5. Removal of primitive fibrils from mixture of fibrinogen and thrombin by stirring with a glass rod. Ordinates represent the number of fibrils appearing in samples removed after varying periods of stirring; abscissae represent time in minutes. Details of experiments are given in text. The curve is constructed from values of  $k=1.6$  and  $a=-6.58$ .

It will be seen from Tables III and IV that fibrils may be formed, not only in undiluted plasma (*Exp.* 1), but also when only 20 % of plasma is present (*Exp.* 9). In the former experiment the maximum concentration of both disperse and continuous phases obtains; in the latter the disperse phase has nearly completely disappeared and the continuous phase has only 33 % of its maximum value (transition point of curve at 61 % of plasma). If an

<sup>1</sup> In mixing fibrinogen and thrombin solutions stirring should be as far as possible avoided in experiments in which fibril counting is attempted.

endeavour is made to count the fibrils present<sup>1</sup>, as in the experiments recorded in Table II, it is found that the approximate number present does not at first exhibit any marked decrease, but the fibrils become very fine and when dilution of the continuous phase is considerable cease to be visible owing to their extreme tenuity. Below a series of experiments performed under the same conditions as in Table III (the concentration of thrombin being constant, while fibrinogen is employed in varying degrees of dilution) is given, in which an attempt is made to enumerate the fibrils present in a given small volume of each mixture of fibrinogen and thrombin. These results are plotted in Fig. 6. The higher

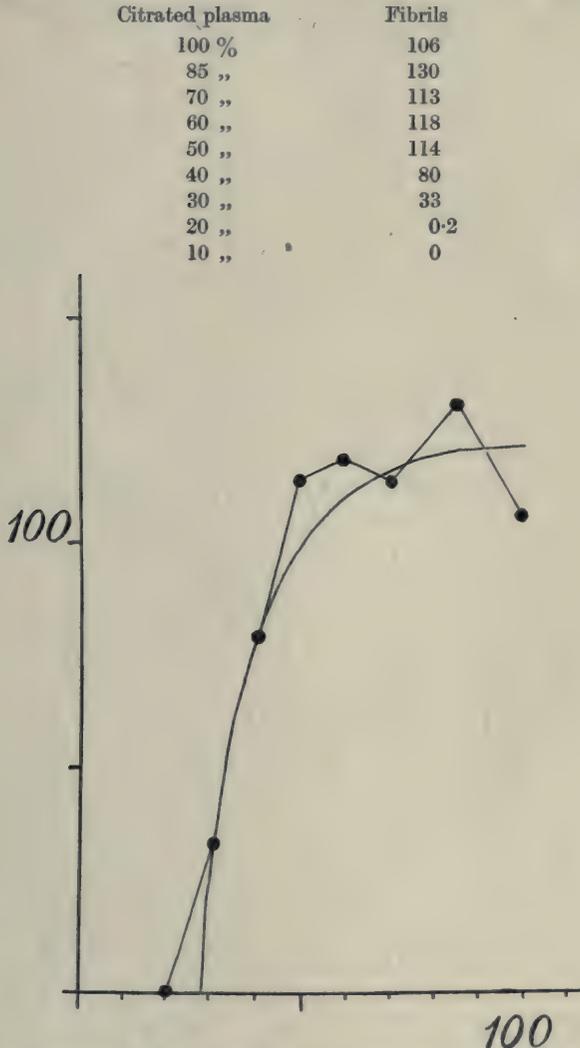


Fig. 6. Approximate number of fibrils obtained on coagulation of varying dilutions of citrated human plasma. Ordinates represent fibrils; abscissae represent percentage of plasma. Details of experiments are given in text. The fibril counts indicate a curve of the type shown.

<sup>1</sup> The enumeration is approximate: the error is considerable when fibrils are abundant.

counts are only approximate, owing to difficulties already referred to, but the type of curve formed by the fibrils is indicated in the figure: the dilution of plasma at which visible fibrils cease to be obtainable is determined graphically to correspond to 28 % of plasma. Since the maximum concentration of the continuous phase of fibrinogen in these experiments was found to be present in 75 % of plasma and in all concentrations of plasma above this point, it follows that, in this series of experiments, fibrils cease to be visible when the continuous phase is reduced to 37 % of its saturation concentration. This result and those of three other similar determinations are given below:

Series	Continuous phase of fibrinogen
<i>A</i>	26 %
<i>B</i>	30 „
<i>C</i>	40 „
<i>E</i>	37 „

The mean of the series corresponds to 33 % of the maximum concentration of the continuous phase.

The action of thrombin in converting fibrinogen in the concentrated phase into fibrin is not exerted solely upon the dispersed particles of this phase, as is shown by the following experiment.

*Exp. C.* Precipitated fibrinogen, obtained by adding to plasma twenty volumes of distilled water through which a stream of carbon dioxide had been passed, was suspended in distilled water so as to form a thick emulsion, composed of closely packed masses of fine motionless granules,  $0.4\mu$  or less in diameter. This emulsion was divided into two parts, one of which (*B*) was used for control experiments; to the other venom, contained in distilled water, was added, the concentration of venom in the mixture being about 1 in 1,000,000. At the end of forty-eight hours the mixture had formed a soft, semi-solid, friable mass, consisting of densely packed fine granules free from fibrils: this was broken up as completely as possible and suspended in distilled water (*A*). Portions of *A* and *B* were used for experiment as follows:

	1 part	+4 parts citrated 0.85 % NaCl containing	Microscopic appearance with dark ground illumination
1.	<i>A</i>	—	No dispersion. No fibrils.
2.	<i>A</i>	venom 1 in 30,000	Marked dispersion. No fibrils.
3.	<i>A</i>	„ 2.3 %	Nearly complete dispersion. No fibrils.
4.	<i>B</i>	—	Complete dispersion. No fibrils.
5.	<i>B</i>	venom 1 in 30,000	Complete dispersion. Fibrils fairly numerous.

The last observation was repeated in a small test-tube: a loose coagulum formed, which had already retracted at the end of three hours.

In the above experiment it is seen that under the action of thrombin precipitated particles of fibrinogen are converted into fibrin without any

change of form, fibril formation being impossible owing to the immobility of the particles. The particles of fibrinogen, however, adhere to each other and to the numerous adventitious particles which accompany the precipitated fibrinogen, so that the latter cannot be dispersed on adding 0.85 % sodium chloride solution. If, however, the sodium chloride solution contains venom in sufficient concentration to dissolve the altered fibrinogen particles, then dispersion occurs. That the fibrinogen is converted into fibrin is indicated by the effect of relatively concentrated venom in producing lysis (see p. 209). It does not appear that the presence of sodium chloride is necessary for the transformation of fibrinogen into fibrin. Direct conversion of some of the dispersed particles of fibrinogen in the concentrated phase does not seem inherently improbable, but investigation of this point is attended with difficulty owing to the presence of numerous adventitious dancing particles in liquids containing fibrinogen.

That the suspended particles of the concentrated phase of fibrinogen are unequal, presumably, in size is shown by the circumstance that (1) primitive fibrils are formed at different periods of time (p. 201), while visible fibrils (2) are of different size (Figs. 1-3), (3) do not all appear at the same time (p. 190), and (4) are reduced in number when highly dilute thrombin is employed (Table II; also p. 202).

The size of the particles which give rise to primitive fibrils does not appear to be capable of measurement, nor can the diameter of primitive fibrils be at present determined, but it is nevertheless possible to give, for both of these, upper limits of size, which cannot be exceeded. Thus the finest fibril which could be recognised with the illumination employed in these experiments had an apparent diameter of about  $0.15\mu$ . Assuming the length of such a fibril to be  $50\mu$ , its volume would be represented by a sphere of  $0.46\mu$  radius. Such a relatively large particle, however, though possessing oscillatory movement, would be much too defective in translatory motion to give rise to a primitive fibril  $50\mu$  long. On the other hand the smallest recognisable particle, having an apparent diameter of  $0.15\mu$ , though still possessing insufficient translatory movement, would have the same volume as a fibril  $50\mu$  long and  $0.0067\mu$  in diameter. These two values, therefore, may be taken to represent maximum limits for the diameter of a particle of origin and for the thickness of a primitive fibril respectively.

The very irregular translatory movement of an oscillating particle having an apparent diameter of  $0.15\mu$  did not, as a rule, exceed  $10\mu$  in the course of a minute. Since fibrin fibrils may reach  $400\mu$  or more in length and more than four-fifths of the primitive fibrils may be already formed within a minute of the addition of thrombin (p. 201), it follows that the average size of the particles of origin of the fibrils must be much less than that of the smallest visible particles observable in these experiments. Several observers have attempted to measure the mean amplitude of oscillation and degree of translation of particles of known diameter [*cp.* R. Zsigmondy, 1905, pp. 104-111],

but sufficient data do not appear to be yet available to enable the limits of size of such colloidal particles of fibrinogen as possess enough translatory motion to be capable of giving rise to primitive fibrils to be determined.

The maximum or saturation concentration of the continuous phase of fibrinogen in plasma obtained from the same source must be assumed to be constant under the same conditions of experiment (concentration of thrombin and temperature) since the time of first appearance of visible fibrils remains unchanged<sup>1</sup>. It is therefore possible, when the transition point of the reaction rate curve has been determined (*cp.* Table IV, Fig. 4 *b*), to express the concentration of the disperse phase in terms of the saturation concentration of the continuous phase. If this is done in respect of the same sample of plasma at different periods after collection, it is found that the disperse phase of fibrinogen gradually diminishes in amount on keeping. This disappearance of the disperse phase is exhibited in the following series of experiments each series being carried out as indicated in Table III:

Series	Age of plasma	Concentration of disperse phase of fibrinogen		
		observed	calculated	in percentage form
—	0 days	—	219	100
<i>A</i>	6 "	163	162	74
<i>B</i>	12 "	120	120	55
<i>C</i>	18 "	92	89	41
<i>D</i>	25 "	64	63	29
<i>E</i>	39 "	33	31	14

In the second column the period after bleeding, at which the experiments were made, is shown; in the third column the concentration of the disperse phase is given in terms of the continuous phase, represented by 100%; in the fourth column these concentrations (and also the initial concentration) are calculated on the assumption that the change of the disperse phase proceeds at a logarithmic rate, the constants of the reaction being estimated at  $k = 0.05$  (time being measured in days) and  $a = -5.38$ ; in the fifth column the concentrations given in the fourth are presented in percentage form. The close correspondence between observed and calculated values, exhibited graphically in Fig. 7, establishes the truth of the assumption that the disappearance of the disperse phase proceeds at a monomolecular reaction rate. This occurs equally when the colloidal particles of this phase are suspended in undiluted or in diluted plasma, thus being independent, within the limits of experiment, of the dilution. The continuous phase of fibrinogen does not appear to be affected by the addition of thrombin: if any disappearance of this phase occurred it

<sup>1</sup> In Series *B*, *C*, *D*, the same stock solution of thrombin (kept at 0°) was employed: the time of first appearance of visible fibrils in undiluted plasma or in plasma dilutions lying above the transition point of the corresponding curve was 8.5 minutes. In Series *A* and *E* in which freshly prepared thrombin solutions were used this period was slightly reduced.

<sup>2</sup> In the series of experiments given in Table III, for example, the concentration of the disperse phase would be represented by  $\frac{39 \times 100}{61} = 63.9$ .

would presumably proceed at a constant rate in all concentrations of plasma above the transition point, in other words so long as the maximum concentration of the continuous phase is maintained, but of this there is no evidence in experiments of the type of those given in Table III.

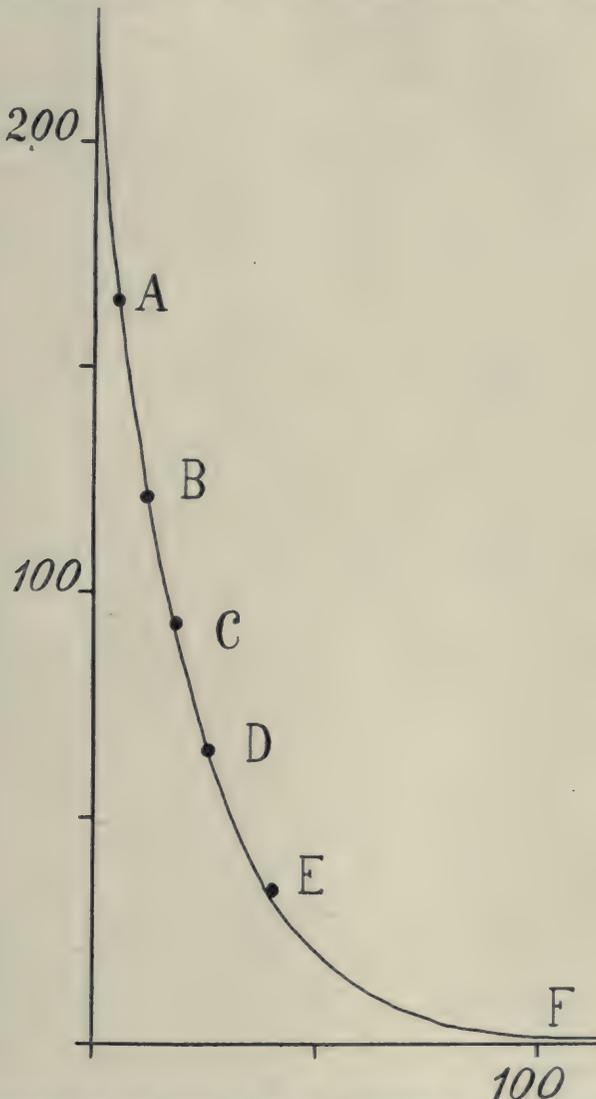


Fig. 7. Disappearance of the disperse phase of fibrinogen, in citrated plasma, on keeping. Ordinates represent concentration of disperse phase in terms of maximum concentration of continuous phase, represented by 100; abscissae represent time in days. Details of experiments are given in text. The curve is constructed from values of  $k=0.05$  and  $a=-5.38$ .

The disappearance of the disperse phase on keeping is in part, if not wholly, due to fibril formation, for when this disappearance is considerable, flocculent masses, which are found on microscopical examination to consist of radiating collections of coarse fibrils mixed with aggregations of granules,

are observed in the plasma, which has become very turbid though still liquid. Whether in addition any of the particles of the disperse phase, in consequence, it may be, of their relatively large size or of an accidental failure to collide with a surface before conversion into fibrin was complete, become directly converted into fibrin is uncertain, the presence of numerous dancing particles, as already pointed out, making it difficult to determine this point.

The particles of the disperse phase, so long as the change of fibrinogen into fibrin is incomplete, consist of a mixture of fibrin and fibrinogen. If neither of these substances is soluble in or miscible with the other no explanation is forthcoming of the failure of the disperse particles to disappear completely at the transition point of the curve, nor is the growth of primitive fibrils accounted for. If, however, it is assumed that fibrin, or any product intermediate between fibrin and fibrinogen, is soluble in fibrinogen in the disperse phase, then the concentration of fibrinogen in disperse particles in which partial change into fibrin had occurred would be lowered and would no longer be in equilibrium with the continuous phase when the latter was saturated. In consequence of this disturbance of equilibrium fibrinogen would pass from the continuous phase into the particles of the disperse phase (or into the primitive fibrils, if the particles had already given rise to fibrils). In this way a new equilibrium between the disperse phase (or primitive fibrils) and the continuous phase would be reached with increasing dilution of the latter. As more of the disperse phase (or primitive fibrils) became converted, under the further action of thrombin, into fibrin this new equilibrium would, in its turn, be disturbed and a further transfer of fibrinogen from the continuous phase into the disperse particles (or fibrils) would occur, these processes continuing until finally all the fibrinogen had been converted into fibrin.

The above appears to be the simplest hypothesis which will account both for the absence of complete disappearance of disperse particles of fibrinogen at the transition point of the reaction rate curve (Fig. 4) and also for the growth of primitive fibrils: it may therefore on this account be, provisionally at any rate, adopted. Two corollaries, it may be pointed out, follow from this hypothesis. The first is that the concentration of the continuous phase below the transition point is not strictly proportional to the further dilution: since, however, the amount of fibrinogen present in the disperse phase, when determined by experiments of the type of those shown in Table III, appears to be very small, it may be surmised that the difference in question is also inconsiderable. The second corollary is that if any of the disperse particles are too large to possess the degree of translatory movement necessary for their conversion into primitive fibrils, undergoing in consequence direct conversion into fibrin, such particles must also subsequently increase in size at the expense of fibrinogen present in the continuous phase, in the same manner as primitive fibrils.

When relatively concentrated venom of *Echis carinatus* is employed as source of thrombin a further action occurs: the fibrils which make their

appearance subsequently disappear, undergoing lysis<sup>1</sup>. Thus a 1 in 3000 solution of venom, added to an equal volume of undiluted plasma, leads to abundant fibril formation in about three minutes. At the end of two hours at room temperature (16°) most of the fibrils have disappeared, those remaining being thinned and for the most part broken, forming débris which falls to the bottom of the layer of fluid examined and is later completely dissolved. The occurrence of this lysis in particles of precipitated fibrinogen, which have undergone direct conversion into fibrin, has been already described (p. 204). On the hypothesis that lysis of fibrin is not due to an adventitious proteolytic ferment, but is a true thrombin effect, the change of fibrinogen in the concentrated phase into fibrin, under the action of thrombin, would appear to be merely a preliminary stage of the lytic action of thrombin.

#### BEARING ON GEL FORMATION IN GENERAL.

The behaviour of fibrinogen under the action of thrombin suggests that in any colloidal solution the disperse phase, if it consists of liquid particles, may, when passing into the solid state under the influence of a fall of temperature or of ferment action, give rise to fibrils, if the particles are sufficiently small, or form a suspension of solid particles, if of relatively large size. In this way may be explained the formation of the characteristic structure, consisting of a mixture of network and granules, sometimes the one sometimes the other predominating or being present exclusively, met with in cell plasm, subjected to the action of fixatives [see Wilson, 1904], and also in gels after similar fixation [Hardy, 1899].

#### SUMMARY.

1. Fibrinogen in solution is diphasic, consisting of a concentrated phase, which is dispersed, and a dilute phase, which is continuous.
2. Under the action of thrombin fibrin is formed out of fibrinogen in the concentrated phase: if this phase is in the form of a precipitate its particles become directly converted into fibrin; if this phase is dispersed its suspended particles give rise to fibrin fibrils, forming a coagulum.
3. The action of thrombin upon fibrinogen in the concentrated phase is purely catalytic. No effect appears to be exerted by thrombin upon fibrinogen in the continuous phase.

<sup>1</sup> Cp. Houssay and Negrete [1918]. These authors observe that solid proteins with the exception of fibrin are not dissolved: the action of venom thus appears to be specifically directed to fibrin.

4. The following hypothesis of the mode of formation of fibrin fibrils is advanced:

Fibrin fibrils are particulate in origin.

The particles of origin, consisting of fibrinogen in relatively high concentration, contained in a solution of fibrinogen of low concentration, and possessing oscillatory and translatory movement, (1) become, under the action of thrombin, semi-solid, viscid and sticky, and (2) form, as the result of collisions, adhesions to solid surfaces, with the result that they (3) become drawn out into primitive fibrils.

Increase in thickness of the primitive fibrils takes place by addition of fibrinogen, derived from the solution of fibrinogen of low concentration.

5. When fibrinogen in the concentrated phase has become partially changed into fibrin, the concentration of fibrinogen in this phase is lowered, disturbance of equilibrium of the two phases resulting, and fibrinogen passing from the dilute into the concentrated phase. In this way may be explained the "growth" of fibrin fibrils. This growth is observed to occur at a monomolecular rate.

6. If, as source of thrombin, venom in sufficient concentration is used, the fibrin formed, whether consisting of fibrils or of particles of fibrinogen in the concentrated phase which have been directly transformed into fibrin, later undergoes lysis.

7. The action of thrombin upon fibrinogen affords an explanation of the mechanism of production of the reticular structure observed in gels and cell plasm.

#### REFERENCES.

- Barratt (1915). *Proc. Roy. Soc. B.* **87**, 179.  
 Cesana (1908). *Arch. Fisiol.* **5**, 180.  
 Hardy (1899). *J. Physiol.* **24**, 158.  
 Houssay and Negrete (1918). *Revista del Instituto Bacteriologico del Departamento Nacional de Higiene*, Buenos Aires, **1**, 341.  
 Howell (1910). *Amer. J. Physiol.* **26**, 253.  
 — (1914). *Amer. J. Physiol.* **35**, 143.  
 — (1916). *Amer. J. Physiol.* **40**, 526.  
 Mayer (1907). *Compt. rend. Soc. Biol.* **63**, 658.  
 Stübel (1914). *Pflüger's Arch.* **156**, 361.  
 Wilson (1904). *The Cell* (London, Macmillan).  
 Zsigmondy (1905). *Zur Erkenntnis der Kolloide* (Jena).

## XXIII. EFFECT OF HEAT ON THE ANTI-SCORBUTIC ACCESSORY FACTOR OF VEGETABLE AND FRUIT JUICES.

BY ELLEN MARION DELF.

*From the Department of Experimental Pathology, Lister Institute.*

*(Received March 2nd, 1920.)*

A KNOWLEDGE of the properties of the accessory food factors is of especial importance in connection with the cooking of food and with its successful preservation. In the case of the best known accessory factors—the water-soluble, the fat-soluble and the anti-scorbutic vitamins—the effect of exposure to heat for shorter or longer periods is not similar, but comparatively little quantitative work has been done on these lines.

The water-soluble factor is now commonly identified with the substance which has been shown to cure polyneuritis of pigeons. Chick and Hume [1917] were unable to detect that the anti-neuritic factor present in wheat germ and in yeast extract was diminished after exposure to a temperature of 100° for two hours, but a temperature of 113° prolonged for 40 minutes destroyed about half the activity of these substances, the rate of destruction being increased at higher temperatures. More recently it has been asserted by McCollum and Simmonds [1918] that the water-soluble vitamin extracted from "navy beans" is destroyed if heated for an hour at 100° in an alkaline medium (0.28 % NaOH).

The fat-soluble vitamin is more sensitive to heat but the evidence on this point is not yet conclusive. According to Steenbock, Boutwell and Kent [1918], butter fat loses nearly all its growth-promoting power after being heated for 4 hours at 100°, and Drummond detected some loss in value after 1 hour's heating at 100° [1919]; moreover the hydrogenation of whale oil at high temperatures completely destroys the fat-soluble content of the original oil [Halliburton, 1919]. On the other hand, the results of numerous observations by workers at the Lister Institute upon milk heated in an autoclave at 15 lb. pressure (*i.e.* at about 120° in absence of air) show that after heating for an hour it still retains the greater part of its growth-promoting efficiency.

More is known as to the effect of heat on the anti-scorbutic accessory factor as it occurs in green cabbage leaves [Delf and Tozer, 1918], and in germinating pulses [Delf and Chick, 1919]. In the former case it was shown that fresh green cabbage leaves lose about four-fifths of their anti-scorbutic value when heated in steam for only 20 minutes to a temperature of 100°.

In the experiments to be recorded in this communication, freshly expressed juices of cabbage, swede and orange were contrasted as to their anti-scorbutic values in the raw state and after heating. In all these cases, it was necessary first to find the minimal daily ration of the fresh raw juice which would just afford protection from scurvy when fed to animals on an otherwise scorbutic diet. The technique was that which has been described at length elsewhere [Chick and Hume, 1917; Delf and Tozer, 1918]. Young guinea-pigs were used as the experimental animals and their basal diet was in all cases oats and bran *ad libitum*, and milk previously autoclaved at a pressure of 15 lb., *i.e.* about 120°. The milk ration was given more liberally than hitherto,—to the extent of 60–90 cc. daily, according to the age and appetite of the animal. This increase of the milk ration was justified by control experiments as yet unpublished. The onset of scurvy is not appreciably affected by the increased allowance and healthier animals more resistant to intercurrent disease can be produced, especially in the case of limiting doses which are often difficult to determine on account of secondary infections, as in the case of the orange juice animals which are quoted in Table V.

In Tables I–IV, the data listed in the columns headed “Histology of rib-junctions” were kindly supplied by Miss F. M. Tozer and the diagnoses made as explained by her in a previous communication [Delf and Tozer, 1918]. In our experience, the histology of the rib-junctions may show a subnormal condition (see No. 933, Table IV) or even definite disorganisation of the tissues (as No. 932, Table IV), when the typical clinical symptoms of scurvy in life have been entirely absent. The histological diagnosis then probably indicates a critical condition (which we have called “incipient”), which, if the experiment were sufficiently prolonged, might develop into the typical disease. Similar pre-scorbutic symptoms have been described by McCarrison in connection with the adrenals and the muscular walls of the alimentary tract, both of which suffer marked changes of structure or function when guinea-pigs are kept for short periods on an entirely scorbutic diet, before the usual symptoms of scurvy can be observed [McCarrison, 1919]. He further regards these pre-scorbutic changes as particularly characteristic of a dietary of low vitamine content, and this is in accord with our impression.

Experiments about to be published by Miss F. M. Tozer prove, as we had formerly expected [Delf and Tozer, 1918], that when the anti-scorbutic element is provided in excess, a diet deficient only in the fat-soluble constituent results in a number of changes in the structure of the rib-junctions of guinea-pigs very similar to those observed in the less severe cases of scurvy. In the experiments described below, the amount of milk consumed by the animals satisfies us that the appearances described are primarily due to the lack of sufficient anti-scorbutic in the diet, even when the animal survived the experimental period in apparent health.

Table I. *Experiments with raw green filtered Cabbage Juice.*

No. of animal	Ration of juice cc.	Average amount food consumed daily			Body weight		Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
		Oats & bran g.	Milk cc.	Initial g.	Final g.						
998	10	19	66	325	638	None	86	Normal	"Incipient" scurvy	Protection	
999	10	34	77	345	570	"	90	"	"	"	
999 C	5	28	76	325	660	"	90	"	Nearly normal	"	
999 D	5	32	72	315	580	"	90	"	"	"	
999 E	5	47	75	340	617	Tender joints	90	"	"	"	
999 F	2.5	45	74	340	605	None	86	"	"	"	
999 G	2.5	33	68	335	375*	"	74	Haemorrhage on stomach and caecum	Definite scurvy	Died of visceral haemorrhage. (?) Scurvy	
999 H	2.5	48	67	320	620	"	87	Normal	Nearly normal	Protection	
1105	1.5	57	89	335	745	"	90	"	"	"	
1106	1.5	51	88	335	695	"	90	"	"	"	
1118	0.75	30	63	352	405	Knees swollen, tender; very lame	42	Haemorrhages: fractured knees, Enlarged rib-junctions	Not examined	Typical severe scurvy	
1119	0.75	28	72	334	445	"	42	"	"	"	
1130	1.0	40	79	345	510	One knee swollen: abscess	83	Haemorrhage severe in one knee. Tibia fragile	Incipient—Definite	Mild scurvy	
1131	1.0	16	61	335	355	Knees swollen, lame	81	Haemorrhage slight. Bones brittle, ill—pleural adhesion	"	Scurvy	
1132	1.0	24	83	345	675	One knee swollen, teeth loose	81	Teeth loose, otherwise normal	Nearly normal	Protection	

\* Maximum body weight, 510.

## EFFECT OF HEAT ON THE ANTI-SCORBUTIC PROPERTIES OF THE JUICE OF GREEN CABBAGE.

In the case of the cabbage juice, at first only the green outer leaves of fresh cabbages were used. The stouter part of the midrib of each leaf was removed and the remaining part passed through an ordinary mincing machine. The pulp was then wrapped in two thicknesses of stout muslin (mosquito netting) and submitted to a hand-press. The thick green juice was afterwards passed through filter paper, when a clear brownish-green liquid was obtained. Later, by a similar process, the juice was expressed also from the white interior leaves of the cabbages and the freshly prepared green or white juices were fed to different experimental animals.

In the case of the green raw juice, daily rations of 10 cc., 5 cc., 2.5 cc. and 1.5 cc. were all found to give perfect protection from symptoms of scurvy during the experimental period (60–90 days, Table I). It was remarkable that the strongest and best grown animals were on the smallest of these doses (1.5 cc., Fig. 1 *E*), but these two had a large appetite for autoclaved milk (Table I). When this dose was halved and fed to another set of animals, typical and severe scurvy developed after a brief period of normal growth (35–45 days, Fig. 1 *F*). Three animals were subsequently given a ration of 1 cc. fresh juice. Two of these showed slight signs of scurvy at 80 days; the third was normal, except for loose teeth. The minimum raw protective dose is therefore just above 1 cc. daily. Parallel experiments with juice from the white leaves lead to the conclusion that the anti-scorbutic value of the two kinds of juices is not very different, 1.5 cc. affording apparent protection from scurvy.

The juice prepared as described above was placed in small flasks stoppered with cotton-wool plugs and heated in steam at 100° for an hour, or for twenty minutes in the different experiments. A soft whitish coagulum was produced by the heating and this was included in the doses fed to the animals.

Successive experiments with the juice heated for one hour showed that on a daily dose of 5 cc. or less all the animals developed severe scurvy (Table II *a*). Of two animals on a 7.5 cc. dose one remained in perfect health for 90 days when the experiment was terminated, and one developed severe scurvy after 20 days. The latter animal survived in fair condition for 90 days, but was badly crippled in the hind-quarters which at the post mortem showed typical scurvy lesions. Two others on a 10 cc. ration were protected. Evidently a ration of 7.5 cc. is near the limit required for complete protection and perhaps may be regarded as the equivalent of 1 cc. of the raw juice. This indicates a loss of about 80 % of the original value during the hour of heating.

Experiments have also been made in which animals were fed on the juice heated for 20 minutes. Two animals on a 5 cc. dose early developed symptoms of scurvy—No. 1127 on the 26th and No. 1128 on the 28th day. Three other

Table II. *Experiments with green filtered Cabbage Juice heated to 100°.*

No. of animal	Ration of juice cc.	Average amount food consumed daily		Body weight		Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
		Oats & bran g.	Milk cc.	Initial g.	Final g.					
1107	3.0	29	64	325	400	Both knees swollen, sore; lame	29	Haemorrhages extensive: fractured knees; enlarged rib-junctions	Seury	Severe seury
1108	3.0	32	82	330	540	" "	32	" "	Severe seury	" "
1100	5	19	69	336	463	" "	90	Haemorrhages extensive: loose teeth	Definite "	Seury; some protection
1101	5	40	79	325	525	Left knee swollen, sore, lame	91	" "	Incipient "	" "
1102	5	31	76	330	527	Both knees swollen, sore, lame	89	Haemorrhages extensive: loose teeth, rib-junctions enlarged	Incipient—definite seury	" "
1122	5	19	55	335	366	" "	35	Haemorrhages severe, knees fractured	Severe seury	Severe seury
1120	7.5	25	83	335	550	No symptoms	90	Normal. 3 rib junctions enlarged	Definite seury	Seury
1121	7.5	20	79	340	380	Knees swollen: crippled	65	Severe haemorrhages: fractured knees, enlarged rib-junctions	Definite—severe	Severe seury
1139	10	20	83	350	615	Slight soreness, improved	60	Still alive	—	Alive, doing well
1140	10	27	83	320	540	Lame, probable injury	60	Abscess in lung	Not examined	Protected
1127	5	18	75	335	340	Right knee swollen, scury position	62	Severe haemorrhages: fractured knees, enlarged rib-junctions	Not examined	Severe seury
1128	5	20	77	328	465	Right knee swollen, lame	89	Severe haemorrhages, bones fragile	Incipient—Definite	Seury
1129	7.5	16	83	335	475	Both knees swollen	83	Haemorrhages slight—rib junctions enlarged	Severe seury	" "
1137	7.5	15	58	342	385	One knee swollen	54	Haemorrhages, fragile tibia, teeth loose	Incipient	" "
1138	7.5	25	69	340	480	" "	46	" "	" "	" "

(b) Heated for 20 minutes.

animals received a ration of 7.5 cc. daily; one of these became scorbutic after 28 days; the other two were killed at 54 and 46 days respectively and both showed distinct signs of scurvy.

These results fall into line with those previously obtained for the green leaves [Delf and Tozer, 1918], in indicating a considerable destruction of the anti-scorbutic value after heating, and the destruction is, if anything, greater in the expressed juice than in the leaf tissue. The experiments with the juice heated for only 20 minutes show how great an amount of destruction of anti-scorbutic substance occurs at the beginning of heating, the loss after an hour being indistinguishable from that after twenty minutes' heating.

#### THE EFFECT OF HEAT ON THE ANTI-SCORBUTIC PROPERTIES OF THE JUICE OF THE TURNIP-SWEDE (*BRASSICA CAMPESTRIS* var. *NAPO-BRASSICA*).

An account has already been published of results of experiments made by Chick and Rhodes [1918], working in this laboratory, which establish that 2.5 cc. may be regarded as the minimum daily protective ration of raw swede juice. In the winter of 1918-1919, these results were confirmed and extended to the heated juices as summarised in Tables III and IV. I am indebted to Miss D. Gardiner of Girton College for much assistance in the preparation of the juices and in the care of the experimental animals.

The juice was prepared daily by the method (previously adopted by Chick and Rhodes) of rubbing a fresh surface of the root on an ordinary kitchen grater, and squeezing the pulp thus obtained by hand through coarse muslin. Out of four animals given 2.5 cc. daily as the sole anti-scorbutic in their diet, two remained in perfect health throughout the experiment, another was slightly scorbutic but was otherwise in fairly good condition (No. 953, Table III), and the fourth died after 36 days of some intercurrent disease, with accompanying symptoms of mild scurvy. On smaller doses severe scurvy always appeared and on a larger dose protection was achieved in every case.

The fresh swede juice was heated for an hour either in test-tubes plugged with cotton-wool and nearly submerged in a water-bath kept at 80°, or in small flasks also plugged with cotton-wool but heated in a steam chamber at 100°, or in an autoclave at about 10 lb. pressure, *i.e.* about 110°, or at 25 lb. pressure, *i.e.* about 130°. Perfect protection from scurvy was given by a 5 cc. dose of the juice previously heated at either 80° or 100° or even 110°. This dose failed to protect from scurvy if previously heated for an hour at 130° (Table IV), but when the dose was increased to 10 cc., protection was achieved in the case of two experimental animals (Table IV).

Swede juice is thus much more stable at high temperatures than is cabbage juice, double the amount of the raw minimal dose giving protection after heating for an hour at 100° or 110°, whereas with raw cabbage juice about seven times the raw minimal dose is necessary to give this protection after heating for an hour at 100°.

Table III. *Experiments with raw Swede Juice.*

No. of animal	Ration of swede juice	Average amount food consumed daily			Body weight		Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
		Oats & bran g.	Milk cc.	Final g.	Initial g.						
938	5	21	69	325	543	None	88	Teeth loose, brittle	Normal	Protection	
939	5	23	53	330	387	"	51	Tibia somewhat brittle	Nearly normal	"	
968	5	21	74	350	487	"	91	Teeth brittle: rib-junctions somewhat enlarged	"	"	
971	5	24	60	335	512	"	91	Rib-junctions slightly enlarged	"	"	
972	5	52	75	345	617	"	91	Teeth rather brittle	"	"	
952	2.5	18	55	330	325	Joints swollen; infection	36	Muscular and subcutaneous haemorrhages—slight	Incipient scurvy	Infection, mild scurvy	
953	2.5	17	59	330	375	Limbs tender when examined	90	Slight subcutaneous haemorrhages	Scurvy	Protection incomplete, mild scurvy	
970	2.5	19	75	325	530	"	90	Normal	Normal	Protection	
973	2.5	30	75	340	550	None	91	"	Nearly normal	"	
955	1.5	31	70	320	505	"	90	Brittle teeth	"	"	
956	1.5	16	58	320	350	"	45	Haemorrhage on gut	"	"	
962	1.5	12	70	320	388	Scurvy position, knees swollen	90	Subcutaneous haemorrhages; fractured knees, etc.	Scurvy	Scurvy	
978	1.5	15	56	320	340	Limbs very tender on handling	71	Muscular haemorrhages; rib-junctions enlarged	"	"	
979	1.5	21	54	325	395	Lame, body very thin	71	Muscular haemorrhages; rib-junctions enlarged; knees fractured	"	"	
980	0.75	15	58	320	315	Lame; swollen knees	42	Severe haemorrhage, etc.	"	Severe scurvy	
981	0.75	16	59	320	320	"	41	"	"	"	
982	0.75	14	51	325	290	"	34	"	"	"	

## EFFECT OF HEAT ON THE ANTI-SCORBUTIC VALUE OF FRESH ORANGE JUICE.

I am indebted to Miss E. M. Hume and Miss A. J. Davey for permission to publish the data quoted in Table V, from which may be seen the results of their experiments with raw orange juice. From the experiments of Miss Hume carried out in 1917, it will be seen that 3 cc. of the juice affords ample protection from scurvy in young guinea-pigs and that fairly good growth was made when autoclaved milk was included in the dietary. The results of a dose of 1.5 cc. were variable, only five out of eleven animals receiving adequate protection from scurvy (Table V, Nos. 720, 721, 723, 744, and 1000). Of these only No. 1000, which had the largest appetite for milk, made a practically normal growth curve (Fig. 4 B). Two of the animals (Nos. 743 and 1001) were presumably in a "pre-scorbutic" condition, showing histological deformities without any clinical symptoms of scurvy. On the whole, we may conclude from these experiments that 1.5 cc. is the minimal protective ration of the freshly squeezed juice from oranges in good condition when fed to animals which can be kept free from other forms of disease.

Freshly squeezed orange juice was heated for an hour at three different temperatures. At 70° in a water-bath as for the swede juice; at higher temperatures in a steam chamber or autoclave as already described for the other juices.

One case of protection was observed on a 1.5 cc. dose, heated at 70°, but this was probably an exceptional animal since a larger ration given later in the year failed to give complete protection (3 cc., Table VI). Animals were kept successfully in good health throughout the experimental period both on 3 cc. rations and on 1.5 cc. rations heated at 100°; but in the former case two out of four animals were found to exhibit post mortem and histological signs of scurvy (perhaps attributable to the difficulty of getting good oranges at that time), whilst in the latter case two out of three animals showed similar signs of a scorbutic or rather pre-scorbutic condition (weight charts, Fig. 4 C, D). Two animals on a 3 cc. dose heated at 130° were protected from scurvy and a third nearly protected, but three other animals on a 2 cc. ration of this juice developed severe scurvy. Orange juice is therefore still more stable than swede juice, less than double the raw minimal dose affording protection after an hour of heating at 130°.

This stability of the orange juice to heat suggested the possibility that the acidity present in the juice might have a stabilising effect on the anti-scorbutic substance, and led to experiments carried out mainly by Miss B. F. Runge in which the juice was nearly neutralised with 10 % sodium carbonate prior to heating. The alkaline solution was added drop by drop until the liquid was just acid to a standard solution of dibromo-*o*-cresolsulphonaphthalein ( $P_H$  5.2-6.8). The residual acidity of the juice was then about the same as the slight natural acidity of the swede and cabbage juices used in the other experiments.

Table IV. *Experiments with Swede Juice heated for 1 hour.*

No. of animal	Ration of juice heated	Temp. of heating, °C.	Average amount food consumed daily			Body weight		Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
			Oats & bran, cc.	Milk, cc.	Initial, g.	Final, g.						
931	5	80	22	67	337	410	No symptoms	84	Haemorrhage on small intestine	Incipient scurvy	Protection, possible infection	
932	5	80	21	71	355	465	"	82	Normal	Scurvy	"	
940	5	80	13	66	322	390	Lung trouble; scurvy also	92	Subcutaneous and muscular haemorrhages; bones fragile	"	Partial protection	
933	5	100	18	69	320	480	No symptoms	90	Teeth brittle	Incipient scurvy	Protection	
934	5	100	20	72	340	517	"	92	"	"	"	
941 A	5	100	26	68	330	412	"	90	One rib-junction much enlarged	"	"	
943	5	110	10	60	330	370	Limbs tender after 38th day	85	Lungs inflamed; intestinal ulcers	Nearly normal	"	
944	5	110	12	75	335	485	"	91	Teeth brittle	Incipient scurvy	"	
945	5	110	16	68	322	485	"	92	Rib-junctions ridged	"	"	
936	10	130	18	67	350	498	"	88	Slight muscular haemorrhages; intestinal ulcers	"	"	
937	10	130	30	74	310	540	"	90	Teeth brittle	Normal	"	
974	5	130	13	53	341	320	Scurvy position, lame after 21st day	33	Muscular haemorrhages, fragile bones, etc.	Not examined	Typical acute scurvy	
975	5	130	15	58	340	335	"	34	"	"	"	
976	5	130	17	58	320	287	"	31	"	"	"	

Three animals given 3 cc. of this juice heated for an hour at 100° and three others given only 1.5 cc. daily were successfully protected from scurvy during the experimental period, the condition of those on the larger ration being rather better than of those on the smaller. Neutralising nearly all the excess of acid in orange juice therefore does not appreciably reduce the stability of the anti-scorbutic constituent of the juice at a temperature of 100°.

Experiments were also made with the juice of oranges which had been canned and stored at laboratory temperatures for five months. The canning process involved heating in closed cans for 20–30 minutes, the temperature gradually rising from about 80° to 100° and remaining at 100° for not more than five minutes. A certain amount of water was added to the fruit after packing the cans and this was included in the juice afterwards expressed by hand from the fruit when the cans were opened. Allowing for the added water, a dose equivalent as nearly as possible to 1.5 cc. of the fresh juice was given daily to three experimental animals and afforded them adequate protection from scurvy for 88 days, when the experiment was terminated. Canned oranges produce a bitter juice and are probably of no commercial value, but this result with fruit in which the value of the raw juice is well known indicates the possibilities of investigations with other fruit juices.

#### DISCUSSION OF RESULTS.

Experiments have been described in which the juice of fresh cabbage, swede and orange has been fed to animals as the sole anti-scorbutic element in a basal diet of oats and bran *ad libitum*, and 60–90 cc. autoclaved milk daily. From these experiments it can be affirmed that under these conditions the minimal daily dose of the raw juice for the adequate protection from scurvy of young guinea-pigs is about 1.0 cc. cabbage, 2.5 cc. swede, and 1.5 cc. orange juice respectively.

When these juices are subjected to temperatures up to 130° a corresponding reduction of their anti-scorbutic properties appears. This reduction is greatest in the case of cabbage juice and is least in the case of orange juice. After being heated at 100° for an hour, at least 7½ times the raw dose in the case of cabbage, or twice the raw dose in the case of swede, is necessary to give protection from scurvy, whereas orange juice does not appear to have deteriorated appreciably during this period of heating. Only at 130° was any definite loss of value detected in the orange juice; 2 cc. was then an insufficient dose for protection, but 3 cc. gave the necessary protection after heating at this temperature.

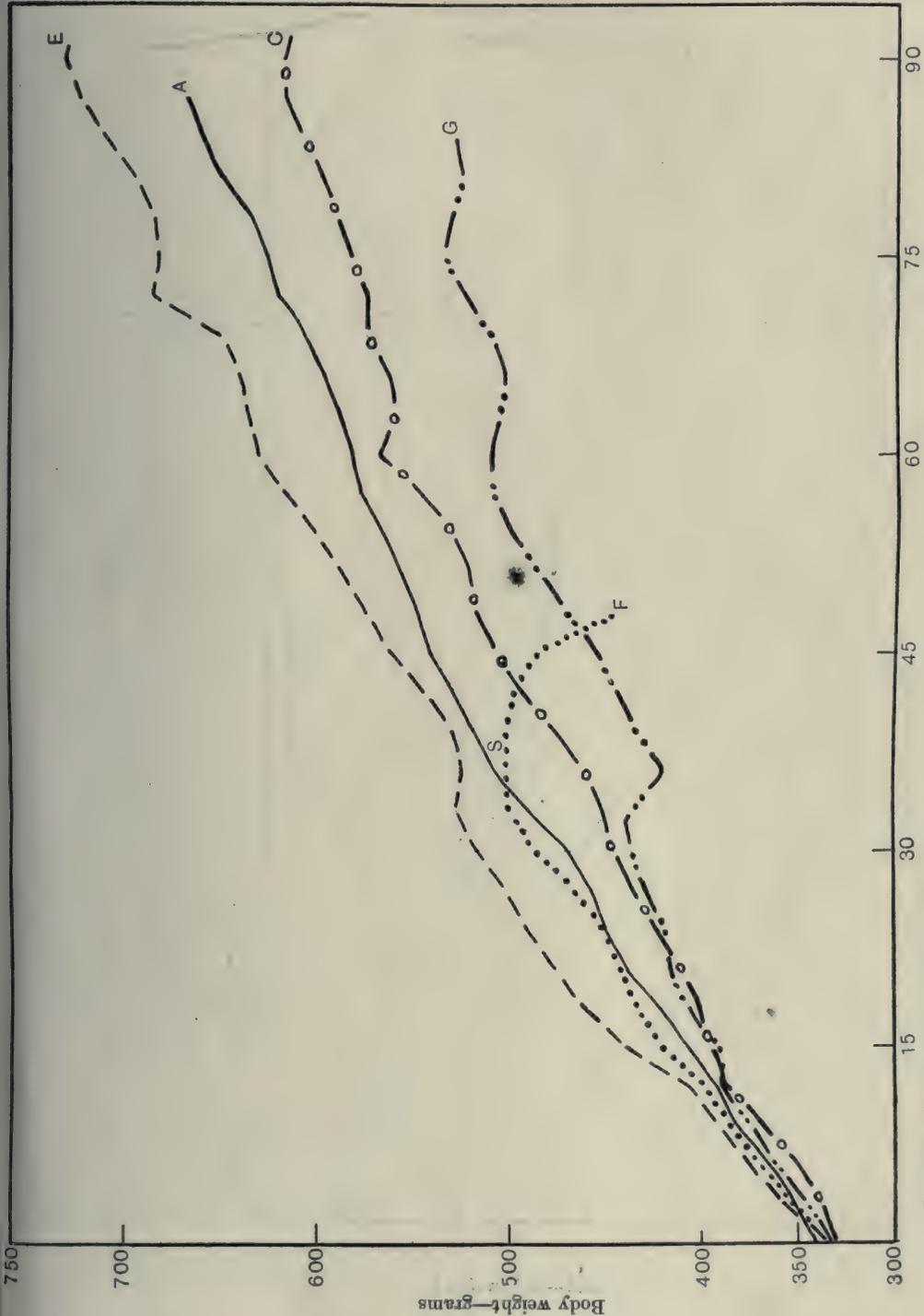
In considering these results, it is interesting to find that orange juice, which is the most stable of the juices tested, is also the most acid in reaction. As long ago as 1912, Holst noticed that fruit juices were more heat-stable than vegetable juices, and he suggested that the stability was due to the presence of acid [Holst and Frölich, 1912]. That the hydrogen ion concentration may have an important influence on the stability of this vitamine is

Table V. Experiments with raw Orange Juice: basal diet oats, bran and autoclaved milk.

No. of animal	Date of starting experiment	Ration of orange juice cc.	Average amount food consumed daily			Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
			Oats & bran lb.	Milk cc.	Body weight g.					
79	21.i.17	5	35	60	290	420	106	Normal	Incipient scurvy	Protection
111	25.i.17	5	30	74	315	520	89	Rib-junctions slightly ridged	Normal	"
239	1.v.17	3	52	55	365	598	93	Normal	"	"
240	3.v.17	3	65	64	300	365	74	Practically normal	Nearly normal	"
1013	13.i.19	3	52	76	336	605	91	Normal	Normal	"
1014	25.i.19	3	41	60	342	514	91	"	"	"
720	9.iii.18	1.5	63	57	342(490)	378	62	Intestine probably infected; spots on liver	Nearly normal	"
721	11.iii.18	1.5	85	60	335	510	90	Normal	Incipient scurvy	"
722	13.iii.18	1.5	66	54	360	372	46	Haemorrhages in stomach and caecum; two rib-junctions enlarged	Severe scurvy	Scurvy
723	13.iii.18	1.5	39	51	336(473)	245	68	Tibia firm, femur brittle; digestive tract unhealthy	Definite scurvy	Protection
742	11.v.18	1.5	25	53	340	260	50	Fragile bones; intramuscular haemorrhages on knees	Severe scurvy	Scurvy
743	17.v.18	1.5	43	59	341	318	83	Bones slightly fragile	Definite scurvy	"
744	6.vi.18	1.5	49	58	337(472)	368	62	Teeth brittle	Incipient scurvy	Protection—died of unknown illness
1000	6.i.19	1.5	56	78	337	651	90	Normal	Nearly normal	Protection
1001	9.i.19	1.5	43	58	334	287	73	Tibiae fragile; rib-junctions ridged, one heavily	Severe scurvy	Partial protection
1002	11.i.19	1.5	36	62	339	340	65	Liquid in pleural cavity; haemorrhages on caecum	Definite scurvy	Partial protection; pneumonia
1003	11.i.19	1.5	24	53	338	263	88	Dark inflammatory patches on lungs	"	Partial protection; lung trouble
745	14.vi.18	0.5	37	58	340(474)	400	59	Haemorrhages in muscles of knees; rib-junctions slightly enlarged	Incipient scurvy	Slight scurvy
1025	3.iii.19	0.5	22	64	339	267	75	Swollen knees on 22nd day, scurvy position	Severe scurvy	Severe scurvy
1026	4.iii.19	0.5	22	63	331	360	91	Swollen knees after 38 days, scurvy position	Definite scurvy	Scurvy
1027	6.iii.19	0.5	30	56	331	309	58	Knees swollen after 34 days	"	"
1028	8.iii.19	0.5	24	59	330	464	89	Knees swollen after 43 days	Haemorrhages; bones slightly fragile	"
1029	11.iii.19	0.5	40	59	340	311	43	Knees swollen after 29 days	Bones slightly fragile; rib-junctions much swollen	Severe scurvy
1030	17.iii.19	0.5	18	59	343	230	66	Knees swollen after 20 days	Haemorrhages; bones fragile; rib-junctions swollen	"

Table VI. Experiments with heated Orange Juice: basal diet oats, bran and autoclaved milk.

No. of animal	Date of starting of experiment	Ratio of orange juice cc.	Temp. of heating °C.	Average amount food consumed daily			Body weight		Symptoms during life	Duration of experiment, days	Histology of rib-junctions	General result
				Oats & bran g.	Milk cc.	Final g.	Initial g.					
A. Heated orange juice.												
871	10. vi. 18	3	70	37	59	330	550	Joints slightly tender	90	Slight muscular haemorrhage; fragile bones	Severe scurvy	Scurvy, but good health
887	23. iv. 18	1.5	70	37	57	331	652	" "	94	Slight haemorrhage on intestine	Nearly normal	Protection
858	23. iv. 18	1.5	70	29	47	326	318	" "	26	" "	" "	Protection; death from unknown cause
905	7. ix. 18	3	100	36	60	350	593	" "	90	Teeth loose; tibiae brittle	Incipient scurvy	Protection
906	7. ix. 18	3	100	18	60	345	500	" "	90	Rib-junctions swollen; slight muscular haemorrhage	Definite scurvy	Partial protection; slight scurvy
949	1. xii. 18	3	100	22	68	320	630	No symptoms	90	Normal	Normal	Protection
950	2. i. 19	3	100	24	70	325	598	" "	89	" "	" "	" "
986	25. iv. 19	1.5	100	22	75	320	610	" "	92	One rib-junction enlarged; the rest normal	Enlarged junction fractured; the rest normal	Protection; trace scurvy (?)
987	21. iv. 19	1.5	100	14	63	328	485	" "	94	Slight muscular haemorrhage	" "	" "
988	25. iv. 19	1.5	100	22	74	320	670	" "	89	Rib-junctions slightly enlarged	Nearly normal	" "
951	4. i. 19	3	130	10	60	325	335	Knees tender, a little lame	72	Emaciated; lungs inflamed	Definite scurvy	Protection incomplete
959	13. i. 19	3	130	17	63	320	525	Knees tender	90	Rib-junctions slightly ridged	Normal	Protection
960	19. i. 19	3	130	15	66	340	402	" "	90	" "	Incipient scurvy	" "
983	8. iv. 19	2	130	23	60	325	350	Knees much swollen; very painful	40	Severe haemorrhages; fragile bones	Severe scurvy	" "
984	8. iv. 19	2	130	16	69	330	430	" "	78	" "	" "	" "
985A	9. iv. 19	2	130	15	72	320	450	" "	85	" "	" "	" "
B. Heated nearly neutralised orange juice.												
989	17. v. 19	3	100	17	74	325	535	No symptoms	92	No symptoms	Incipient scurvy	Protection
990	17. v. 19	3	100	23	74	325	670	" "	92	Fatty and subcutaneous tissue injected	Normal	" "
991	21. v. 19	3	100	27	76	322	600	" "	90	Normal	Incipient scurvy	" "
992	27. v. 19	1.5	100	21	72	323	515	" "	89	Jaw and lower teeth somewhat brittle	" "	" "
993	27. v. 19	1.5	100	20	72	325	585	" "	90	Rib-junctions all slightly enlarged	" "	" "
994	27. v. 19	1.5	100	20	74	330	510	" "	89	Normal	" "	" "



Duration of Experiment—Days

Fig. 1. Weight charts showing growth of animals fed on oats, bran, autoclaved milk and raw or heated green cabbage juice.

A = Mean weight chart of four animals: showing standard growth on normal diet of oats, bran and 30 g. fresh cabbage daily and water.  
 C = " " Nos. 999 C, D, E, on 5 cc. raw filtered green cabbage juice.  
 E = " " Nos. 1105, 1106 on 1.5 cc. " "  
 F = " " No. 1119 on 0.75 cc. raw filtered green cabbage juice. "At S symptoms of scurvy first appeared."  
 G = " " No. 1120 on 7.5 cc. green filtered cabbage juice heated for an hour at 100° C.

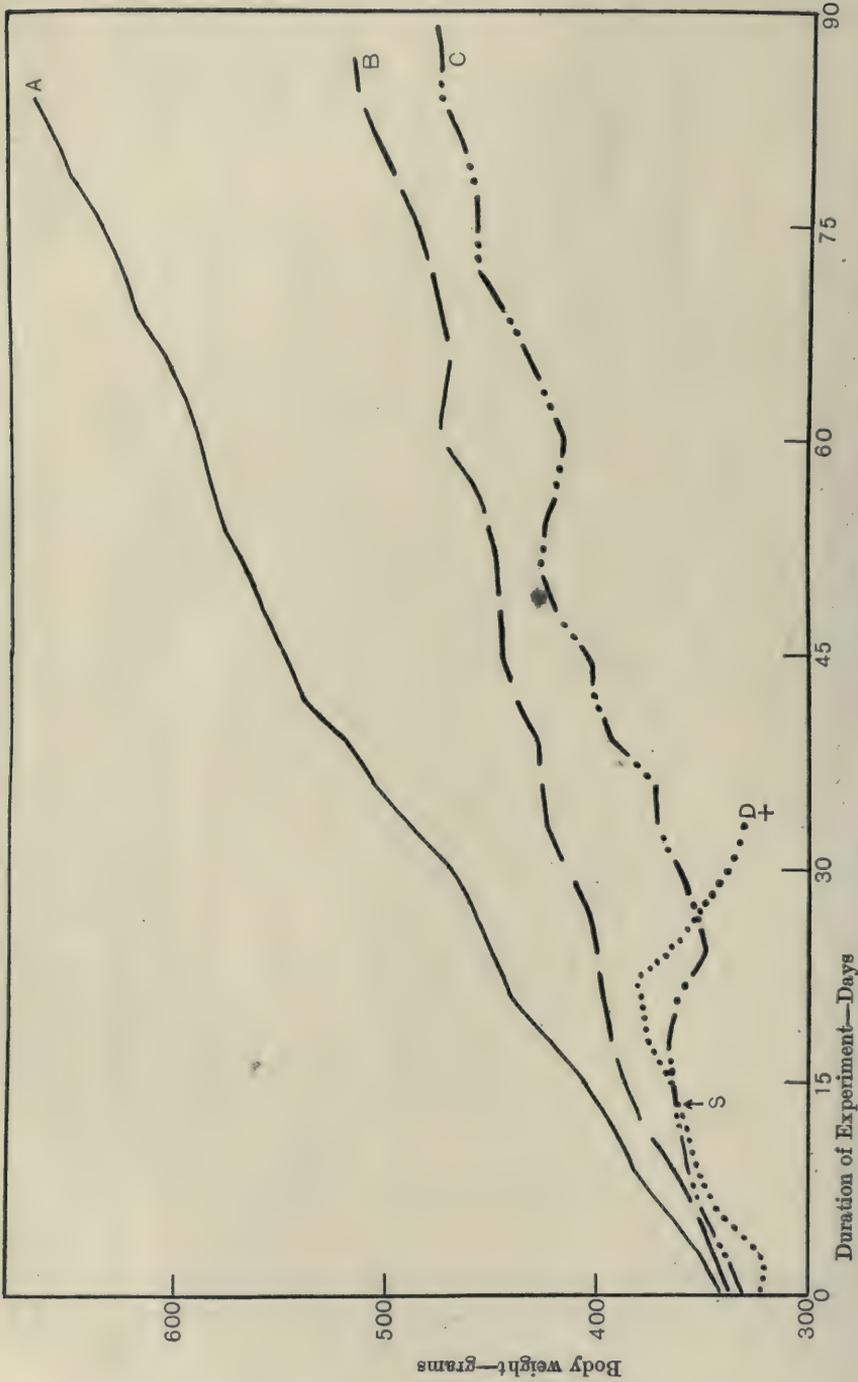


Fig. 2. Weight charts of animals on oats, bran, autoclaved milk and raw swede juice.  
 A = Standard weight chart on diet of oats, bran, water and 30 g. fresh cabbage daily.  
 B = Mean weight chart of Nos. 938, 968, 971, 972, on a ration of 5 cc. raw swede juice.  
 C = " " Nos. 953, 970, 973, " " 2.5 cc. " "  
 D = " " Nos. 980, 981, 982, " " 0.75 cc. " "  
 At S symptoms of scurvy first appeared; at + No. 982 died of scurvy.

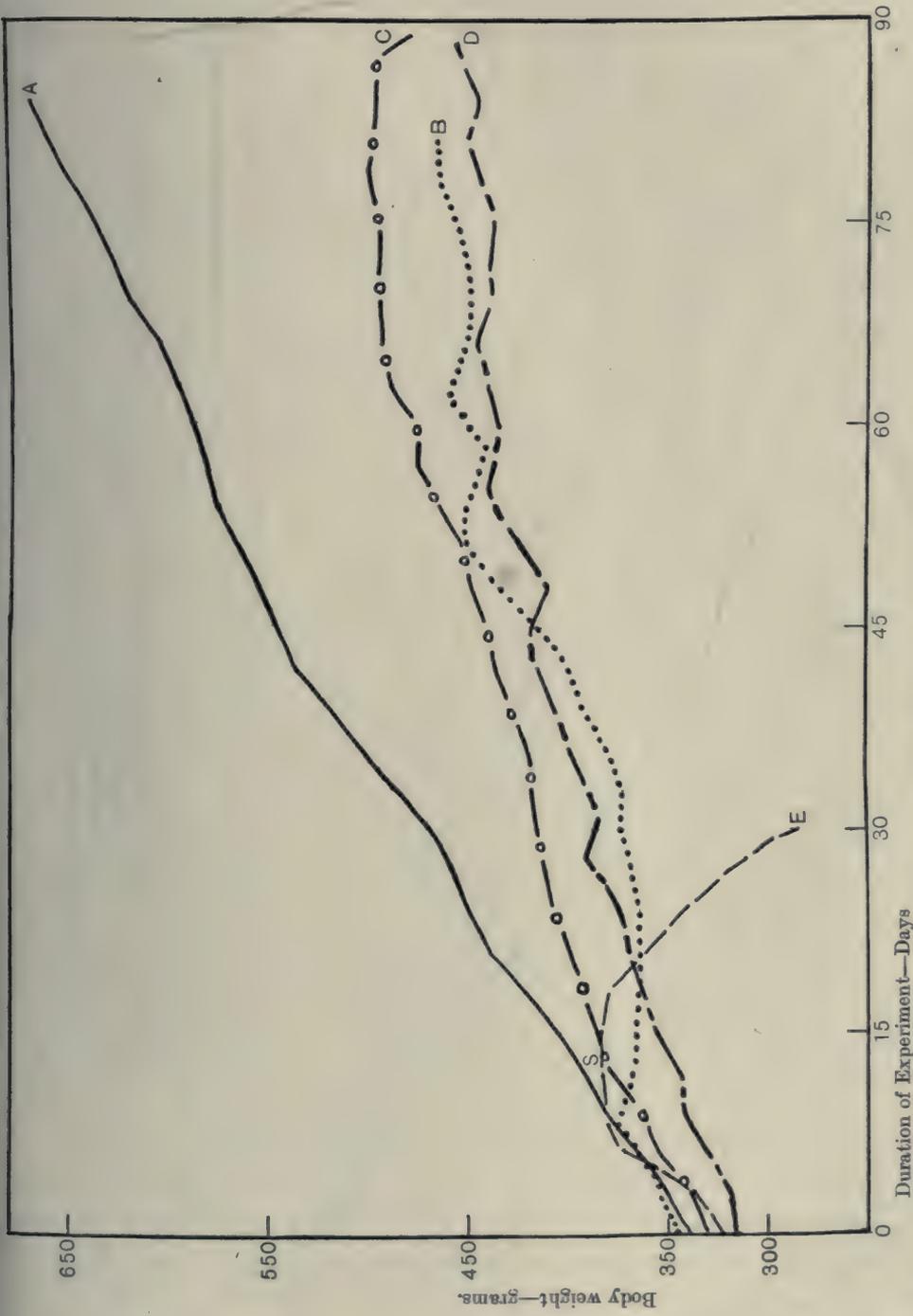


Fig. 3. Weight charts of animals fed on oats, bran, autoclaved milk and heated swede juice.

A = Standard weight chart of normal growth (as for Figs. 1, 2).  
 B = Weight chart of No. 932 on 5 cc. swede juice heated for 1 hour at 80° C.  
 C = Mean weight chart of Nos. 933, 934, 941 A, on 5 cc. swede juice heated for 1 hour at 100° C.  
 D = " " Nos. 937, 961, on 10 cc. swede juice heated for 1 hour at 130° C.  
 E = Weight chart of No. 977, on 5 cc. swede juice heated for 1 hour at 130° C. At S symptoms of scurvy first appeared.



shown by the experiments of Harden and Zilva [1918], who found that orange juice which was made only slightly alkaline lost almost immediately its anti-scorbutic value. In the experiments described above, the raw juices of cabbage and swede have about the same hydrogen ion concentration, but this slight acidity is accompanied by very different degrees of stability in the two cases. The greater stability of the orange juice to heat was maintained even when the juice was nearly neutralised before heating.

A further point of difference between the three juices tested may be found in their effect on the growth of the experimental animals. A glance at Figs. 1-4 (pp. 223-6) will give the general impression that the cabbage juice animals were on the whole the most satisfactory in this respect, and the variability in the consumption of milk does not altogether account for this<sup>1</sup>. It is well known that green vegetables have growth-promoting as well as anti-scorbutic properties and other workers in this Institute have obtained evidence (as yet unpublished) that this growth-promoting power is also shared to some extent by the expressed juice. No appreciable growth is obtained, however, when guinea-pigs are fed on either cabbage, swede or orange juices in doses up to 10 cc. if there is no other source of fat-soluble substance in the diet. In the above experiments therefore, the apparent limitation of growth in the case of certain groups of animals in spite of their considerable allowance of milk suggests that a low ration of anti-scorbutic in a diet may also limit the availability of the fat-soluble substance to the animal, even when protection from scurvy has been secured. In the case of the animals to which swede juice was fed (Table III), if we except No. 939 (on 5 cc.) and No. 952 (on 2.5 cc.), both of which died early in the course of the experiment with some unknown complaint, the amount of milk consumed by the two lots is about the same, but the average rates of growth of the animals are different (Fig. 2 *B*, *C*); whilst in both these cases the growth of none of the animals approached that of the normal growth on a cabbage diet (Fig. 2 *A*). In the case of animals on 3 cc. heated orange juice (Table VI), the consumption of milk was nearly equal in the cases of the two groups of animals, but those on the juice heated to 130° made very little growth although the ration of juice given proved to be just above the scurvy limit (Fig. 4 *C*, *E*). Growth seems therefore to be affected by the limitation of the anti-scorbutic element in the diet apart from the appearance of definite symptoms of scurvy and apart from deficiency in the growth-promoting vitamins.

With regard to the somewhat unexpected stability of swede and orange juice at temperatures above 100°, this is parallel with what was previously found with green cabbage leaves. It must be remembered that the heating at these temperatures was done in a closed autoclave in the absence of air. This may well affect the rate of destruction either directly by retarding oxidation or indirectly by the production of stabilising bodies. The rather

<sup>1</sup> Cp. Nos. 970, 973, Table III with 999 *F*, *H*, Table I, both on 2.5 cc. doses; or Nos. 938, 968, 972, Table III with 999 *C*, *D*, *E*, Table I, both on 5 cc. doses.

surprising stability at 130° in the absence of air suggests that there may be advantage in adopting methods of canning fruit or vegetables at temperatures above boiling-point for as short a time as possible to ensure sterility. Further investigations into the value of canned products would appear to be desirable.

In conclusion I have to thank Dr Harriette Chick for suggestions and advice especially during the earlier stages of the investigation, and Miss F. M. Tozer for permission to quote the results of her histological investigations of the rib-junctions of the animals used in these experiments.

#### REFERENCES.

- Chick and Hume (1917). *J. Soc. Trop. Med. Hyg.* **10**, 141.  
Chick and Rhodes (1918). *Lancet*, ii, 774.  
Delf and Chick (1919). *Biochem. J.* **13**, 201.  
Delf and Tozer (1918). *Biochem. J.* **12**, 416.  
Drummond, J. (1919). *Biochem. J.* **13**, 81.  
Halliburton and Others (1919). *J. Physiol.* **52**, 328.  
Harden and Zilva (1918). *Lancet*, ii, 320.  
Holst and Frölich (1912). *J. Hyg.* **72**, 1.  
McCarrison (1919). *Indian J. Med. Research*, **7**, 188.  
McCollum and Simmonds (1918). *J. Biol. Chem.* **33**, 55.  
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **25**, 517

## XXIV. THE PRODUCTS OF THE "ACETONE: *n*-BUTYL ALCOHOL" FERMENTATION OF CARBOHYDRATE MATERIAL WITH SPECIAL REFERENCE TO SOME OF THE INTERMEDIATE SUBSTANCES PRODUCED.

BY JOSEPH REILLY, WILFRED JOHN HICKINBOTTOM, FRANCIS ROBERT HENLEY, AND AAGE CHRISTIAN THAYSEN.

*From the Royal Naval Cordite Factory, Holton Heath.*

*(Received March 5th, 1920.)*

THE production of butyl alcohol and other higher alcohols from carbohydrate material, directly by fermentation, has been well-established [compare Fitz, 1878, 1882, 1883, 1884; Gruber, 1887; Perdrix, 1891; Grimbert, 1893; Botkin, 1893; Schardinger, 1907; Bredemann, 1909; Buchner and Meisenheimer, 1908].

The presence of iodoform-producing substances among the products of fermentation has been recorded occasionally, but the substance yielding the iodoform has never been identified. The first observation of acetone as the result of fermentation of carbohydrates was made by Schardinger [1905], who obtained acetone together with acetic and formic acids. The isolation of an organism yielding acetone and *n*-butyl alcohol from amylaceous material was due to Fernbach and Strange [1912]. Since this initial discovery other processes have been described which produce acetone and *n*-butyl or ethyl alcohol by the breakdown of carbohydrates under bacterial action [Farbenfabr. vorm F. Bayer & Co.; Weizmann, 1915, 1919; Ricard, 1918; Northrop, 1919, 1 & 2]. While the technique of fermentations yielding acetone and *n*-butyl alcohol has improved so that it is possible to carry them out successfully on a large scale, no investigation on the mechanism of the process appears to have been recorded.

A quantitative examination was therefore undertaken of certain intermediate substances formed. The normal end-products of the fermentation, as estimated during the course of the present work, are shown in the following Table I. The total weight of end-products exceeds the weight of starch fermented, but the total carbon content is nearly equivalent to that of the starch. Hence it may be inferred that the starch undergoes hydrolysis.

Table I.

*From Observations made at the Royal Naval Cordite Factory, Holton Heath.*

1000 lb. maize containing 650 lb. starch. Volume of mash (6.5 % maize) = 1540 gallons.

		lb.	Carbon content
650 lb. starch yield	3410 cu. ft. of CO <sub>2</sub> evolved—measured at 27° and 760 mm.	70 acetone 163 <i>n</i> -butyl alcohol = 390 CO <sub>2</sub> evolved	43.5 105.7 106.3
1 vol. of mash dissolves 0.555 vol. CO <sub>2</sub> at 38°	135 cu. ft. CO <sub>2</sub> in solution at 0° and 760 mm.	= 17 CO <sub>2</sub> in solution	4.6
	2090 cu. ft. H <sub>2</sub> at 27° and 760 mm.	= 11 H <sub>2</sub>	—
Total gas evolved (Composition of gas assumed to be constant throughout the fermentation)	= 5.5 cu. ft. at 27° and 760 mm. of mixed gas per 1 lb. of maize fermented		
	Residual acidity*	= 12.0 containing	5.7
		663.0	265.8

Theoretically 650 lb. of starch is equivalent to 722 lb. of hexose. The carbon content of these amounts to 288.8 lb.

\* The residual acidity is taken as acetic acid 56.5 % by weight, and butyric acid 43.5 % by weight. The non-volatile portion is recorded as butyric acid for the purpose of calculation. (See Table III, p. 232, and pp. 246-7.)

*Calculated as Percentage of Starch Fermented.*

100 g. starch gives 111.1 g. hexose and contains 44.4 g. carbon

	g.	Carbon
100 g. starch gives	10.77 acetone	6.68 g.
" "	25.07 <i>n</i> -butyl alcohol	16.21
" "	62.61 carbon dioxide	17.07
" "	1.60 hydrogen	—
" "	1.80 residual acidity	0.85
	<u>101.85</u>	<u>40.81</u>

In a normal fermentation several remarkable features may be observed. The acidity of the mash increases from a very small initial value until a maximum is reached in from 13-17 hours after inoculation. (The length of time taken to reach this point is influenced by the percentage of inoculant used, temperature of the mash, etc.) When the maximum acidity is reached in 6.5 % maize mash, 3.5-4.5 cc. of *N*/10 alkali are required to neutralise 10 cc. of mash. After this point is reached a very marked acceleration in the rate of production of acetone, *n*-butyl alcohol, carbon dioxide and hydrogen takes place. The acidity gradually falls to a constant value, 1.5-2.5 cc. of *N*/10 alkali being required to neutralise 10 cc. of mash. (See Fig. 1, Curve 2.)

The rate of gas evolution is shown in Curve 1, Fig. 1, and the composition of the gas in Table H, p. 232.

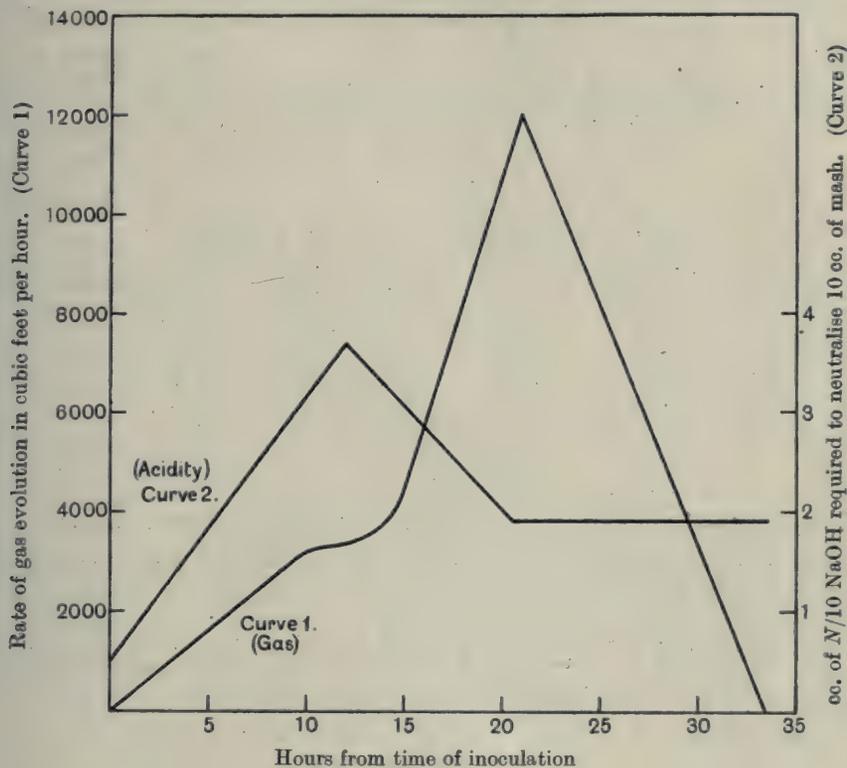


Fig. 1.

#### RATE OF GAS EVOLUTION, AND RISE AND FALL OF ACIDITY THROUGHOUT THE FERMENTATION.

The curves in Fig. 1 show the changes in

(1) the rate of gas evolution, and

(2) the acidity during a fermentation. The figures are the average of 12 fermentations of 6.5 % maize mash (40,000 gallons) with 4.7 % of inoculant.

The acidity is measured in cc. of *N*/10 alkali required to neutralise 10 cc. of mash after boiling the solution to remove carbon dioxide. Phenolphthalein is used as indicator.

It will be noted that the rate of gas evolution rises steadily with the increase of acidity for some time; it then becomes constant (in some cases it even slackens somewhat). As the acidity falls, the rate of gas evolution rises quickly to a maximum, and then falls rapidly until the end of the fermentation. Readings of the gas evolution were taken every hour. The acidity was estimated every three hours. The acidity at the end of the fermentation is generally higher than that of the mash when inoculated.

*Composition of Gas.*

Estimations were made of the composition of the gas evolved during fermentation. The following Table shows the results of one of these experiments.

The fermenting vessel was about two-thirds full of mash, the space above the latter being occupied by air before inoculation.

Table II.

Time	Gas evolved cubic feet per hour	CO <sub>2</sub> %	H <sub>2</sub> %	Air %	
28.vi.'16	—	—	—	—	100 fermentation started
4 p.m.	—	—	—	—	
7 "	253	11.5	38.5	50	
8 "	834	27.1	57.9	15	
9 "	822	40.3	55.2	4.5	
10 "	660	46.0	59.0	3.0	
11 "	760	50.3	47.2	2.5	
29.vi.'16					
9.30 a.m.	1186	62	38	—	

The percentage of carbon dioxide did not vary from 9.30 a.m. 29. vi. '16 to the end of the fermentation, which lasted 36 hours. Total gas evolved 42,694 cubic feet. The high percentage of hydrogen in the gas evolved during the early hours is probably due to the greater solubility of the carbon dioxide.

The production of acetone and *n*-butyl alcohol at various stages in the fermentation is shown in Table III.

Table III.

Time	Acidity	Ratio, acetic to butyric acid	Amount of acetone and <i>n</i> -butyl alcohol mixture in 1 litre of mash
9.0 p.m.	1.0	1 : 0.5	None
11.0 "	1.5	1 : 0.62	None
12.15 a.m.	2.0	1 : 0.9	Trace
5.30 "	3.7	1 : 1.25	1 cc.
3.15 p.m.	2.0	1 : 0.28	4 cc.
4.30 "	1.6	1 : 0.25	5 cc.

Acidity is expressed in cc. of *N*/10 alkali required to neutralise 10 cc. of mash.

There appears to be an intimate connection between the production of acetone and *n*-butyl alcohol, and the fluctuations of acidity and rate of gas evolution.

*Composition of Acids produced during Fermentation.*

The acids present in the mash were examined in order to ascertain their nature and to determine if possible their function in the fermentation. The greater part of these acids was found to be volatile in steam. To study their nature the volatile acids were removed by distillation in a current of steam. The neutralised distillate was concentrated. The acids regenerated from the

concentrated solution were found by Duclaux's method [1895] to be butyric and acetic acids. By fractional crystallisation of the barium salts of the mixed acids, a separation was effected into two acids of different molecular weights. The barium content of each of the two fractions agreed with that for barium acetate and barium butyrate respectively. Neither by the Duclaux method of distillation nor by fractional crystallisation of the barium salts was any indication obtained of the presence of a third volatile acid in more than traces.

*Fermentation in presence of Calcium Carbonate.*

The presence of acetic and butyric acids in the fermenting mash is significant. It lends support to the hypothesis that these acids are intermediate products in the formation of acetone and *n*-butyl alcohol from the carbohydrate of the mash. A fermentation was carried out in the presence of calcium carbonate to determine the effect of neutralising the acids as produced. Under these conditions the production of acetone and *n*-butyl alcohol was almost entirely suppressed, and the calcium salts of acetic and butyric acids were produced usually in the ratio of 1 molecular proportion of acetic acid to 1.8 molecular proportions of butyric acid. Evidence was also obtained of a third acid only very slightly volatile in steam, having a molecular weight equal to or greater than that of butyric acid. The relative proportions of acetic and butyric acids vary under certain conditions and in one experiment a ratio of ten molecules of acetic to nine molecules of butyric acid was obtained.

If it be assumed that two molecules of acetic acid yield one molecule of acetone, and that each molecule of butyric acid yields by reduction one molecule of *n*-butyl alcohol, then the ratio of acetic acid to butyric acid, when the fermentation is conducted in presence of calcium carbonate, should be 1 mol. : 1 mol., provided the balance of the reactions taking place is not upset in these circumstances. The result obtained in practice however is usually 1 molecular proportion of acetic acid to 1.8 molecular proportions of butyric acid.

RELATIVE RATE OF PRODUCTION OF ACETIC AND BUTYRIC ACIDS IN  
THE COURSE OF FERMENTATION.

Further information on the formation of the acids was obtained by determining the relative amounts of acids present at intervals throughout the fermentation. Shortly after inoculation acetic acid is found to be present in considerable amount compared with butyric acid, but as the acidity increases the relative proportion of butyric acid also increases until at the point of maximum acidity the ratio is about 1.1–1.5 molecular proportions of butyric acid to 1 molecular proportion of acetic acid.

From this point onwards the ratio of butyric acid to acetic acid falls progressively, until at the end of the fermentation the acids are in the ratio

of 4-5 molecular proportions of acetic acid to 1 molecular proportion of butyric acid. (See Summary of Results, p. 246, and Fig. 2.)

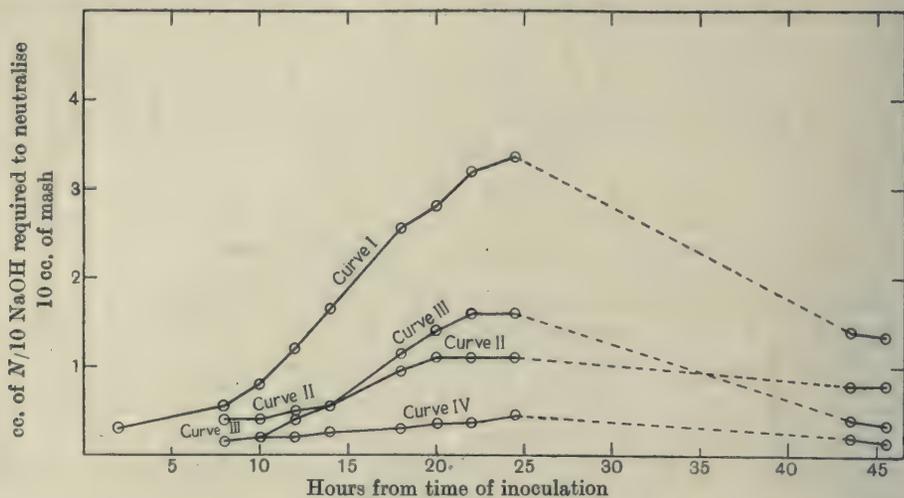


Fig. 2.

**Explanatory:**

- Curve I. Total acidity of mash expressed in cc.  $N/10$  NaOH per 10 cc. of mash.
- Curve II. Acidity due to acetic acid.
- Curve III. Acidity due to butyric acid.
- Curve IV. Acidity due to slightly volatile acid.

A comparison of (a) the relative proportions of the two volatile acids in the mash at different periods and of (b) rise and fall of total acidity shows that during the production of acetone and *n*-butyl alcohol the proportion of butyric acid falls more rapidly than that of acetic acid, agreeing with the formation of a greater proportion of *n*-butyl alcohol than of acetone.

*The addition of Acetic Acid and of other Acids to the Fermenting Mash.*

When acetic acid is added to the fermenting mash an increase takes place in the amount of acetone obtained at the end of the fermentation.

A few preliminary experiments have been made in which other acids were added to the fermenting mash. Formic acid and trichloroacetic acid tended to inhibit the fermentation. Propionic and butyric acids were converted into the corresponding alcohols. Valeric acid appeared to have no effect. The addition of aceto-acetic ester resulted in an increased yield of acetone.

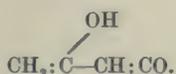
*Production of Ethyl Alcohol.*

A small proportion of ethyl alcohol is usually obtained. It is doubtful whether it is a normal product of the fermentation of the carbohydrate, or a by-product derived from non-carbohydrate material in the mash. The possibility of its production by foreign organisms is not excluded. When infection of the mash has been observed, this has generally been due to lactic acid-producing organisms.

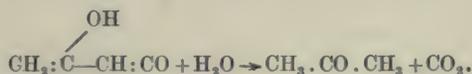
## THE CHEMICAL CHANGES INVOLVED IN THE FERMENTATION.

The acetic acid normally formed in the mash appears to yield acetone, for on neutralising the acid by means of calcium carbonate only traces of acetone are obtained. As already mentioned, the addition of acetic acid to the mash (fermenting in absence of  $\text{CaCO}_3$ ) causes an increase in the yield of acetone. This action might be compared to the production of acetone by distilling calcium acetate, carbon dioxide and water being produced instead of calcium carbonate. The acetic acid may on the other hand be supposed to undergo a condensation to aceto-acetic acid, which subsequently produces acetone and carbon dioxide.

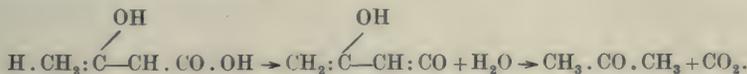
A tentative suggestion to explain the peculiar action on acetic acid is to consider acetic acid as a monoketide,  $\text{H} \cdot \text{CH}_2 \cdot \text{C}(\text{OH}) \cdot \text{CO} \cdot \text{OH}$ . By condensation  $\text{CH}_2 : \text{CO}$  yields



The elimination of  $\text{CO}_2$  and the addition of the elements of water would then yield acetone.



Aceto-acetic acid is a diketide

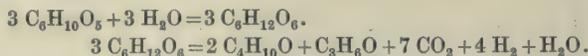


This hypothesis may be extended and is capable of explaining ordinary alcoholic fermentation.

In these reactions acetone and carbon dioxide are produced in the ratio of 58 to 44 by weight. It is obvious therefore from the relative amounts of acetone and carbon dioxide in the end-products of the fermentation that there must be some additional source of carbon dioxide. The 10.77 parts of acetone (see Table I) are only equivalent to 8.2 parts of carbon dioxide, but the total of the latter obtained is 62.61 parts. By comparing Curve 1, Fig. 1 and Table III, it will be seen that a large volume of gas has been evolved before any appreciable amount of acetone or *n*-butyl alcohol has been produced. On the large scale it has been observed that from 20% to 30% of the total volume of gas is evolved before the maximum acidity is reached, and the production of acetone and *n*-butyl alcohol in appreciable amounts begins.

A careful estimation of the total acids, carbon dioxide and hydrogen formed when the fermentation is carried out in presence of calcium carbonate, may throw light on the source of the carbon dioxide and hydrogen. All that can be said in this connection, without further experiments, is, that the weights of acetic and butyric acids calculated from the weight of mixed calcium salts obtained when a fermentation was carried out in presence of calcium carbonate were equivalent to only a part of the starch fermented. No measurements of the gas evolved nor of its composition have been made.

The proportions of the end-products obtained and their relation to the amount of carbohydrate fermented may be fairly accurately represented by the following two equations:

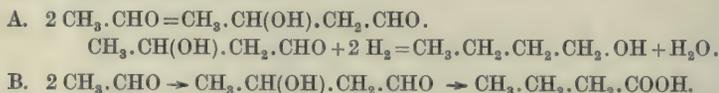


The amounts of the end-products calculated as percentages on the starch fermented from the equations and actually obtained are as follows:

	From the equations	Found (Table I)
Acetone ... ..	11.9	10.77
<i>n</i> -Butyl alcohol ... ..	31.0	25.07
Carbon dioxide ... ..	63.0	62.61
Hydrogen ... ..	1.6	1.60
		(1.80) residual acidity

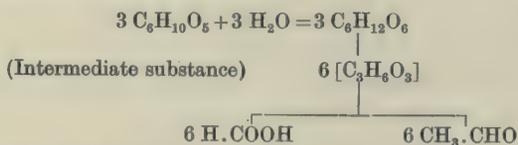
The nature of the reactions taking place to produce these results is obscure. Apparently acetic and butyric acids are intermediate products in the formation of acetone and *n*-butyl alcohol. No information has been obtained concerning the source of the acetic and butyric acids or of the hydrogen and carbon dioxide. A portion of the last-named substance may arise from the conversion of the acetic acid into acetone. The following theories may account for the origin of the carbon dioxide and hydrogen and of part of the *n*-butyl alcohol, but do not explain the production of acetic acid.

Buchner and Meisenheimer [1908], in dealing with a fermentation yielding butyric acid and butyl alcohol together with other products, suggested that acetaldehyde was first produced, which yielded aldol by condensation. The butyl alcohol was obtained by reduction from aldol, while rearrangement of the aldol yielded butyric acid thus:



A similar theory has been put forward by Harden [1901], and Grey [1914], to explain the mechanism of the fermentation produced by *B. coli communis*. According to this theory the carbohydrate is first decomposed into an intermediate substance which gives rise to formic acid and acetaldehyde. The former is converted into carbon dioxide and hydrogen; the latter undergoes the Cannizzaro reaction and yields ethyl alcohol and acetic acid.

The following equations show the application of the above-mentioned theories to the acetone fermentation:



No direct evidence has yet been found of the formation of acetaldehyde or of formic acid in the acetone fermentation. It may, however, be pointed

out that the following empirical equations, in which the end-products are underlined, agree closely with the quantities actually found.

1.  $6 \text{ H. COOH} = 6 \text{ CO}_2 + 4 \text{ H}_2 + 2 \text{ H}_2$ ;
2.  $2 \text{ C}_2\text{H}_4\text{O} + 2 \text{ H}_2 = \text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH} + \text{H}_2\text{O}$ ;
3.  $4 \text{ C}_2\text{H}_4\text{O} = \text{C}_2\text{H}_6\text{O} + \text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{OH} + \text{CO}_2$ .

Many bacteria are known to produce the change represented in equation (1), but the organism employed in this work has not been tested in this respect. Equation (2) corresponds with Buchner's theory of the formation of butyl alcohol. As to the mechanism of equation (3) nothing is known.

The fact that formic acid was not found when the fermentation was carried out in presence of calcium carbonate is not necessarily a proof that this theory is incorrect. It has been shown by Pakes and Jollyman [1901] and Grey [1914] that formic acid and formates are decomposed by *B. coli* and possibly the acetone-producing organism may act similarly. It is intended to make experiments to discover whether this is the case by the addition of formates to the fermenting mash. Careful measurements of the composition of the gas evolved when the fermentation takes place in presence of calcium carbonate are also necessary, and the effect of additions of acetaldehyde should be studied.

The possibility of the production of *n*-butyl alcohol from amino-acids is excluded, as the quantity of amino-acids present is totally insufficient to account for the quantity of alcohol produced.

### EXPERIMENTAL.

#### *Investigation of Acids produced during the Acetone: n-Butyl Alcohol Fermentation Process.*

A portion of the mash was subjected to a steam distillation. 250 cc. of liquid, having an acidity equivalent to 43.8 cc. *N*/10 alkali, gave 450 cc. of distillate having an acidity equal to 27.4 cc. *N*/10 alkali. The presence therefore of a volatile acid or acids is evident.

In order to obtain larger quantities of the volatile acids free from sugars and other impurities present in the fermentation liquid, a larger quantity of the mash was steam distilled. The distillate was neutralised by means of a saturated solution of barium hydroxide and the solution evaporated to a small bulk. A stream of carbon dioxide was passed through the solution to precipitate any slight excess of barium hydroxide, and the solution was next raised to boiling point, filtered and afterwards evaporated to dryness. During evaporation of the neutralised distillate it became darker in colour, changing from light to a darker brown. The barium salt was usually obtained as an amorphous brown mass which only crystallised with difficulty. On warming with dilute sulphuric acid an odour resembling that of acetic acid was obtained. 0.1957 g. of salt dried in the oven gave 0.1599 g. of  $\text{BaSO}_4$ ; Ba = 48.1 %.

With another sample, 0.1500 g. gave 0.1206 BaSO<sub>4</sub>; Ba = 47.3 %.

The steam distillate of a sample of fermented acetone mash usually gave a barium salt which only crystallised slowly. In one experiment however a crystalline product was readily obtained and after one crystallisation it yielded a white salt which contained 45.85 % of Ba. By crystallising the mother-liquors a salt was obtained in the form of plates which contained 49.90 % of Ba, and on further crystallisation the barium content was reduced to 45.57 %. The barium salt on warming with dilute sulphuric acid gave an odour somewhat similar to acetic acid and the free acid was stable to oxidising agents and bromine water.

The quantity of material available was not sufficient for further crystallisations but from the general properties of the compound it would appear to be mainly barium butyrate (requires Ba = 44.1 %). The other barium salt, if it is a salt of a fatty acid, would appear to be an acetate rather than a propionate, for even by crystallising the mother-liquors containing a relatively high percentage of the butyrate, a salt is obtained containing 49.9 % of Ba (barium propionate requires Ba = 48.4 %; barium acetate, Ba = 53.7 %; barium formate, Ba = 60.35 %).

Another crystallisation brings the barium content of this salt nearer to that of barium butyrate owing to the somewhat smaller solubility of this salt in water than of the acetate. These figures indicate the probable absence of a large proportion of barium propionate. It should be noted that a phenylhydrazine compound was obtained as described below. On heating the crude crystalline barium salt under diminished pressure with syrupy phosphoric acid, an acid distillate was obtained which gave with phenylhydrazine a small amount of a white precipitate which was insoluble in water and ether, but soluble in absolute alcohol. It was purified by dissolving in absolute alcohol and precipitating with ether. It melted at 235–238°, yielding a brown oil. Evaporation of the alcoholic solution mentioned above gave the substance in the form of small needles. In order to determine if there were any  $\beta$ -hydroxybutyric acid present the crude barium salt was dissolved in a small amount of water, the barium removed by the addition of slight excess of sulphuric acid, and the solution extracted several times with ethyl acetate. On evaporating the solvent a thick brown oil remained which did not solidify on cooling. It had an acid reaction and was stable towards bromine water and Fehling's solution, but was decomposed by chromic acid and concentrated sulphuric acid. It yielded the iodoform reaction with sodium hydroxide and iodine and also dissolved silver oxide, yielding a solution which rapidly darkened on exposure to light. Extraction with amyl acetate instead of ethyl acetate yielded an oil similar in properties to that described above. An acetone extraction followed by a salting out with calcium chloride gave a solution which contained some mineral matter. On evaporation to dryness followed by a further extraction with acetone or ether a light coloured oil was obtained. By acidifying the barium salt with sulphuric

acid, absorbing the mixture in anhydrous sodium sulphate, powdering and extracting with ether in a Soxhlet, a brown ethereal extract was obtained which had an odour similar to butyric acid. After removing most of the volatile fatty acids, the residue was tested for  $\beta$ -hydroxybutyric acid according to the method of Black [1908] using hydrogen peroxide and ferric chloride, and also by oxidising it with dichromate in presence of a mercuric salt. Neither method gave any positive result. These experiments would indicate the absence of  $\beta$ -hydroxybutyric acid.

In order to identify the volatile acids present, the barium salt dissolved in water was treated with the calculated amount of *N*/1 sulphuric acid to precipitate the barium, filtered from barium sulphate, and distilled according to Duclaux's method:

Vol. of distillate c.c.	<i>N</i> /10 KOH cc.	Percentage	Calculated for 1 mol. acetic acid: 1 mol. butyric acid
30	19.75	35.0	35.2
40	25.57	45.3	46.0
50	31.17	55.3	55.7
60	36.35	64.4	65.0
70	41.45	73.5	73.7
80	46.33	82.1	82.2
100	56.43	100.0	—

The results of the Duclaux estimation would appear to confirm our original observations that acetic and butyric acids were the only volatile acids present. The ratio of acetic acid to butyric was also determined by Dyer's method [1917]. About 1 gram of the salt dissolved in water was treated with a slight excess of *N*/1 sulphuric acid, the volume made up to 150 cc. and steam distilled.

The first 100 cc. of distillate required 29.52 cc. *N*/10 alkali for neutralisation and the second 100 cc. 14.9 cc. The distilling constant is 49.5.

Dyer's figures give 1 mol. acetic acid, 0.94 mol. butyric acid.

Stein's figures [1913] give 1 mol. acetic acid, 0.78 mol. butyric acid.

10 g. of the finely powdered barium salt were treated with a slight excess of phosphoric acid and distilled. The distillate was dried over phosphorus pentoxide and again distilled. It was collected in two fractions — 130° and 130–160°. Each fraction was then submitted to a Duclaux distillation.

Fraction — 130°			Fraction 130–160°		
Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage	Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage
30	4.75	34.4	30	19.95	38.1
40	6.18	44.8	40	25.45	48.5
50	7.53	54.6	50	31.15	59.4
60	8.90	64.5	60	35.85	68.3
70	10.05	72.8	70	40.55	77.3
80	11.25	81.5	80	44.75	85.3
100	13.80	100.0	100	52.47	100.0

1 mol. of acetic acid to 0.83 mol. of butyric acid.

1 mol. of acetic acid to 1.8 mols. of butyric acid.

Thus by distilling the volatile acids and by collecting the distillate in two fractions the ratio in which the acids are present has been altered, and no appreciable amount of a third volatile acid was shown to be present.

*Fermentation in presence of Calcium Carbonate.*

For further examination of the acid produced, the fermentation was allowed to proceed in presence of calcium carbonate. A quantity of 2 litres of 5 % maize mash, sterilised at 30 lb. pressure for 2 hours, was inoculated with 2 % acetone inoculant. A slight excess of calcium carbonate was added, and the acidity was prevented from developing by the frequent addition of calcium carbonate, during the fermentation. Throughout the fermentation, the organism, examined microscopically, appeared normal. After 7 days' fermentation the suspended matter was removed, and the filtrate reserved for the examination of the calcium salts. 300 cc. of the filtrate were distilled, and the first 50 cc. of distillate shaken with an excess of anhydrous potassium carbonate. On "salting out," about 2 cc. of an oil were obtained. It had an odour similar to *n*-butyl alcohol and the oil also gave the iodoform test, when both sodium carbonate and ammonia were used.

For a further examination of its properties a fresh amount of calcium salt was made: 2 litres of 5 % maize mash were prepared as in the previous case. To this was added 40 cc. of inoculant and an excess of calcium carbonate. Probably the excess of calcium carbonate was too great, since the fermentation was not very satisfactory until a further amount of 40 cc. of acetone inoculant had been added. The fermentation was allowed to proceed for 7 days, and the liquid neutralised periodically by the addition of small amounts of calcium carbonate. The soluble calcium salts were filtered off, the insoluble portion washed with water and the washings added to the above solution. On evaporating to dryness (on the water-bath) the solution containing the calcium salts, 45 g. of solid were obtained (containing  $H_2O = 7.6\%$ ,  $Ca = 17.7\%$ ). The amount of sugar present was very small.

10 g. were distilled with an excess of glacial phosphoric acid—there was some charring and frothing. The distillate was strongly acid and was dried with calcium chloride and finally with phosphorus pentoxide. On redistillation it gave a fraction at  $115-140^\circ$  smelling strongly of acetic acid, whilst at  $140-160^\circ$  a second fraction was obtained, having an odour similar to butyric acid. From these two fractions the barium salts were prepared by neutralising with barium carbonate, filtering, and evaporating to dryness. The lower boiling fraction yielded a barium salt which contained  $Ba = 47.95\%$ . On extracting the anhydrous salt several times with absolute alcohol to remove higher fatty acids if present, the barium content was increased to  $51.57\%$ . By recrystallising the barium salt of the higher boiling fraction from water a salt was obtained containing  $45.99\%$  Ba.

The results obtained by the fractional crystallisation of the barium salts indicate the presence of butyric and acetic acids. This was confirmed by a

Duclaux estimation. A weighed amount of the original calcium salt was dissolved in water, decomposed by the calculated amount of sulphuric acid and distilled:

Vol. of distillate cc.	N/10 KOH cc.	Percentage	Ratio, acetic acid : butyric acid
20	11.12	26.3	1 : 1.7
30	16.12	38.2	1 : 1.7
40	20.90	49.5	1 : 1.8
60	28.70	68.0	1 : 1.8
70	32.85	77.8	1 : 1.9
100	42.20	100.0	—
Average ...			1 : 1.8
Ratio, acetic acid : butyric acid* ...			1 : 1.8 mols. 1 : 2.6 by weight.

The ratio was also determined from the constants given by Dyer and by Stein [1913]. For this purpose two determinations were made. From the mean of these 58.3 % of the total acid distilled over in the first 100 cc. of distillate, giving from Dyer's constants, 1 mol. acetic acid : 2.4 mols. butyric acid; from Stein's constants, 1 mol. acetic acid : 2.0 mols. butyric acid.

In order to check these methods, 1 g. of calcium salt, after acidifying with  $\text{NH}_2\text{SO}_4$ , was steam distilled, the distillate being neutralised with a saturated solution of barium hydroxide. When all the volatile acid had been driven over, the neutralised distillate was evaporated to a small bulk. A stream of carbon dioxide was passed through the solution to remove any slight excess of barium hydroxide: the insoluble barium carbonate was removed by filtration after boiling, the filtrate evaporated to dryness and the residue heated in an oven until constant in weight. 0.1500 g. gave 0.1202 g.  $\text{BaSO}_4$ ; Ba = 47.20 %.

This figure corresponds with the presence of acetic acid and butyric acid in the ratio 1 mol. acetic acid to 1.75 mols. of butyric acid.

#### Summary.

Method	Dyer	Stein	Duclaux	Ba salt
Ratio, acetic acid : butyric acid, mols. ...	1 : 2.4	1 : 2.0	1 : 1.8	1 : 1.8
Ratio, acetic acid : butyric acid, by weight ...	1 : 3.5	1 : 2.9	1 : 2.6	1 : 2.6

Disregarding the Dyer determinations it would seem as if the proportion in which acetic and butyric acids were present is approximately 1 : 1.8 (mol.) or 1 : 2.6 (weight). It should be pointed out that the above workers carried out their investigations on prepared mixtures of the pure acids. In our investigation, the mash liquor and a non-volatile acid have probably some effects on the distillation figures and the results may not be so uniform. In some other experiments higher proportions of acetic acid were obtained. Evidence was also obtained of the presence of a third acid only slightly volatile in steam.

1 g. of the calcium salt was dissolved in a small amount of water, and a solution of 4 g. of quinine sulphate dissolved in absolute alcohol added. After heating for a short time to 100° the mixture was allowed to stand for a few days. The precipitate was removed by filtration and the filtrate evaporated to dryness in vacuo and finally extracted with chloroform.

Although the quinine salts have not yet been examined in detail, a preliminary investigation points to the presence of another acid in addition to acetic and butyric acids. Some other evidence on this point is given later.

*Nature of the Acids present during the course of a Normal Fermentation.*

In order to investigate the nature of these acids samples were withdrawn periodically from a fermentation and a Duclaux estimation was carried out directly on the mash after determining the total acidity. This method was adopted as it was imperative to carry out all the determinations on the same fermentation, and it appeared to yield the most concordant results, while the time required to carry out a determination was short. The results are comparable among themselves, since the influence of substances in the solution is approximately constant. The suspension of carbohydrate material was allowed to settle, as far as possible, and the supernatant liquid decanted through purified glass-wool to remove any further amount of suspended matter. This investigation was only undertaken in the case of one or two fermentations and the results obtained may not be general. The following figures are therefore of the nature of a preliminary investigation.

In order to test the accuracy of the results obtained by direct distillation of the mash two experiments were carried out with the same sample of mash as follows:

A Duclaux estimation was carried out on the mash.

Acidity of mash, 5.1 cc. <i>N</i> /10 <sup>1</sup> .			
Duclaux estimation			
Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage	
10	4.90	12.6	1.0
20	9.48	24.1	0.9
30	13.93	35.7	1.1
40	18.03	46.3	1.1
50	21.86	56.1	1.1
60	25.41	65.2	1.0
80	31.90	81.9	0.9
100	38.96	—	—

Ratio  
acetic : butyric  
1 : 1.0

Another portion was distilled in steam at about 60° under diminished pressure. The distillate was neutralised and concentrated; the calculated amount of sulphuric acid added, and the ratio of butyric and acetic acids determined by distillation at constant volume by a method which has been recently described by Reilly and Hickinbottom [1919].

<sup>1</sup> The values recorded for the acidity of the mash refer to the number of cc. of *N*/10 NaOH required to neutralise 10 cc. of mash.

200 cc. of mash on steam distillation gave a distillate requiring 89.0 cc. *N*/10 Ba(OH)<sub>2</sub> for neutralisation. The volatile acid is 87% of the total acidity.

*Estimation of ratio of acetic and butyric acids by new method.*

Vol. of distillate cc.	Percentage of total acid distilled	Ratio	
		acetic acid	butyric acid
20	16.5	1	1.14
30	23.3	1	1.15
40	29.2	1	1.10
50	35.1	1	1.14
60	40.1	1	1.13
70	45.7	1	1.13
80	48.7	1	1.12
90	52.4	1	1.13
100	55.6	1	1.10
		Av. 1	1.12

The agreement between the two series is good. The difference is not more than that due to experimental error in determining the ratio by the Duclaux method.

The estimation on the mash was checked by distilling 1 litre to a small bulk, when it was diluted with distilled water, and again distilled. The distillate so obtained was neutralised with normal sodium hydroxide solution and evaporated to a small bulk. The volatile acids in the neutralised concentrated liquor were estimated by Dyer's method and the results calculated, using both Stein's and Dyer's figures.

During the removal of the volatile fatty acids from the mash, it is extremely difficult to distil all the acetic acid over without carbonising the residue and consequently the results from the Dyer estimations may be somewhat too low for acetic acid.

Sample I ...	... 7.30 p.m.	} The composition of the mixture of acids present in these three samples was not estimated
Acidity ...	... 0.33 cc. <i>N</i> /10	
Sample II...	... 9.30 p.m.	
Acidity ...	... 0.28 cc. <i>N</i> /10	
Sample III	... 11.30 p.m.	
Acidity ...	... 0.32 cc. <i>N</i> /10	
Sample IV	... 1.30 p.m.	
Acidity ...	... 0.51 cc. <i>N</i> /10	

Duclaux estimation

Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage	Calc. for 1 mol. acetic acid : 0.33 mol. butyric acid
30	1.51	29.7	29.4
40	1.98	39.0	39.0
50	2.47	48.6	48.8
60	2.95	58.1	57.7
80	3.85	75.8	77.0
100	5.08	100.0	—

## J. REILLY AND OTHERS

Sample V ... 3.30 a.m.  
Acidity... ... 0.77 cc. *N*/10

## Duclaux estimation

Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage	Calc. for acetic acid 1 mol. : butyric acid 0.5 mol.
30	1.85	31.8	31.4
40	2.42	41.6	41.3
50	2.97	51.0	50.8
60	3.51	60.3	60.2
70	4.10	70.5	69.4
80	4.62	79.4	78.8
100	5.82	100.0	—

Sample VI ... 5.30 a.m.  
Acidity... ... 1.20 cc. *N*/10

## Duclaux estimation

			Ratio, acetic acid : butyric acid	
30	3.15	33.7	1 : 0.71	} The average proportion is 1 mol. of acetic acid : 0.83 mol. of butyric acid
40	4.15	44.4	1 : 0.71	
50	5.09	54.5	1 : 0.84	
60	5.94	63.6	1 : 0.77	
70	6.89	73.8	1 : 1.0	
80	7.67	82.1	1 : 1.0	
100	9.34	100.0	—	

Sample VII ... 7.30 a.m.  
Acidity... ... 1.63 cc. *N*/10

## Duclaux estimation

			Percentage calc. for 1 mol. acetic acid : 1 mol. butyric acid
30	4.58	34.6	35.2
40	6.01	45.5	46.0
50	7.31	55.3	55.7
60	8.60	65.1	65.0
70	9.80	74.1	73.7
80	11.01	83.3	82.2
100	13.22	100.0	—

Sample VIII ... 11.30 a.m.  
Acidity... ... 2.55 cc. *N*/10

## Duclaux estimation

			Ratio, acetic acid : butyric acid	
30	7.53	35.7	1 : 1.1	} 1 mol. of acetic acid to 1.2 mols. of butyric acid
50	11.93	56.5	1 : 1.2	
60	13.89	65.8	1 : 1.2	
70	15.78	74.8	1 : 1.2	
80	17.55	83.1	1 : 1.3	
100	21.10	100.0	—	

Sample IX ... 1.40 p.m.  
Acidity... .. 2.80 cc. *N*/10

Duclaux estimation

Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage	Ratio, acetic acid : butyric acid
30	8.78	36.0	1 : 1.2
40	11.60	47.6	1 : 1.3
50	14.00	57.4	1 : 1.3
60	16.30	66.8	1 : 1.3
70	18.45	75.6	1 : 1.4
80	20.50	84.0	1 : 1.5
100	24.40	100.0	—

} 1 mol. of acetic acid to 1.30 mols. of butyric acid

A Dyer estimation of the distillate from 1000 cc. required 27.8 cc. *N*/10 KOH to neutralise acid in 1st 100 cc.  
12.45 " " " " 2nd "

These figures give a distilling constant of 55.2 which from Dyer's figures give a ratio of 1 mol. of acetic acid : 1.7 mols. of butyric acid.  
Stein's " " " " " " 1.4 " " "

Sample X ... 3.30 p.m.  
Acidity... .. 3.25 cc. *N*/10

Duclaux estimation

			Ratio, acetic acid : butyric acid
30	9.68	36.1	1 : 1.2
40	12.78	47.6	1 : 1.3
50	15.65	58.3	1 : 1.3
60	18.10	67.4	1 : 1.5
70	20.40	76.0	1 : 1.5
80	22.61	84.2	1 : 1.6
100	26.85	100.0	—

} 1 mol. of acetic acid to 1.4 mols. of butyric acid

A Dyer estimation on the distillate from 1000 cc. required 52.6 cc. *N*/10 KOH to neutralise the acid in 1st 100 cc.  
23.8 " " " " 2nd "  
Distilling constant = 54.8.

Dyer's figures give a ratio of 1 mol. of acetic acid : 1.6 mols. of butyric acid.  
Stein's " " " " " " 1.4 " " "

Sample XI ... 6.0 p.m.  
Acidity... .. 3.35 cc. *N*/10

Duclaux estimation

			Ratio, acetic acid : butyric acid
30	10.10	35.9	1 : 1.2
40	13.20	47.0	1 : 1.2
50	16.00	56.9	1 : 1.3
60	18.72	66.6	1 : 1.3
70	21.30	75.9	1 : 1.5
80	23.60	84.0	1 : 1.5
100	28.10	100.0	—

} 1 mol. of acetic acid to 1.3 mols. of butyric acid

A Dyer estimation on the distillate from 1000 cc. required 33.0 cc. *N*/10 alkali to neutralise acid in 1st 100 cc.  
14.0 " " " " 2nd "  
Distilling constant = 57.6.

Dyer's figures give a ratio of 1 mol. of acetic acid : 2.1 mols. of butyric acid.  
Stein's " " " 1 " " 1.9 " "

Sample XII ... 1.0 p.m.  
Acidity... .. 1.4 cc. *N*/10

## Duclaux estimation

Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage	
30	2.43	27.3	1 mol. of acetic acid to 0.22 mol. of butyric acid
40	3.35	37.6	
50	4.15	46.6	
60	4.98	56.0	
70	5.80	65.1	
80	6.75	75.8	
100	8.90	100.0	

A Dyer estimation on the distillate from 1000 cc. required

11.1 cc. *N*/10 alkali to neutralise acid in 1st 100 cc.

6.4 " " " " 2nd "

Distilling constant = 42.4.

Dyer's figures give the ratio 1 mol. of acetic acid : 0.40 mol. of butyric acid

Stein's " " " 1 " " 0.33 " " "

Sample XIII ... 3.0 p.m.  
Acidity ... .. 1.35 cc. *N*/10

## Duclaux estimation

30	2.52	28.0	1 mol. of acetic acid to 0.2 mol. of butyric acid
40	3.45	38.3	
50	4.09	45.4	
60	4.85	53.9	
70	5.85	65.0	
80	6.71	74.5	
100	9.00	100.0	

*Summary of Results.*

Age of fermenting mash in hours	Acidity*	Ratio acetic acid : butyric acid as determined by method of			
		Duclaux Determined in the mash	Dyer	Duclaux Determined in the distillate from the mash	Stein
2	0.33	—	—	—	—
4	0.28	—	—	—	—
6	0.32	—	—	—	—
8	0.51	1 : 0.33	—	—	—
10	0.77	1 : 0.5	—	—	—
12	1.20	1 : 0.83	—	—	—
14	1.63	1 : 1	—	—	—
18	2.55	1 : 1.2	—	—	—
20	2.80	1 : 1.3	1 : 1.7	—	1 : 1.4
22	3.25	1 : 1.4	1 : 1.6	—	1 : 1.4
24½	3.35	1 : 1.3	1 : 2.1	—	1 : 1.9
43½	1.4	1 : 0.22	1 : 0.43	(1 : 0.4)	1 : 0.33
45½	1.35	1 : 0.20	—	—	—

\* Expressed in cc. of *N*/10 alkali required to neutralise 10 cc. of mash.

To test the view that the production of "oil" and the diminution of butyric acid were connected, the amount of "oil" produced was determined on samples taken during the progress of a fermentation. The ratios in which the volatile acids were present in the same sample were also determined.

Time	Acidity	Ratio determined by Duclaux estimation, acetic acid : butyric acid	Volume of oil from one litre of mash
9.0 p.m.	1.0	1 : 0.5	None
11.0 "	1.5	1 : 0.62	"
12.15 a.m.	2.0	1 : 0.9	Trace
5.30 "	3.7	1 : 1.25	1 cc.
3.15 p.m.	2.0	1 : 0.28	4 "
4.30 "	1.6	1 : 0.25	5 "

These results indicate that traces of acetone and *n*-butyl alcohol are produced before the maximum acidity has been attained. The production of "oil" in the fermentation using calcium carbonate appears to confirm this result.

As far as can be judged from the limited number of experiments carried out, these ratios indicate that while the acidity of the mash is increasing previous to the production of *n*-butyl alcohol and acetone, the production of butyric acid proceeds at a faster rate than that of acetic acid. In the particular fermentation examined the maximum amount of butyric acid appears to be present at the time of maximum acidity, and when the production of acetone and *n*-butyl alcohol takes place, the amount of butyric acid diminishes. It would seem that the production of butyric and acetic acids, of *n*-butyl alcohol, and of acetone is intimately connected.

In the presence of calcium carbonate fermentation apparently proceeds as far as the point corresponding to the maximum acidity in a normal fermentation.

*Evidence of the presence of a Third Acid only slightly volatile in Steam.*

Indications have been obtained of the presence of another acid in the mash; it has not been isolated. In determining the ratio of butyric acid and acetic acid by Duclaux's method, it was observed that the amount of acid present in the original mash, calculated from Duclaux's table, did not agree with the total acid present, as estimated by titration.

The following tables and Fig. 2 show the percentage of the total acidity which is accounted for by butyric and acetic acids:

Acidity of the mash	Acetic and butyric acid, percentage of total acidity
1.0	105 (carbon dioxide present in addition to fatty acids)
1.5	—
2.0	90
3.7	86
2.0	70
1.6	74

Another mash gave the following results:

Age of fermenting mash in hours	Acidity of the mash	Acetic and butyric acids, percentage of total acidity
8	0.51	107 (carbon dioxide present)
10	0.77	80
12	1.20	80
14	1.63	83
18	2.55	84
20	2.80	89
22	3.25	83
24½	3.35	88
43½	1.4	70
45½	1.35	73

This agreement between two series points to the existence of an acid not readily volatile in steam which increases in proportion to the volatile acids after the maximum acidity has been reached. The calcium salt obtained by fermenting in presence of chalk, on distillation by the Dyer method, yields only about 53–55 % of its weight as volatile acid. The moisture and calcium oxide only account for about 30–35 % of the salt. The amount of sugar present in the crude salt was extremely small. There was, therefore, about 10–15 % not accounted for.

The quinine salt prepared from the calcium salt, on hydrolysis with baryta, gives a mixture of barium salts which points to the existence of another acid with an equivalent equal to or greater than that of butyric acid.

*Addition of Acetic Acid to the Fermenting Mash.* [See Desborough etc., 1918.]

105 lb. of maize meal were used to prepare 168 gallons of mash: the mash was inoculated with half a gallon of fermenting mash. Total volume of mash 168.5 gallons.

The acetic acid was added to the mash in the concentrated form, but dilute acid may equally well be used. During the addition of the acid the mash was vigorously stirred and the stirring was continued for 15 minutes after the acid had been added. The first lot of 500 cc. of acid was added 24 hours after inoculation, as soon as the normal acidity had passed its maximum and was beginning to fall.

Subsequent additions of 500 cc. of acid were made as soon as the acidity of the mash had fallen to the same point at which the first lot of acid was added. In all, seven lots of 500 cc. of acid were added, making a total of 3500 cc. of acid added.

Specific gravity of 97 % acetic acid used, 1.0625.

Total weight added, 3718.7 g.; so that 3607.1 g. of acetic acid were added.

The total acetone produced at the end of the fermentation was 27.27 g. per gallon. The volume of fermented mash at the end of the fermentation amounted to 163 gallons, as 4 gallons were taken out for a control im-

mediately after inoculation and 1.5 gallons were drawn off at intervals for estimating the acidity.

The total acetone produced was therefore  $163 \times 27.27 = 4445.01$  g.

The acetone produced in the control to which acetic acid was not added amounted to 19.54 g. per gallon. The total acetone produced in 163 gallons of mash to which acetic acid was not added would be  $163 \times 19.54$  g. = 3185.02 g.

Hence the acetone produced from the acetic acid =  $4445.01 - 3185.02 = 1260$  g.

Assuming that two molecules of acetic acid produce one molecule of acetone, the 3607 g. of acetic acid added should give 1743.4 g. of acetone. The amount actually found was 1260 g. or 72.2 % of the theoretical amount.

The yield would probably have been better had less acetic acid been added, as apparently more acetic acid was added to the mash than could be converted.

The acidity of the mash at the end of the fermentation remained abnormally high, 10 cc. of mash requiring 3.0 cc. *N*/10 alkali for neutralisation, instead of the normal amount of 1.9 cc.

In another experiment made about the same time, a similar result was obtained. In this case also, too much acetic acid was added, the yield of acetone was 79.9 % of the theoretical amount that should have been obtained from the total acetic acid added.

There were no indications of infection by lactic acid producing organisms in either of these fermentations. The volume of gas evolved was slightly above the normal.

A large scale experiment was carried out in which over a ton of acetic acid was added to 86,000 gallons of fermenting mash.

Unfortunately the mash became infected by lactic acid producing organisms before the fermentation was completed. In spite of this the weight of acetone produced was much above the normal amount, the excess of acetone obtained, over the normal yield from the grain, showing a yield of 61.27 % of the theoretical from the acetic acid added.

#### *Examination of an Infected Mash.*

A sample was taken from a fermentation in which the acidity had become high. The sample contained "oil" and had an acidity = 6.3 cc. *N*/10 NaOH. Part of the acidity was due to the presence of volatile acids, which were estimated by the Duclaux method.

Vol. of distillate cc.	<i>N</i> /10 KOH cc.	Percentage	
30	9.20	28.9	} The distillation figures agree with a mixture of butyric and acetic acids in the ratio of approximately 3 mols. of acetic acid to 1 mol. of butyric acid
40	12.20	38.4	
50	15.30	48.2	
60	18.20	57.3	
70	21.35	67.3	
80	24.50	77.5	
100	31.75	100.0	

From this estimation approximately 55 % of the total acidity is accounted for as acetic and butyric acids. For the examination of the non-volatile acid, about 3 litres of the mash were evaporated down to a very small bulk. The residue, which was very dark in colour and sticky when cold, was mixed with fine silica while still hot, and finally made into a hard mass by stirring in finely-powdered anhydrous sodium sulphate. The mass was then broken up and extracted with ether in a Soxhlet extraction apparatus. A brown syrupy extract was obtained, which had an acid reaction. Oxidation by means of dilute potassium permanganate solution in presence of sulphuric acid yielded acetaldehyde. The brown liquid gave a *zinc* salt containing  $\text{Zn} = 26.6 \%$ . (Zinc lactate requires  $\text{Zn} = 26.9 \%$ .)

Another portion gave a *barium* salt which did not crystallise. The *calcium* salt crystallised in small nodules. A sample of the calcium salt after 3 crystallisations showed a slight laevo-rotation  $[\alpha]_{\text{D}} = -0.73^{\circ}$ ; after 8 crystallisations  $[\alpha]_{\text{D}} = -1.34^{\circ}$ . The *copper* salt crystallised in long blue prisms.

By distilling the acid under moderately diminished pressure a solid distillate was obtained having a constant m.p. of  $125^{\circ}$  after 3 crystallisations from chloroform (lactide from lactic acid, m.p.  $125^{\circ}$ ). A mixture with a specimen of pure lactide made from lactic acid melted at the same temperature. The acid cooled in ice was oxidised by the action of dilute hydrogen peroxide solution, in presence of a trace of ferrous acetate, in the manner described by Fenton and Jones [1900]. On the addition of a few drops of phenylhydrazine acetate solution, a red precipitate was produced which after washing well with water and small amounts of ether melted at  $177-178^{\circ}$  with decomposition. The melting point was not depressed by the addition of some pure pyruvic phenylhydrazone. The acid is therefore lactic acid.

A high acidity mash contains lactic, acetic and butyric acids.

#### SUMMARY.

In the fermented mash, acetic and butyric acids are present in varying proportions. During the progress of the fermentation the ratio of butyric acid to acetic acid increases with the increase in the acidity of the mash, reaching a maximum when the acidity is at its highest. With the production of "oil" the ratio of butyric acid to acetic acid diminishes, until the mash contains an excess of acetic acid. If the acidity is neutralised by the addition of calcium carbonate, the calcium salts of the acids present in the mash replace the acetone and *n*-butyl alcohol, and these latter are produced in almost negligible amount. In the presence of calcium carbonate, however, the fermentation proceeds as far as the point corresponding to the maximum acidity in a normal fermentation. It is extremely probable that acetic and butyric acids are not the only acids present. The type of infection most frequently observed in the fermenting mash produces lactic acid. When the mash is

infected in this way the amount of volatile acid accounted for by the Duclaux estimation is lower than in the case of a normal fermentation.

When acetic acid is added to the fermenting mash an increased yield of acetone is obtained; the yield of *n*-butyl alcohol being unchanged. Propionic and butyric acids when added appear to be converted into the corresponding alcohols. An increased yield of acetone has been obtained by the addition of aceto-acetic ester.

In conclusion the authors take this opportunity of expressing their thanks to Capt. Desborough, R.A., Superintendent, and Mr W. T. Thomson, Manager of the Royal Naval Cordite Factory, Dorset, both for the interest they have taken in this investigation, and for granting permission to publish the results obtained. In addition we are indebted to Dr A. Harden for suggestions which may help to explain the mechanism of the reactions taking place.

## REFERENCES.

- Black (1908). *J. Biol. Chem.* **5**, 207.  
 Botkin (1893). *Zeitsch. Hyg.* **11**, 421.  
 Bredemann (1909). *Centr. Bakt. Par.* **II**, **23**, 385.  
 Buchner and Meisenheimer (1908). *Ber.* **41**, 1410.  
 Desborough and Others (1918). Eng. Patent 128403.  
 Duclaux (1895). *Ann. Inst. Past.* **9**, 269.  
 Dyer (1917). *J. Biol. Chem.* **28**, 445.  
 Farbenfabr. vorm F. Bayer & Co. German Patents 289687, 294683, 19143.  
 Fenton and Jones (1900). *J. Chem. Soc.* **77**, 71.  
 Fernbach and Strange (1912). Eng. Patent 21073.  
 Fitz (1878). *Ber.* **11**, 42.  
 — (1882). *Ber.* **15**, 867.  
 — (1883). *Ber.* **16**, 844.  
 — (1884). *Ber.* **17**, 1188.  
 Grey (1914). *Proc. Roy. Soc. B.* **87**, 470, 472.  
 Grimbert (1893). *Ann. Inst. Past.* **7**, 353.  
 Gruber (1887). *Centr. Bakt. Par.* **1**, 367.  
 Harden (1901). *J. Chem. Soc.* **79**, 610.  
 Northrop (1919, 1). *J. Biol. Chem.* **39**, 1.  
 — (1919, 2). U.S. Amer. Patent 1293172.  
 Pakes and Jollyman (1901). *J. Chem. Soc.* **79**, 386.  
 Perdrix (1891). *Ann. Inst. Past.* **5**, 287.  
 Reilly and Hickinbottom (1919). *Roy. Soc. Dublin*, **15**, 513.  
 Ricard (1918). Eng. Patent 130666.  
 Schardinger (1905). *Centr. Bakt. Par.* **II**, **14**, 772.  
 — (1907). *Centr. Bakt. Par.* **II**, **18**, 748.  
 Stein (1913). *J. Prakt. Chem.* **88**, 83.  
 Weizmann (1915). Eng. Patent 4845.  
 — (1919). U.S. Amer. Patent 1315585.

## XXV. A NOTE ON BRAUNSTEIN'S MODIFICATION OF THE MÖRNER-SJÖQVIST PROCESS FOR THE ESTIMATION OF UREA.

BY ALAN HERAPATH TODD.

*From the Physiological Laboratory, Guy's Hospital.*

(Received March 8th, 1920.)

AN oft-repeated objection to the Mörner-Sjöqvist process is the fact that hippuric acid and creatinine escape precipitation, and appear in the final result as urea. Braunstein suggested that this might be avoided by using crystalline or syrupy phosphoric acid instead of sulphuric acid at the "Kjeldahl" stage, and as this proposal has gained added interest in view of the large amount of work now being done upon creatinine, the matter has been re-investigated.

The fundamental point in the whole process is the assumption that urea is completely incinerated under the conditions prescribed by Braunstein, viz., heating at 145° for 4½ hrs. To test this, an aqueous solution of known strength was used:

*Expt. 1.* Urea, re-crystallised from water, dried over H<sub>2</sub>SO<sub>4</sub> *in vacuo*; solution contained N = 0.327 %, by ordinary Kjeldahl. 10 cc. heated in an oil-bath for various periods with Kahlbaum's crystalline phosphoric acid:

	Wt. acid used	Heated for	Temp. °C.	%N found	Remarks
	g.	hrs.			
(a)	10	4½	145	0.220	Braunstein's conditions
	—	—	—	0.220	
	—	—	—	0.261	
(b)	10	5	175	0.316	Results better, but N values
	—	—	—	0.317	still too low
	—	—	—	0.318	
(c)	10	6	178	0.318	No improvement with longer
	—	—	—	0.318	time
(d)	20	5	150	0.260	10 g. acid are enough
(e)	30	5	150	0.291	" "

It is evident, from consideration of the three experiments included in Group (a), that urea-estimation is by no means quantitative under the original conditions. This might have been due to insufficiency of acid, too low a temperature, or too short a period of heating. The results of varying these conditions are seen in the experiments cited above in Groups (b), (c), (d), and (e), from which it appears that the temperature is the most important factor.

There remained the possibility that the small quantity of creatinine and other reducing bodies present in normal urine might in some way facilitate the process, so it was decided to incinerate entire urine with phosphoric acid and with sulphuric acid, and to compare the results.

*Expt. 2.* Normal urine, boiled and preserved with chloroform, N = 0.955 %, by sulphuric acid. 5 cc. used for each determination.

	Wt. acid used	Heated for	Temp. °C.	% N found	Remarks
	g.	hrs.			
(a)	10	10	180	0.166	Acid insufficient
(b)	15	5	175	0.855	
	—	—	—	0.863	
	—	—	—	0.868	
(c)	15	10	180	0.896	
	—	—	—	0.900	
(d)	15	14	185	0.899	Liquid in flask after ignition
	—	—	—	0.903	was dark brown in colour,
	—	—	—	0.904	with white crystals on cooling

The last four values, though not equalling the figure for the total nitrogen, yet approximate so closely to one another as to suggest that the ignition of the urea is complete, and therefore that the ratio .904/.955 represents the proportion of the urea-nitrogen to the total nitrogen. To decide whether this was the case or not, an "artificial urine" was made up, containing:

Creatine, dried at 100° and <i>in vacuo</i> , over H <sub>2</sub> SO <sub>4</sub>	N = 0.0233 %
Hippuric acid	" " " " N = 0.0030
Urea	" " " " N = 0.8937

Total N is ∴ = 0.9200 %, both by calculation and by Kjeldahl; of which urea = 97.14%

(It was found impossible, in practice, to dry the urea to a constant weight, as was done with the other two substances; it was therefore dried until its loss of weight began to be uniform, and then the exact amount of it in solution was calculated from the difference between the total N and the (creatin + hippuric) N.)

*Expt. 3.* 5 cc. of the above artificial urine, with 15 g. of phosphoric acid, heated at 185° for 14 hrs.; the % N found was: 0.900, 0.882, 0.880, 0.873, 0.900, 0.895. Average = 0.892 %, as compared with 0.894, calculated.

The values given in the last group of six experiments are perhaps not quite as concordant as might be wished, but owing to the accidental destruction of notes in which other series of experiments were recorded, they are the only ones available at the moment. However, they are not far out, and if care be taken at the beginning of distillation, much better results can be obtained. *These figures prove that incineration of the Mörner-Sjöqvist filtrate for 14 hours at 185° with phosphoric acid gives an accurate, quantitative estimation of the urea, and the urea only.*

One possible fallacy remains, perhaps, and that is, the occurrence of a double compensatory error, a slight loss on the urea being repeatedly and exactly balanced by a slight incineration of the creatine and hippuric acid. This difficulty was readily disposed of by heating suitable quantities of these latter bodies with phosphoric acid under identical experimental conditions, when it was found that not a trace of incineration occurred.

In conclusion, it may be useful to add a few practical notes on the technique of the method. In the first place, the time taken in ignition is no objection to the process, since these experiments are always performed in a series, and so one day's material can be heated whilst another lot is being distilled and titrated. The actual heating is best performed in a bath of "government" colza oil, the height of the gas-flame being regulated by a regulator, so that, once adjusted, it is only necessary to turn the gas full on to secure the exact degree of heating required. It is best to incinerate in Jena flasks of the ordinary shape, so as to avoid transference before distillation, and as soon as ignition is completed, 100 cc. or more of distilled water should be added; if this is not done, a hard white mass forms on cooling, which it is most difficult to re-dissolve. Mica scales having been added, strong soda solution may be run in fairly quickly, as there is no heat evolved. As soon as the distillation-tube has been adjusted, the two layers of liquid should be gently mixed, and then be heated *very slowly* to boiling-point: in this way, much of the ammonia is driven over before rapid ebullition begins, and the rapid "surging" of the liquid, and the loss of ammonia from sudden mixing of the two hot liquids (that were so troublesome in the older method), are completely avoided. Lastly, it may be mentioned that the most convenient way of weighing out the crystalline phosphoric acid is to melt it in a water-oven, and then to pour it out into a weighed glass evaporating-basin, placed upon an ordinary spring "letter-balance"; the exact quantity used does not matter very much, provided that it is enough, as Kahlbaum's acid, at any rate, is quite free from nitrogen.

In conclusion, I take the opportunity to tender to Professor Pembrey my most sincere thanks for his help and encouragement in this and all my studies, and for the generosity with which he placed all the resources of his laboratory at my disposal.

#### SUMMARY.

1. The conditions originally suggested by Braunstein in his modification<sup>1</sup> of the Mörner-Sjöqvist process do not give a quantitative yield of the urea-nitrogen.

2. Ignition of the filtrate for 14 hours at 185° with 15 grams of Kahlbaum's crystalline phosphoric acid gives an accurate quantitative result.

<sup>1</sup> In the Mörner-Sjöqvist process, 5 cc. of urine are mixed with 5 cc. of a saturated solution of barium chloride, containing 5 % of barium hydrate; 100 cc. of a mixture of ether and alcohol, in the proportion of 1 : 2, are then added. This is stated, erroneously, to precipitate all the nitrogenous matter, but, as stated above, hippuric acid and creatinine escape. This is the whole point of the present communication.

After leaving the mixture to stand for 24 hrs., the liquor is filtered off, the precipitate is washed with 50 cc. of the alcohol-ether mixture, the washings are added to the filtrate, and a little magnesia is added. The alcohol and ether are driven off at a temperature of 55°, and evaporation is continued until the residue measures 10-15 cc. The nitrogen in this is then estimated by the Kjeldahl method; the result, multiplied by 2.143, is said to equal the amount of urea.

In this paper, it is shown that quantitative estimation of the urea, apart from all other nitrogen, can be obtained, provided that certain experimental conditions are complied with.

## XXVI. THE IODOMETRIC ESTIMATION OF SUGARS.

BY HILDA MARY JUDD.

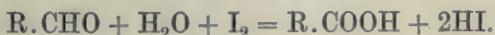
*From the Department of Plant Physiology and Pathology, Imperial College of Science and Technology, S. Kensington.*

*(Received March 6th, 1920.)*

IN the course of a series of investigations of the changes that take place in the nature and amount of the carbohydrates of apples during the process of ripening, both in ordinary storage and in cold storage, it has been found that the usual method of estimating fructose by means of the polarimeter is very unsatisfactory. In the first place, the optical activity of apple juice is very low; the sugars present appear to consist mainly of saccharose, glucose, and fructose, the last being in considerable excess, with the result that the juice gives a very small polarimetric reading. Moreover, the juice is always coloured, and is frequently so viscous that it is necessary to dilute it very considerably before it is possible to clear the solution by means of basic lead acetate and alumina cream. The resulting solution seldom contains more than 0.5 % or 1 % of sugars, giving in a 2 decimetre tube a maximum reading of one degree. Obviously the error involved in calculating the proportion of each of the sugars present from such small readings is very large. It becomes clear then that some other method is required.

Several papers dealing with the action of bromine and iodine in alkaline solution on various sugars have been published, some of them quite recently. It has been definitely established that under suitable conditions, both bromine and iodine bring about the oxidation of most sugars, the main product being the corresponding monobasic acid. Bromine in cold aqueous solution slowly oxidises aldoses, and has a still slower action on ketoses. In a mixture of aldose and ketose, however, it is not possible so to control the experiment that only the aldose is attacked.

Bougault [1917] suggests the use of weakly alkaline solutions of iodine. Glucose is then oxidised according to the equation:



According to Bougault, fructose when treated in the same way is not appreciably attacked; he admits however that his results are not consistent, and show many discrepancies.

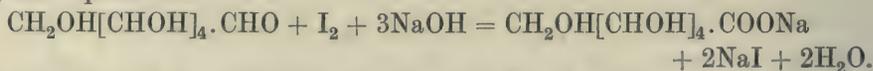
Colin and Lièvin [1918] have attempted to modify and improve Bougault's method. They point out that the degree of alkalinity of the solution may influence the result obtained. While Bougault used a mixture of sodium carbonate and sodium bicarbonate, Colin and Lièvin prefer a solution containing per litre 35 g. of disodium phosphate and 50 cc. of *N* sodium hydroxide.

Romijn [1897] recommends the use of alkaline iodine solutions for the estimation of glucose in presence of fructose. He describes experiments with sodium carbonate, ammonium carbonate, trisodium phosphate, and borax, and prefers the last, as being only very weakly alkaline, and having a less destructive action on the sugar molecule.

The objections to the use of the borax-iodine solution advocated by Romijn are: (1) below 25°, the action is unduly prolonged; (2) at temperatures above 25°, fructose is attacked; (3) in such weakly alkaline solutions, the alkalinity is appreciably reduced by the formation of acid from the sugar.

According to Romijn, all the aldoses behave like glucose, and such di- and tri-saccharides as saccharose and raffinose, which do not reduce Fehling's solution, are also attacked.

Willstätter and Schudel [1918] comment on Romijn's work, and point out that the course of the reaction depends on the amount and on the concentration of the alkali present. There must be sufficient alkali to neutralise the acid formed as a result of the oxidation, but if this amount is exceeded the reaction is not complete. According to Willstätter and Schudel the reaction goes best with decinormal solutions, and in the proportions required by the equation:



They state that if these conditions are fulfilled the error with glucose is not more than 1%. They point out however that in the presence of free alkali the sugars undergo the Lobry de Bruyn transformation.

They find that cane sugar is not acted upon. They also give results with invert sugar, which approximate closely to theory; and they state that under the same conditions fructose is not attacked; no figures however are given for fructose alone.

The following experiments have been carried out in order to determine whether any of the methods mentioned above are applicable to the estimation of the amounts of glucose and fructose present in fruit juices. It has been found, as the result of a large number of determinations carried out with solutions of pure sugars, that neither the method of Colin and Lièvin nor that of Willstätter and Schudel is exact, and that in no case is the sugar oxidised quantitatively to the corresponding monobasic acid; on the other hand, there is always a very definite attack on the fructose. The results obtained with invert sugar are misleading since the low result obtained from the glucose is masked by the partial oxidation of the fructose.

It has further been noticed that the reaction mixture always smells strongly of iodoform, suggesting that some secondary change is taking place, Romijn also draws attention to the fact that a large number of organic substances which give the iodoform reaction are readily oxidised by alkaline iodine solutions.

It seems probable however that the chief source of error is the action of dilute alkalis on sugars, investigated by Lobry de Bruyn and van Ekenstein [1895, 1896, 1897]. Since even such weak alkalis as lead hydroxide bring about this change, it is certain that it would occur in a solution containing a considerable excess of sodium hydroxide. Starting therefore with a solution of pure glucose, part of this sugar would be converted by the alkali into mannose (which is also oxidised by iodine) and part into fructose. Consequently the whole of the sugar present would not be oxidisable by iodine, and the result obtained for the glucose would be too low. Conversely, a solution of fructose would yield a certain proportion of both glucose and mannose, and this probably accounts for the apparent partial oxidation of the fructose.

It may be assumed therefore that the behaviour of the above sugars towards alkaline iodine solutions is in some measure dependent on the Lobry de Bruyn transformation, and that in the presence of iodine some definite equilibrium is reached.

The equilibrium obtained in this way must be a very complex one. According to the observations of Lobry de Bruyn and van Ekenstein, the composition of the almost inactive syrup obtained by the action of dilute alkalis on glucose, mannose, or fructose depends on the nature of the sugar which was used at the start. It appears also from the present series of experiments that the removal of the aldoses present by oxidation does not, as would be expected, tend to convert more ketose into aldose.

It is however possible to utilise the method of Willstätter and Schudel for the analysis of mixtures of glucose and fructose. A large number of experiments show that a given weight of glucose always uses up a definite and constant weight of iodine, although not the amount theoretically required. The same statement holds good for fructose. Neither of these reactions is in any way affected by the presence of other sugars. Experiments with mixtures of glucose and fructose in varying proportions show that the iodine equivalent of the mixture calculated from the two constants in all cases shows very close agreement with that found by experiment. This being the case, it is possible to calculate from the copper reducing power of a mixture of glucose and fructose, and from the amount of iodine used up by the mixture, the proportion of each sugar present.

#### *Experimental details.*

The solution of sodium thiosulphate used was carefully standardised by Volhard's method against *N*/10 potassium permanganate. The latter had been standardised in the usual way with ferrous ammonium sulphate and also

against oxalic acid; the values obtained by these two methods showed very close agreement.

The concentration of the sugar solutions used was determined by Bertrand's method, using the same permanganate solution, and the purity of the sugar was also tested by means of the polarimeter. The reaction was carried out in well-stoppered wide-mouthed bottles.

1 cc. *N*/10 iodine contained 0.01293 g. iodine.

1 cc. *N*/10 thiosulphate was equivalent to 0.0128 g. iodine.

(20 cc. iodine = 20.2 cc. thiosulphate.)

#### A. Method of Willstätter and Schudel.

(1) 10 cc. glucose solution (0.978 %).

20 „ *N*/10 iodine.

30 „ *N*/10 NaOH.

The mixture was allowed to stand 15–20 minutes at the ordinary temperature, then acidified with dilute sulphuric acid and titrated with thiosulphate.

*Titrations*: 10.2, 10.1, 10.1, 10.2, 10.15. *Mean*: 10.15.

From this, it follows that 1 g. of glucose requires 1.315 g. of iodine.

(2) 10 cc. fructose solution (1.012 %).

10 „ *N*/10 iodine.

20 „ *N*/10 NaOH.

*Titrations*: 9.3, 9.3, 9.3, 9.3.

From the above it follows that 1 g. of fructose takes up 0.1028 g. of iodine.

#### B. Method of Colin and Lièvin.

Three different solutions were tried, containing varying amounts of alkali, in order to see whether a concentration could be found at which the glucose would be completely oxidised and the fructose remain unaffected.

Phosphate solution I contained 35 g. of sodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ ) and 50 cc. of *N* NaOH per litre.

Phosphate solution II contained 35 g. of sodium phosphate and 100 cc. of *N* NaOH per litre.

Phosphate solution III contained 35 g. of sodium phosphate and 25 cc. of *N* NaOH per litre.

The mixtures used were:

10 cc. sugar solution.

20 „ *N*/10 iodine.

40 „ phosphate solution.

The mixture was allowed to stand for one hour at the temperature of the laboratory, then acidified with dilute sulphuric acid, and titrated with thiosulphate solution.

The results are given below (Table I). It will be noted that phosphate solutions I and II give results identical with that obtained by Willstätter's method, although solution II contains twice as much sodium hydroxide as

solution I. The third mixture did not contain enough alkali to neutralise the gluconic acid formed. It was repeated, and the results obtained are given under nos. (4), (5) and (6).

(4) Three of the samples were left for two hours to see if the reaction would go further, but the result was negative.

(5) and (6) To each of six other samples was added 10 cc. of *N*/10 sodium hydroxide. Three of them were left for one additional hour, and the rest for two additional hours.

(7) The mixture of glucose and phosphate solution I was heated to 37° for one hour, to see if the reaction would be more complete at a higher temperature. Increase of temperature was however also found to be without influence on the result.

C. *Method of Colin and Lièvin (fructose).*

10 cc. fructose solution (1.012 %).

10 ,, *N*/10 iodine.

20 ,, phosphate solution.

In all cases the numbers given are the mean of five closely agreeing titrations

Table I.

A. *Willstätter's Method.*

Glucose ... 10.15

Fructose... 9.3

B. *Method of Colin and Lièvin (glucose).*

- (1) Glucose + phosphate I (1 hr.), 10.15
- (2) " " II " 10.15
- (3) " " III " 12.62
- (4) " " III (2 hrs.), 12.65
- (5) " " + 10 cc. *N*/10 NaOH (3 hrs.), 10.18
- (6) " " + 10 cc. *N*/10 NaOH (4 hrs.), 10.18
- (7) " " I at 37° (1 hr.), 10.13

C. *Method of Colin and Lièvin (fructose).*

Fructose + phosphate I (50 cc. *N* NaOH per litre), 9.6

" " II (100 cc. " " " " ), 9.32

The titration corresponding to the complete and quantitative oxidation of glucose to gluconic acid is 9.55. The titration required if fructose is completely unchanged is 10.1. It appears from the above results that glucose is oxidised by alkaline iodine solutions to the extent of about 95 %. The reaction proceeds smoothly, provided that sufficient alkali is present to neutralise the gluconic acid formed. Increasing the amount of alkali does not appear to influence the result, which is also unaffected by heating to 37°.

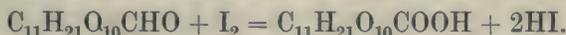


be due to the steric influence of the methyl group. Moreover, the fact that rhamnose does not when oxidised yield the very unstable rhammonic acid, but a lactone, may influence the reaction.

The results obtained with the disaccharides are remarkable.

The action on saccharose is negligible. In actual experiments the titration differed by 0.1 cc. from the blank, but this is within the limits of experimental error and, further, saccharose almost always contains a trace of reducing sugar.

Maltose behaves quite normally, and the iodine value found by experiment agrees closely with that calculated from the equation:



This is also in agreement with the copper-reducing power of maltose.

Lactose, on the other hand, takes up twice as much iodine as is required by the above equation, which fact suggests that the sugar is hydrolysed, and that both the glucose and the galactose molecules are oxidised.

In this connection it is interesting to note that Lobry de Bruyn and van Ekenstein [1895, 1896, 1897] found that when lactose is subjected to prolonged treatment with dilute alkali, it is hydrolysed, as much galactose being produced as in ordinary inversion, together with a little pseudo-tagatose—but no glucose. They assumed that the latter was split off in the form of an anhydride; this does not appear to be the case when iodine is present.

The numerical results are shown in Table III. The solutions used were approximately 2 %, but the actual concentration of the solutions was estimated by Bertrand's method, and checked by means of the polarimeter, except in the case of rhamnose, of which only a small sample was obtainable. In this case however the specimen was very well crystallised, and appeared to be pure, and its copper-reducing power was quite normal.

Table III.

Sugar	Wt. of sugar in 5 cc.	Titration N/10 Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> Mean	Diff. from blank	Iodine equivalent of 1 g. sugar	
				Found	Calculated
Mannose ...	0.108	11.83	8.17	0.977	1.509
Galactose ...	0.107	8.25	11.75	1.418	1.509
Arabinose ...	0.1004	6.65	13.35	1.702	1.69
Rhamnose ...	0.1087	12.15	7.85	0.9243	1.509
Saccharose ...	0.100	9.95	0.05	—	—
Maltose ...	0.0462	14.4	5.6	0.7456	0.75
Lactose ...	0.104	7.80	12.2	1.502	0.75

SUMMARY.

In dealing with fruit juices, the ordinary method of estimating fructose by means of the polarimeter is unsatisfactory, on account of the low polarimetric readings obtained.

Various iodometric methods for the estimation of aldoses in presence of ketoses have been tested, and none of them has been found to give quantitative results. Experiments with pure sugars show that while the oxidation of glucose is never complete there is always a partial action on fructose. The attempt to find experimental conditions which will ensure the complete oxidation of the glucose while the fructose remains unchanged has been unsuccessful.

It is suggested that the results obtained are due to the behaviour of sugars towards dilute alkalis, which has been investigated by Lobry de Bruyn and van Ekenstein.

In using either the method of Colin and Lièvin or that of Willstätter and Schudel, a definite and constant weight of iodine always reacts with a given weight of glucose. This weight of iodine is not affected by changes in the amount of alkali present, or by the presence of other sugars in the solution. The above statement is also true of fructose. It is therefore possible, using either of the above methods, to calculate from the copper-reducing power of a solution containing glucose and fructose, and from its action on iodine in alkaline solution, the amount of each sugar present in the solution.

The method has also been applied to other sugars. It is shown that each sugar has its own characteristic iodine value. The behaviour of rhamnose is abnormal. The three disaccharides investigated give very different results. Saccharose is not oxidised under the conditions of the experiment. Lactose and maltose are both oxidised, but lactose requires twice as much iodine as maltose.

The work described above has been undertaken in connection with investigations of Cold Storage problems which are being carried out for the Food Investigation Board of the Department of Scientific and Industrial Research.

#### REFERENCES.

- Bougault (1917). *J. Pharm. Chim.* (vii), **16**, 97.  
Colin and Lièvin (1918). *Bull. Soc. Chim.* **47**, 403.  
Lobry de Bruyn and van Ekenstein (1895). *Rec. Trav. Chim.* **14**, 156, 203.  
— (1896). *Rev. Trav. Chim.* **15**, 92.  
— (1897). *Rec. Trav. Chim.* **16**, 257.  
Romijn (1897). *Zeitsch. anal. Chem.* **36**, 18, 349.  
Willstätter and Schudel (1918). *Ber.* **51**, 780.

## XXVII. DIETETIC EXPERIMENTS WITH FROGS.

BY ARTHUR HARDEN AND SYLVESTER SOLOMON ZILVA.

*From the Biochemical Department, Lister Institute.*

*(Received March 8th, 1920.)*

THE preliminary experiments of which the results are recorded in the present paper were instituted with the object of ascertaining whether the accessory food factors had any influence on the development and growth of the frog. Both the larval and adult forms of *Rana temporaria* were investigated.

### 1. *Experiments with Tadpoles.*

These experiments were not entirely satisfactory and are therefore not described in detail. Sets of 6 tadpoles were kept in 200 cc. of boiled tap-water in Erlenmeyer flasks, and were transferred into fresh water every day. The diets were added to the water on five days during the week. They consisted of a basal diet of purified caseinogen (20 %), starch (75 %) and salts (5 %), to which the accessory factors were added in the form of fat-soluble *A*, 4 drops of butter-fat; water-soluble *B*, 0.5 cc. of 1 % marmite; anti-scorbutic *C*, 1 cc. of lemon juice freed from citric acid. For comparison a set of 11 tadpoles was kept in a small aquarium, to which small pieces of raw meat were occasionally added. These control animals thrived well, passed through the normal stages and emerged as frogs in the course of 6–9 weeks (with the exception of three which were accidentally lost). On the other hand the experimental animals on the complete diet (Basal + *A* + *B* + *C*) did not succeed nearly so well—only 2 out of 6 reaching the mature state, the others dying on the 15th, 40th, 81st and 155th day in various stages. Similar results were obtained with the diets Basal + *A* and Basal + *A* + *B*, two frogs being produced in the former and one in the latter case.

It is obvious that under these circumstances observations on rates of growth are of no great value and it is proposed to repeat the experiments when a thoroughly satisfactory form of experimental diet has been found. It may however be pointed out that no tadpole reached the mature state which had not had butter-fat in its diet.

The experiments of Emmett and Allen [1919] which were published while our experiments were in progress show that the problem is a very difficult one as the rate of growth and development is greatly affected by the amount and nature of the fat, carbohydrate and protein as well as by the presence or absence of accessory food factors.

2. *Experiments with adult Frogs.*

The frogs were kept in glass basins covered by book muslin and containing a shallow layer of water. They were fed by hand two or three times a week, a bolus of about 0.5–1 g. of the diet being administered. The diets employed were identical with those commonly used for dietetic experiments. The basal mixture consisted of a mixture of caseinogen (20 %), starch (75 %) and salts (5 %), and the accessory factors were added in the form of clarified butter-fat (*A*), autolysed yeast (*B*) and lemon juice freed from citric acid (*C*).

The following diets were used, the composition being in every case adjusted to give a mass of suitable consistency.

	Control	<i>ABC</i>	<i>AB</i>	<i>AC</i>	<i>BC</i>	<i>A</i>	<i>B</i>	<i>C</i>
Basal mixture, g. ...	10	10	10	10	10	10	10	10
Butter-fat, g. ...	—	1.6	1.85	1.8	—	1.3	—	—
Autolysed yeast, cc. ...	—	4.7	5	—	4	—	4	—
Anti-scorbutic equivalent to lemon juice, cc. ...	—	4.7	—	5	4	—	—	4
Olive oil, cc. ...	3.1	0.24	2.5	1.6	0.6	—	3.1	3.1
Water, cc. ...	5	—	—	—	—	4	2	2

A disturbing factor was introduced by the fact that some of the animals were attacked by a fungoid disease which caused ulceration of the flesh. The animals which suffered in this way are noted in the tables of results. The frogs were weighed weekly, but it was found that a considerable casual variation occurred. Most of them gained weight during the course of the experiment.

Diet	No.	Orig. wt.		Final wt.		Duration of experiment	Result
		g.	g.	g.	g.	days	
<i>ABC</i>	...	5	18.8	26.4	371	371	Survived
	...	10	10	26.8	369	369	"
	...	11	10.2	14	54	54	Disease; chloroformed
	...	27	10	21.4	348	348	Survived
<i>BC</i>	...	2	38	47.5	361	361	"
	...	4	23.1	35.5	339	339	Disease; died
	...	23	10.7	14.5	267	267	" "
<i>AC</i>	...	15	17.3	21	82	82	Died
	...	16	12.7	18	74	74	"
	...	13*	17	23.2	155	155	"
	...	3	17	30.8	42	42	"
<i>AB</i>	...	17	15.9	32.1	369	369	Survived
<i>A</i>	...	21	17.5	24.5	56	56	Died
	...	25	9.8	11.8	37	37	"
<i>B</i>	...	19	14.6	20	70	70	"
	...	20	15	27.6	369	369	Survived
	...	22*	18.2	25.3	91	91	Died
<i>C</i>	...	26*	13.9	20.6	73	73	"
	...	6	10.2	15.1	52	52	"
	...	7	27.1	31	112	112	Disease; died
	...	12*	9.2	19.1	26	26	Died
Control	...	9*	13.2	12.8	22	22	"
	...	28	12.4	16.8	111	111	"

In the cases marked \* (Nos. 13, 22, 26, 12 and 9) the animals took up a characteristic attitude before death, which is often adopted by moribund frogs, with the hind legs extended and the fore part of the body raised on the fore limbs.

In several cases (Nos. 6, 15, 16, 22, 25, 26) a nerve was removed and submitted to Miss E. M. Tozer to whom we are indebted for their microscopic examination. In only one case (No. 6) was any abnormality observable. The nerve (the sciatic) had very few nodes and the sheath had an eroded appearance.

In spite of the small number of animals on some of the diets, the following conclusions appear to be justified:

1. All the 7 frogs deprived of both *A* and *B* died within four months. Of these animals 4 showed the "extended legs" symptom; one suffered from the fungus disease.

2. Of the 5 animals receiving both *A* and *B*, with or without *C*, only one died during a whole year and this one was attacked by the fungus.

3. Of the 6 animals receiving *A* (with or without *C*) but not *B*, all died within 155 days, one showing the extended legs symptom.

4. Of the 5 animals receiving *B* (with or without *C*) but not *A*, 4 survived for 250 days. Two of these subsequently died (at 267 and 339 days), both having shown signs of external haemorrhage.

5. Considering the whole set of animals, 11 received *A* in their diet and 12 did not. After 6 months 7 of those receiving *A* and 9 of those not receiving *A* were dead. On the other hand 10 received *B* in their diet and 13 did not. After 6 months all those not receiving *B* and only 2 of those receiving *B* were dead.

This affords clear evidence that *B* is necessary for the continued existence of adult frogs. On the other hand the effect of absence of *A* does not show itself definitely within 6 months. The absence of *C* in these experiments did not produce any definite effect.

In order to obtain definite confirmation of this result 6 frogs were kept on a complete *ABC* diet and 6 on an *AC* diet, with the following results:

Diet	No.	Orig. wt.	Final wt.	Duration of experiment		Result	
				days			
<i>ABC</i>	...	...	29	26.9	28.7	273	Disease; survived
"	...	...	30	26.9	28.3	273	Survived
"	...	...	31	24	20.8	154	Disease; died
"	...	...	32	30.2	30.9	273	Survived
"	...	...	33	23.2	23.9	273	"
"	...	...	34	21.2	21.4	272	Disease; survived
<i>AC</i>	...	...	35	26.7	28.5	255	Died
"	...	...	36	26.7	26.5	241	"
"	...	...	37	27.3	27.3	229	"
"	...	...	38	24.7	22.2	146	Disease; chloroformed
"	...	...	39	20.7	19.8	149	Died
"	...	...	40	18.6	19.8	219	"

In this set, not considering two frogs (31 and 38), one on each diet, which died while suffering from the fungus, we have 5 on *ABC* diet, all of which survived 272 days, and 5 on *AC* diet, all of which succumbed within 255 days, the actual duration of life being 255, 241, 229, 149 and 219 days respectively.

Characteristic symptoms were only noticed in two cases. No. 39 adopted the "extended legs" position 3 days before death. No. 40 became rigid after being fed on the 14th July, gradually recovered, but had a second similar attack, preceded by a series of convulsions on the 17th July, and was found dead on the 18th. The sciatic nerve was examined in both these cases with the result that the nerve of No. 39 was normal, whereas that of No. 40 contained several degenerating fibres.

These results show that the addition of autolysed yeast juice to the *AC* diet employed in these experiments greatly prolongs the life of the frog. This is most probably due to the presence of the water-soluble *B* factor, but it must be remembered that the autolysed yeast juice also contains a considerable proportion of easily absorbable nitrogen which may also have some effect. Further experiments in which a basal diet richer in protein is being employed are however yielding precisely similar results to those described above.

Part of the expenses of the research was defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

#### SUMMARY.

A series of feeding experiments with synthetic diets indicates that the water-soluble *B* factor is necessary for the continued life of the adult frog.

#### REFERENCE.

Emmett and Allen (1919). *J. Biol. Chem.* **38**, 325.

## XXVIII. A GAS ANALYSIS APPARATUS ACCURATE TO 0.001 %. MAINLY DESIGNED FOR RESPIRATORY EXCHANGE WORK.

BY AUGUST KROGH.

*From the Laboratory of Zoophysiology, University of Copenhagen.*

*(Received August 26th, 1919.)*

THE safest and by far the simplest method of determining the total respiratory exchange of large animals and of man is that introduced by Jaquet, according to which a respiration chamber is ventilated by a current of pure air which is maintained and measured by a gas meter working as a pump. From the current of outgoing air an average sample is collected and analysed, and from the analytical data combined with the ventilation the gas exchange can be calculated. As the determination of the ventilation can easily be made more than accurate enough for the purpose the total accuracy depends on the sampling and the gas analysis.

In gas analysis by current methods [Haldane, 1912; Petterson-Sondén, described by Sondén and Tigerstedt, 1895], the carbon dioxide can be determined with any desired accuracy (to 0.0005 %), but to obtain accurate oxygen determinations is more difficult and even for the best instruments it is not claimed that they can accomplish more than 0.005 % while in actual practice errors of 0.01 % are common enough.

As the oxygen deficit in the air leaving a respiration chamber cannot be increased beyond 2 % and ought not for several reasons to be increased beyond 1 % the accuracy obtainable is scarcely satisfactory, though it must be admitted that, even as it stands, the accuracy of the Jaquet method is certainly not inferior but on the whole superior to that of the Regnault method, the more so as the respiratory quotients obtained with large Regnault apparatus are rather unreliable as shown by the researches of Carpenter [1915].

A great deal would be gained if the accuracy of the gas analysis could be increased sufficiently to allow an oxygen deficit of 0.5-1 % to be analysed without causing errors on the gas exchange exceeding 0.2 %, and this is what I have endeavoured to attain.

The sources of error which prevent the oxygen analyses from being as accurate as the CO<sub>2</sub> determinations are intimately connected with the presence of water and dirt in the gas burette. Water must be present to insure the saturation of the gas with vapour, and dirt will accumulate rather rapidly from the contact of the mercury with the rubber tubing and with oxygen.

In analyses of atmospheric air or the outgoing air from respiration chambers the volume of the burette between the 0 mark at the lower end and the reading after the absorption of oxygen is always about 21 %, and this volume is usually partly expanded into a bulb, as a cylindrical stem with divisions capable of being read to 0.001 % would become inconveniently long. The amount of water and dirt present in this bulb cannot be constant, and this is the chief reason why individual analyses differ. If the burette is kept scrupulously clean and is moistened with water in a constant manner before each analysis, as done by Benedict [1912], the variation of the results can be kept well below  $\pm 0.01$  %, and the results will remain comparable over long periods, but when this precaution is not taken the variations become larger, and two analyses of the same air made with an interval during which 5–10 other analyses are carried out may differ considerably. This is due, as pointed out before by A. and M. Krogh [1913], chiefly to the amount of water in the burette which does not remain constant, but decreases steadily by distillation into the absorption pipettes which contain strong solutions of alkali, possessing low vapour tensions<sup>1</sup>. I attempted to obviate the difficulties by measuring the air in a state of complete dryness, but this involved so many difficulties that I had to abandon the plan.

#### DESCRIPTION OF APPARATUS.

I have now attained the end desired by two improvements on the usual arrangements.

The most important of these is to use three separate gas burettes (Fig. 1 (1, 2 and 3)) of which one (1) is employed exclusively for moving the air to and from the absorption pipettes, while the second (2) is of a suitable size to measure the air before and after the absorption of  $\text{CO}_2$  and the third (3) to measure it after the absorption of  $\text{O}_2$ . The water vapour necessary for saturating the sample of air, when it has become partially dried in the absorption pipettes, will be supplied by the first burette, and the variations in the amount of water present in this burette will have no influence upon the accuracy of the measurements. The two other burettes (2 and 3) contain just enough water to insure that the sample remains saturated. This water cannot interfere with the readings, because it does not enter the divided stems of the burettes, and its amount will remain constant over a long series of analyses, because the air is always saturated before being brought in contact with it.

The second improvement is that in my apparatus the mercury is raised and lowered in the burettes not by raising and lowering a mercury reservoir but by means of air pressure, an arrangement which obviates the use of rubber connections between the burettes and the reservoirs and moreover facilitates considerably the analytical manipulations.

<sup>1</sup> The soda or potash lye for  $\text{CO}_2$  absorption should never be more concentrated than 10 % which is amply sufficient to absorb the  $\text{CO}_2$  from an air sample completely and rapidly.

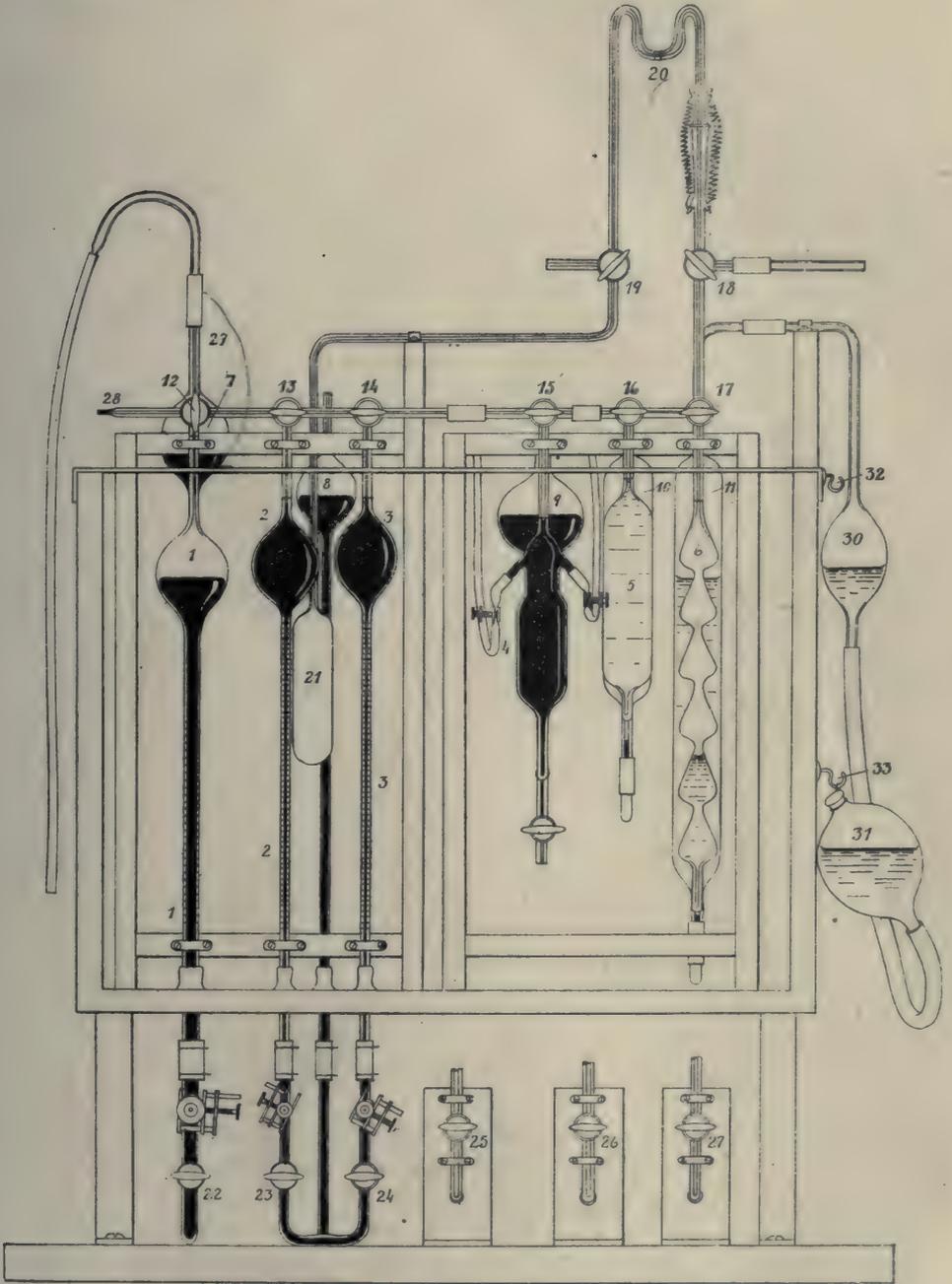


Fig. 1.

The details of the arrangement for raising and lowering the mercury in one of the burettes is shown in Fig. 2 and parts of it also in Fig. 1.

In Fig. 2 (1) is the burette which is mounted together with the other two and the corresponding reservoirs on a wooden stand (2, comp. Fig. 1). The stems of the burettes and reservoirs are carried through short wide metal tubes (3) in the bottom of the water bath, and a watertight connection which does not expose the glass tubes to any undue strain is brought about by means of soft rubber in the manner shown (4). Below the water bath the burette is connected with the reservoir (9) by means of the U-tube (5) which is provided with the stopcock (6) and the side tube (7). On the side tube is a short piece of stout rubber tubing stoppered with a glass stopper (8). By a screw clip on the rubber a fine adjustment of the level of mercury can be brought about, when the tap (6) is closed. The top of the reservoir (9) is connected by the wide  $\cap$ -tube (10) and rubber tubing (11) to the three-way tap (12)<sup>1</sup>. The  $\cap$ -tube (10) is interposed to prevent an accidental sucking over of the mercury. The tap (12) is connected through the bottle (13) to a filter pump (14). The same bottle is connected through separate three-way taps with the other reservoirs.

When the tap (12) is open to the atmosphere as shown in Fig. 2 the mercury can rise in the burette to the zero mark at the top, and when by turning tap (12) 90° connection is established with the filter pump, the mercury in the burette can be lowered. The height of the reservoir (9) and the quantity of mercury put into it are so adjusted that the position of equilibrium will bring the meniscus in the burette very near to the zero mark.

Water which is just acidulated by sulphuric acid is introduced into the burettes by taking out the stopcocks (13) and (14) (Fig. 1). In burette 1 about 30 cubic mm. are introduced, and this quantity will last for about 20 analyses (of CO<sub>2</sub> and O<sub>2</sub>) corresponding to a loss by distillation of 1.5 cubic mm. during each analysis. In burettes 2 and 3 the quantity of water must be so small that it will always remain in the bulb and not be carried down into the stem, where it would hinder the accurate reading of the mercury meniscus. On the other hand so much water must be present that it is visible as a distinct line along the mercury meniscus when this is anywhere in the bulb. Air is never introduced into any of these burettes directly from the absorption pipettes or from outside but always from burette 1 where it is saturated with moisture.

Burette 2 will in the course of time become a little dirty while 3 will remain clean. The dirt must therefore be caused mainly by oxidation, but we have not been able to detect any diminution in volume when air is left in the burette for a number of minutes.

When a burette has to be cleaned the mercury is lowered to a point just below the side tube (7, Fig. 2) the stopper (8) is removed and a glass tube

<sup>1</sup> The taps (12) are not really placed behind the burettes and reservoirs, but on a line with the taps (6) (see Fig. 1) so as to be easily accessible.

bent at a right angle inserted. Tube (29, Fig. 1) is connected through tap (25) with the filter pump and cleaning fluid (potassium dichromate in dilute sulphuric acid) drawn up. The burette is finally washed with distilled water and dried by a current of air filtered through cotton wool.

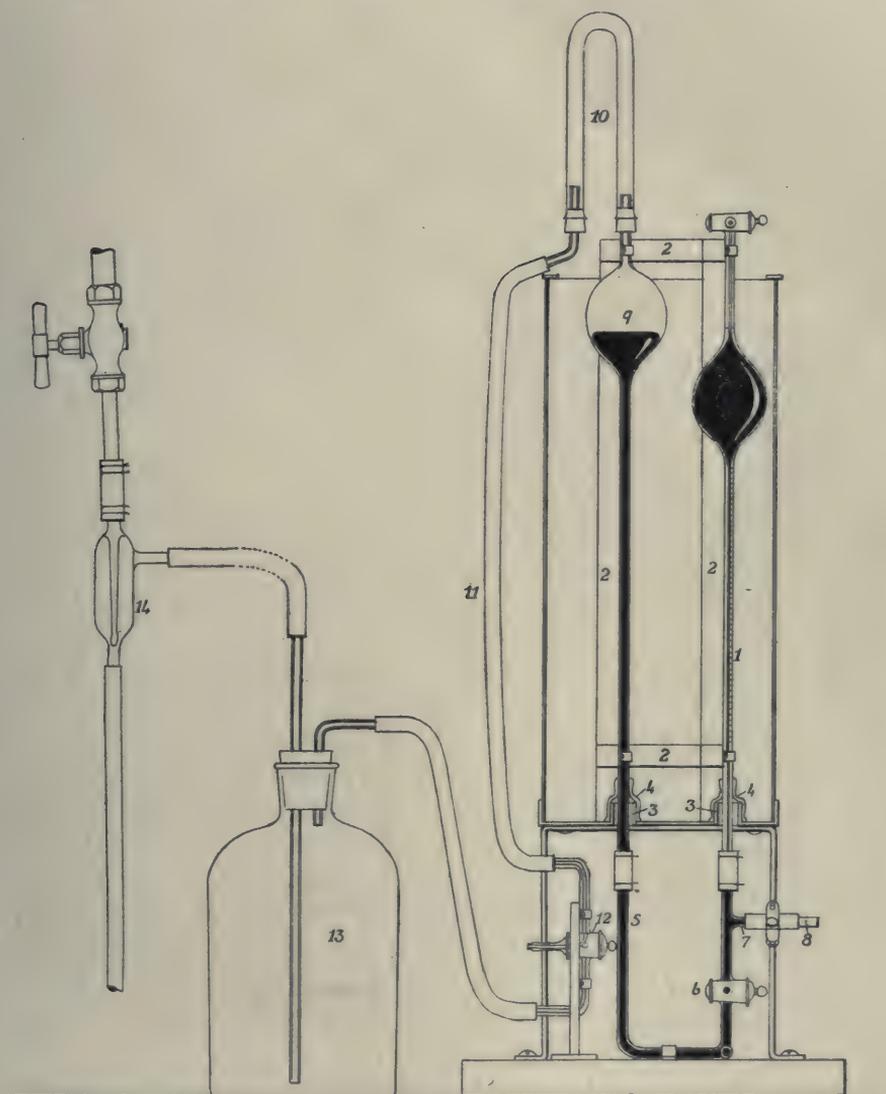


Fig. 2.

The  $\text{CO}_2$  absorption pipette (10, Fig. 1), the  $\text{O}_2$  absorption pipette (11) and the combustion pipette (9) are mounted together on a wooden stand placed in the same water bath with the burettes. The arrangement of the pipettes has been dictated by several considerations. The main point is to avoid any diffusion of oxygen beyond tap (17) before the absorption takes

place, and this end is fully attained in ordinary analyses in which only  $\text{CO}_2$  and  $\text{O}_2$  are determined. When, after the absorption of  $\text{CO}_2$ , connection is established through taps (16, 17 and 18) with the pressure index (20) oxygen will diffuse into the space between taps (16) and (17) but before it can get beyond tap (17) this tap is closed and is not opened again before all (or practically all) the oxygen has been absorbed. If, however, repeated absorptions of  $\text{CO}_2$  and corresponding readings become necessary, as in combustion analyses, the possibility cannot be avoided that a trace of oxygen may get beyond tap (17) and escape absorption. Combustion analysis cannot therefore be carried out together with oxygen analysis on the same sample if the greatest possible accuracy of the latter determination is desired.

As the air between tap (17) and the pressure index is always pure nitrogen there is not the slightest danger of contamination of the analysis from this side.

Logically the combustion pipette (4) ought to be placed between the pipettes for  $\text{CO}_2$  (5) and for  $\text{O}_2$  absorption (6), since combustion is always carried out after the absorption of  $\text{CO}_2$ . I did not venture however to have the heavy (about 1 kg.) combustion pipette fused on to the other two and therefore preferred to arrange it as shown.

The  $\text{CO}_2$  absorption pipette (5) is a plain cylinder with a cylindrical reservoir (10). It is charged with about 10 % potassium hydroxide, carefully saturated with air at the temperature of the bath. It is filled to such a height that the fluid will stand at the mark when the pressures on both sides are equal.

The  $\text{O}_2$  absorption pipette (6) is of a peculiar shape designed to insure a maximum of absorption without any danger of small air bubbles being trapped as often happens in pipettes filled with vertical glass tubes. The absorbing fluid for oxygen is Haldane's potash pyrogallate (23 g. of pyrogallic acid of the best quality dissolved in 230 cc. saturated potassium hydroxide, specific gravity 1.55). When air is introduced rapidly into the pipette a considerable quantity of the viscous fluid remains above each contraction and flows slowly downwards, thus providing a large absorbing surface which is automatically renewed so long as the fluid is flowing down. This arrangement shortens considerably the time necessary for complete absorption. One charge of the pipette will last for about 150 analyses before the absorption becomes sluggish.

The construction of the combustion pipette (4, Fig. 1) has been altered after the execution of Fig. 1 and the final shape is shown in Fig. 3. A vertical spiral (1) of fine platinum wire (0.1 mm.) is arranged between two stout wires. Current is supplied through a rheostat from a couple of accumulator cells or from the lighting installation. One pole (2) is connected with the mercury in the pipette and the other (3) through a sealed glass tube with the upper one of the two wires (4). The vertical heated wire, which is placed as far down in the pipette as possible, insures a rapid circulation of the inclosed air,

and the combustion can be completed therefore within a very short space of time—one minute or less.

As the available pressure in the burettes is insufficient to drive an air sample over into the combustion pipette the reservoir (5, Fig. 3) is connected through the three-way tap (27, Fig. 1) with the filter pump.

#### METHOD OF CONDUCTING AN ANALYSIS.

An ordinary analysis involving the determination of  $\text{CO}_2$  and  $\text{O}_2$  is performed as follows (Fig. 1). We suppose all the fluids to be brought exactly to the zero marks on the burettes and pipettes, the three-way taps (13–16) to be turned into their normal position  $\perp$ , the index drop of petroleum (20) to stand at 0, the apparatus to be filled with nitrogen and the pressures in the compensating vessel (21) and the apparatus to be equal. We have the sample of air to be analysed in a glass vessel under a positive pressure. The sampling vessel is connected by means of narrow lead or rubber tubing with the horizontal tube to the left (28). The four-way tap (12) is turned so as to connect the tubes (28) and (29)  $\perp$ , and the connections are washed out with the air to be analysed. The tap (21) is next turned so as to connect the sample with the burette (1)  $\Gamma$ . The mercury reservoir (7) is connected with the filter pump through the tap (25). The tap (22) on the burette is opened and the sample drawn in. When the mercury reaches the 50 cc. mark on the burette the tap (22) is closed and (25) opened to the atmosphere. A slight positive pressure should now obtain in the sampling vessel and the burette (1). The lead tube is disconnected from the sampling vessel and while the burette (1) is thus open to the atmosphere the excess of pressure blows itself off. The tap (12) is turned so as to connect the burette (1) with the rest of the apparatus  $\Gamma$ . Tap (13) is turned from the normal position  $\perp$  into this  $\perp$ , thereby establishing connection between

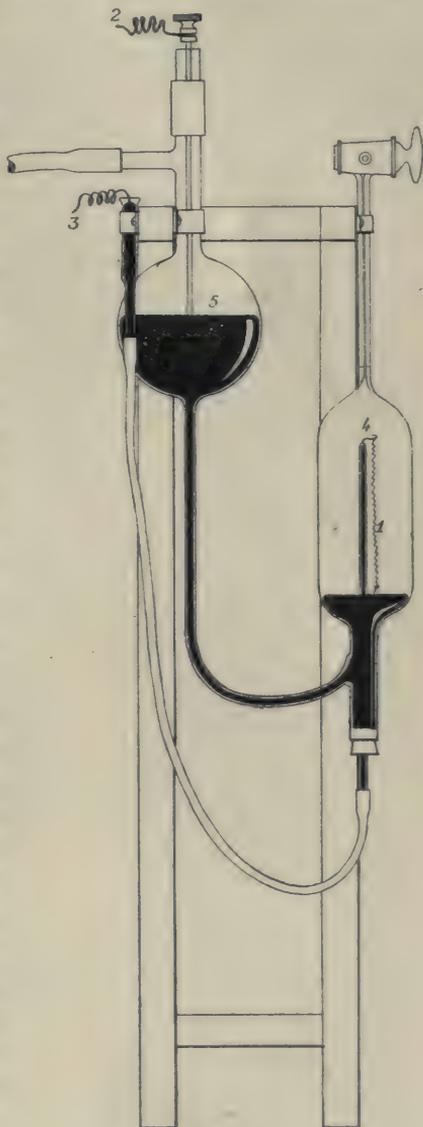


Fig. 3.

the burettes (1) and (2). Taps (22) and (23) are opened, and by opening the tap (26) to the filter pump the sample is transferred to burette (2). When the mercury in (2) has reached the 50 cc. mark on the stem the tap (23) is closed and (26) opened to the atmosphere, and thereupon tap (22) is closed and the water on top of the mercury in burette (1) brought to the zero mark just below tap (12). Tap (13) is turned to the position  $\top$  and the taps (18) and (19) connecting the apparatus and the compensating vessel with the pressure index are opened alternately, while the index is watched and the level of the mercury in burette (2) adjusted according to its movements. Finally both taps (19 and 18) are opened ( $\top \dashv$ ), the index set at 0, the burette read and the taps (19 and 18) closed again ( $\wedge \lambda$ ).

The tap (16) in the potash pipette is now turned from  $\perp$  to  $\dashv$  and tap (23) opened in consequence of which the sample is driven over into the pipette (5). When the mercury in (2) reaches up to the zero mark tap (23) is closed and usually the mercury is raised by the adjusting screw clip to a little below the tap (13). The tap (22) on burette (1) is now opened and by alternately opening the tap (25) to the filter pump and to the atmosphere the mercury in (1) is lowered and raised 4 times, about 10–15 cc. each time to wash out the connections and insure the complete absorption of the  $\text{CO}_2$ . This being finished the sample is drawn into burette (1). The potash in (5) is set at the zero mark and the tap (16) closed ( $\succ$ ). In just the same way as before the sample is transferred from (1) to (2), connection is cautiously established through the index with the compensating vessel, the level of mercury in (2) adjusted and read.

Tap (17) is turned so as to establish connection with the  $\text{O}_2$  absorption pipette (6)  $\neg$ , and by opening tap (23) the sample is transferred to this pipette. As the available mercury pressure is not sufficient to drive the whole of the sample over into (6) the glass bulb (31) is lowered from the hook (32) to (33). The water in bulbs (30) and (31) protects the pyrogallate solution from contact with the atmosphere. As before the mercury in (2) is brought up to a point a little below the tap (13). By means of burette (1) the sample is taken backwards and forwards to hasten the absorption of oxygen. I have found it most effective to keep it constantly moving. After a suitable number of movements (usually 6) the sample is taken back into (1) and the tap (17) closed ( $\succ$ ). Tap (16) is now opened ( $\dashv$ ) and the sample taken once into the potash pipette (5) in order to "fetch" the air between the zero mark and the tap (16). Thereupon it is again taken into (1). The potash is set at zero and (16) turned to the normal position ( $\perp$ ). Tap (17) is opened ( $\neg$ ) and the sample again carried forwards and backwards between burette (1) and pipette (6) to absorb the last traces of oxygen. Finally the sample is taken back into (1), the bulb (31) is raised to (32), the absorbing fluid in (6) set at zero and the tap (17) closed. The water on top of the mercury in burette (2) is now set at zero, the tap (13) turned to the normal position ( $\perp$ ) and thereupon the sample is transferred from (1) through the tap (14) to burette (3), where it is measured for the third time.

An analysis of  $\text{CO}_2$  and  $\text{O}_2$  can be made in about 16 minutes. Before the next analysis is made the taps (18) and (19) are opened to the atmosphere ( $\top \top$ ) and the nitrogen in burette (3) forced out through (18). The water in (3) is set at zero, the tap (14) turned to the normal position while tap (12) is turned so as to establish connection between tubes (28) and (29) ( $\perp$ ). When finally taps (19) and (18) are closed ( $\wedge \lambda$ ) the apparatus is ready for the next analysis.

When a series of analyses is finished the nitrogen is stored in pipette (6), and all taps are closed. Before a new series of analyses is begun all menisci are set and finally the nitrogen is driven from burette (1) through the tap (18), which is turned into position  $\perp$ , and the index tube out through the tap (19) ( $\perp$ ). This is done to insure that the whole system is filled with nitrogen.

A combustion analysis is performed as follows: When the air sample has been measured in burette (2)  $\text{CO}_2$  is absorbed and a second reading is taken. Thereupon connection is established between (2) and the combustion pipette (4, Fig. 1). Tap (23) is opened and afterwards tap (27) connecting the reservoir (9) with the filter pump. Between the reservoir and the tap (27) a piece of very narrow tubing is intercalated. Otherwise the movements of the mercury between the pipette (4) and the reservoir (9) would become too violent. When the mercury in pipette (4) has been lowered sufficiently the reservoir is turned off from the pump and the atmosphere and current put on to the platinum wire for one minute. Hydrogen and carbon monoxide will burn readily when the wire is just visibly glowing, but methane will require a bright red heat. A few cc. air can be drawn backwards and forwards between burette (1) and pipette (4), but with the very low percentage of combustible gases which may be present in an ordinary analysis (0-0.01 %) this will scarcely be necessary. After 1 minute's heating the current is shut off and tap (27) opened to the atmosphere. The sample is drawn into burette (1), the meniscus in pipette (4) set, tap (15) closed, the sample transferred to burette (2) and measured. If there is a contraction in volume an absorption of  $\text{CO}_2$  is thereupon undertaken.

The volume of air between tap (15) and the zero mark in (5) cannot be subjected to combustion. It is however only about 0.3 % of the total sample. If therefore the percentage of combustible gases does not exceed 0.3 the error committed will be imperceptible. With a higher percentage it can easily be allowed for.

#### DIVISION OF BURETTES. CALCULATION OF ANALYSES.

The stems of burettes 2 and 3 have been selected by myself from a large number of glass tubes and are very nearly cylindrical in bore. Their internal diameter is about 2 mm. and they are divided in ten thousandth parts of 50 cc. The distance between two divisions is approximately 1.6 mm. and it is easy by means of a lens to judge  $\frac{1}{10}$  or even  $\frac{1}{20}$  of the division. The burettes have been divided and marked with a view to facilitate as much as possible

the calculation of the analyses and especially to make it possible to perform all calculations by means of a simple slide rule.

On burette 2 the point corresponding to 50 cc. has been marked 100, and when the  $\text{CO}_2$  percentage in the air analysed is less than 1 (100 divisions) the sample taken should correspond as nearly as possible to this volume. If it is desirable, however, a larger volume can be taken, up to 180. Supposing the first reading of the burette to be  $100 + a$  and the second after absorption of the  $\text{CO}_2$  to be  $100 + b$  we have the  $\text{CO}_2$  directly in hundredths % as  $a - b$ . If the initial reading differs considerably from 100 we must apply a correction which is  $-\frac{a(a-b)}{10000}$ .

*Example.* Initial reading 170.0, second reading 20.0. Correction  $-\frac{70 \cdot 150}{10000} = 1.05$ . The corrected figure for  $\text{CO}_2$  is therefore  $170 - 20 - 1.05 = 148.95$ . When the initial reading is between 90 and 110 and the  $\text{CO}_2$  is 50 or less the correction can be omitted altogether, viz.  $\frac{10 \cdot 50}{10000} = 0.05$ . Usually initial readings between 97 and 103 can be secured.

In burette 3 the point corresponding to 79.000 % of 50 cc. is marked 0. If the initial reading is  $100 + a$  and the third reading (after absorption of the oxygen) is  $c$  we have the nitrogen in hundredths % =  $7900 + c - \frac{79}{100} a$ .

*Example:*

$$a = 2.4$$

$$c = 11.7$$

$$\frac{79}{100} a = 1.9$$

$$N_2 = 9.8 \quad \text{Nitrogen percentage } 79.098$$

If the first reading differs considerably from 100 this figure must be corrected by  $-\frac{N_2 \cdot a}{10000}$ .

*Example:*

First reading	175.4	$\text{CO}_2$	$148.3 - \frac{148.3 \times 75.4}{10000} = 147.2$
Second reading	27.1		
Third reading	120.3	$N_2$	$60.8 - \frac{60.8 \times 75.4}{10000} = 60.35$
$\frac{79}{100} a$	59.5		

Or in per cent.  $\text{CO}_2 = 1.472$  %,  $N_2 = 79.6035$  % and  $\text{O}_2 = 18.9245$  %.

In gas exchange work it is never necessary to figure out the actual percentages, because differences only are dealt with.

*Example: Analyses of*

atmospheric air		outgoing air	
1.	99.4	1.	100.1
2.	96.05	2.	42.0
	$\text{CO}_2$ 3.35		$\text{CO}_2$ 58.1
3.	3.95	3.	12.9
	$N_2$ 4.4		$N_2$ 12.8
$\frac{79}{100} a$	+45	$\frac{79}{100} a$	-1
	$\text{CO}_2 + N_2$ 7.75		$\text{CO}_2 + N_2$ 70.9

Differences

$$\begin{aligned} \text{CO}_2 \text{ out.} - \text{CO}_2 \text{ in.} & \quad 58.1 - 3.35 = 54.75 \text{ hundredths of } 1\% \\ \text{N}_2 \text{ out.} - \text{N}_2 \text{ in.} & \quad 12.8 - 4.4 = 8.4 \quad \text{''} \quad \text{''} \\ \text{O}_2 \text{ in.} - \text{O}_2 \text{ out.} & \quad 70.9 - 7.75 = 63.15 \quad \text{''} \quad \text{''} \end{aligned}$$

The oxygen deficit must be corrected in the usual way, assuming the quantity of nitrogen in the outgoing and ingoing air to be the same. The

correction on the ingoing oxygen ( $O_i$ ) is  $O_i \frac{79.00 + N_o}{79.00 + N_i}$ . As the fraction  $\frac{79.00 + N_o}{79.00 + N_i}$  differs very little from unity we can without introducing any perceptible error transform to  $\frac{79.00 + N_o - N_i}{79.00}$  or the corrected ingoing oxygen  $O_i + \frac{O_i}{79.00} (N_o - N_i)$ . For the purposes of the correction  $O_i$  can be taken as constant and  $\frac{O_i}{79} = 0.265$ .

In the example considered the correction on the oxygen deficit is

$$0.265 \times 8.4 = 2.2,$$

the corrected deficit  $63.15 + 2.2 = 65.35$  and the respiratory quotient

$$\frac{54.75}{65.35} = 0.838.$$

ACCURACY OF RELATIVE ANALYSES FOR GAS EXCHANGE WORK.

In gas exchange work the only practical requirement is that analyses of the same air made within a short space of time (say one day) should agree, but whether the apparatus will give identical results when the same air is analysed repeatedly over a prolonged period is irrelevant. That the apparatus fulfils the conditions of gas exchange work has been thoroughly tested by making regular double determinations of the ingoing atmospheric air and a large number of samples of the outgoing air in actual experiments with a Jaquet respiration chamber. The following series of examples have been selected at random from a very large number and show the accuracy attained in respiratory exchange work when the analyses are performed as rapidly as possible (between 3 and 4 per hour).

Table I. Double determinations.

Date	Atmospheric air			Outgoing air		
	CO <sub>2</sub> %	N <sub>2</sub> %	O <sub>2</sub> deficit %	CO <sub>2</sub> %	N <sub>2</sub> %	O <sub>2</sub> deficit %
	0.0	79.0	0.0	0.	79.	0.
8. v. 1917	335	445	78	5775	1315	709
	335	44	775	5785	1295	708
10. v.	32	425	745	5625	1315	694
	33	395	725	563	1315	6945
11. v.	35	42	77	552	125	677
	33	41	74	553	126	679
14. v.	32	405	725	5785	094	6725
	32	—	—	5775	0935	671
15. v.	315	42	735	600	0775	6775
	33	42	75	600	078	678
17. v.	30	41	71	543	089	632
	305	41	715	544	088	632
18. v.	31	40	71	542	127	669
	32	385	705	541	126	667
21. v.	31	465	775	505	192	697
	315	39	705	506	190	696
22. v.	325	485	81	481	1895	6705
	315	45	765	4805	1895	670
24. v.	35	47	82	499	183	682
	30	46	76	4995	184	6835
25. v.	33	45	78	506	1675	6735
	31	45	76	5055	1685	674
Average difference between double determinations	0.0014	0.0012	0.0025	0.0008	0.0009	0.001

Table II. Double determinations.

Date	Atmospheric air			Outgoing air								
	CO <sub>2</sub> %	N <sub>2</sub> %	O <sub>2</sub> deficit %	CO <sub>2</sub> %	N <sub>2</sub> %	O <sub>2</sub> deficit %						
	0·0	79·0	0·0	0·	79·	0·						
7. ii. 1918	34	44	78	4655	138	6035						
	325	445	77	466	1375	6035						
8. ii.	315	51	825	4785	1525	631						
	315	525	84	4795	1545	634						
9. ii.	305	485	79	4575	149	6065						
	31	49	80	456	152	608						
10. ii.	30	525	825	4565	1535	610						
	31	51	82	457	1545	6115						
11. ii.	295	53	825	4685	1235	592						
	30	545	845	469	1225	5915						
12. ii.	38	57	95	485	102	587						
	375	58	955	486	101	587						
14. ii.	36	43	79	500	0705	5705						
	355	43	785	501	0705	5715						
15. ii.	34	455	795	4575	1115	569						
	34	465	805	457	1105	5675						
17. ii.	39	495	88	4605	158	6185						
	395	485	885	460	158	618						
	32	44	76	5125	0865	599						
	32	445	765	512	087	599						
Average difference between } double determinations							0·0005	0·0009	0·0009	0·0008	0·0010	0·0010

Table III. Double determinations.

		Atmospheric air								
Date	CO <sub>2</sub> %	N <sub>2</sub> %	O <sub>2</sub> deficit %	Date	CO <sub>2</sub> %	N <sub>2</sub> %	O <sub>2</sub> deficit %			
1918	0·0	79·0	0·0	1918	0·0	79·0	0·0			
5. iv.	325	555	88	16. iv.	31	485	795			
	32	555	875		305	48	785			
6. iv.	33	54	87	17. iv.	335	535	87			
	325	535	86		33	535	865			
7. iv.	32	54	86	19. iv.	30	55	85			
	325	555	88		305	545	85			
8. iv.	315	48	795	20. iv.	31	525	835			
	31	48	79		305	53	835			
9. iv.	32	40	72	21. iv.	30	525	825			
	32	42	74		295	525	82			
10. iv.	32	435	755	22. iv.	305	525	83			
	315	43	745		31	515	825			
11. iv.	315	465	78	23. iv.	30	525	825			
	32	455	775		305	54	845			
12. iv.	31	45	76	24. iv.	295	52	815			
	315	445	76		30	51	81			
13. iv.	315	49	805	25. iv.	315	515	83			
	315	48	795		32	51	83			
14. iv.	31	455	765	26. iv.	305	515	82			
	315	455	77		30	515	815			
15. iv.	31	48	79	Average } difference		0·0005	0·0006	0·0007		
	315	485	80							

The atmospheric air analysed was taken from a Copenhagen street about 20 cm. in front of a window in the laboratory. For the analyses given in Table I two separate samples were taken with an interval of 1-2 hours. Each

sample was taken in less than half a minute, and between the analyses from 2-10 analyses of outgoing air were made. For the analyses in Tables II and III one sample only was taken in six separate portions of about 20 cc. each during the two hour experiment, but one of the analyses was made before and one after a series of 3-6 samples of outgoing air.

The analyses show irregular variations from one day to another both in the  $\text{CO}_2$  and in the nitrogen percentages, and these variations are sufficiently large to make it necessary to analyse the ingoing atmospheric air each experimental day, if perfectly reliable results of the gas exchange determinations are to be secured.

The variations observed in the carbon dioxide percentage are real and due to the gas exchange of the town, but the variations in nitrogen are to a large extent of instrumental origin and represent variations in the amount of water present in the measuring burettes.

Further details and a discussion on the composition and possible variations of the atmospheric air have been reported in a separate publication [1920].

#### ABSOLUTE DETERMINATIONS OF OXYGEN AND NITROGEN.

While in a series of analyses of the same air extended over a prolonged period the  $\text{CO}_2$  percentage indicated by the apparatus will remain constant indefinitely, the nitrogen and oxygen may show irregular variations amounting to several thousandths of 1 %. When it is desired to avoid these and to make the determinations absolute, as for instance in investigations of the composition of pure atmospheric air, certain further arrangements and precautions are necessary.

1. The measuring burettes must be very carefully calibrated. The calibration is performed as follows. The burettes are disconnected from the tubing below, cleaned and dried completely by a current of air. A tap is put on to each by means of thick rubber tubing. The junction is made rigid by applying a bandage soaked in gypsum. The lower tube of the tap is drawn out to a point (not too fine) and marked with a line about 5 mm. above the point<sup>1</sup>. A basin containing mercury is arranged below the burette to be calibrated, and by means of burette 1 mercury is drawn up to the desired mark on the stem (care must be taken to avoid air between the tap and the burette). The basin is lowered until the line on the tap is just level with the mercury, the tap is then closed the burette read and the basin weighed. Thereupon the process is repeated, the mercury being drawn up to the zero mark at the top. The difference between the two weighings represents the mercury necessary to fill the burette.

<sup>1</sup> When a second apparatus of this type is built, the lower ends of the measuring burettes will be ground to a standard size, and a tap with a corresponding ground joint provided for calibration purposes.

I reproduce as an example the calibrations made in 1918:

Volumes in cc. at 20°.

Burette 2			Burette 3		
Date	T.	0 to 100.0 on stem	Date	T.	0 to 0.0 on stem
13	14.97°	50.0052	13	14.60°	39.4900
14	14.60°	50.0052	13	14.79°	39.4890
14	14.80°	50.0045	14	14.90°	39.4901
<hr/>			<hr/>		
Average 50.0050			39.4897		
Boring of tap 12 0.021			0.021		
<hr/>			<hr/>		
50.0260			39.5107		
<hr/>			<hr/>		
			$\frac{7.0}{100} \times 50.0260 = 39.5205$		
			Difference 0.0098 = 0.0195 %		

I have found that during the first year after the mounting of the apparatus the volumes of the burettes did not remain constant, but changes amounting to several hundredths of 1 % took place. During the second year and a half the changes observed were very slight, and the volumes have now, probably, become constant.

2. When the dry burettes have been connected with the reservoir and filled with pure and dry mercury a measured quantity of water acidulated with sulphuric acid is introduced into each and preferably 10 cubic mm. into burette 2 and 8 into 3 by which the relation between their volumes is not altered.

3. The water bath must be kept at a constant temperature, lower than that of the room—preferably about 15–16°. To accomplish this it is cooled by means of a current of tap water and heated by a small flame regulated through a sensitive regulator. The lower temperature of the bath will prevent the distillation of water from the burettes to the tubing above.

4. The potassium hydroxide solution must be saturated with air and the pyrogallate solution with nitrogen at ordinary barometric pressure and the temperature of the bath.

5. For absolute determinations the potassium pyrogallate must be tested regarding its absolute absorbing power and the formation of carbon monoxide. The absorbing power can be tested by analysing air with a small proportion of carbon monoxide (1–2 %). When after the absorption of oxygen the residual mixture of nitrogen and CO is put to the test of combustion any residue of oxygen left will cause a contraction of the volume and a formation of carbon dioxide. I have never yet performed this test. I have not the slightest doubt that with a good sample of pyrogallic acid the absorption of oxygen is complete, because the readings become absolutely constant after a very short period of absorption.

When the potassium hydroxide used to make up the pyrogallate solution is not concentrated very appreciable amounts of carbon monoxide are produced by the absorption of oxygen, and I have therefore tried to find out

whether a small quantity of the gas might not possibly be produced also by a concentrated solution. This is done by adding air to the residual nitrogen and subjecting the mixture to combustion analysis. As an average of two series of such determinations I have demonstrated the production of 0.0025 % CO by each analysis. The nitrogen percentage read off must therefore be reduced by subtraction of 0.0025.

6. Even with all the precautions mentioned it is not possible to obtain absolutely constant results over a long time, because a slight accumulation of dirt in burette 2 cannot be avoided, while nothing happens in burette 3 where the mercury is in contact with nitrogen only. Burette 2 must therefore be cleaned frequently and a fresh supply of water introduced after careful drying. The results obtained with a burette which is not quite clean or does not contain the exact quantity of water can be corrected by making control analyses from a stock of standard air alternating with the real analyses. Air which is compressed to 3–5 atmospheres can be stored indefinitely in a strong absolutely dry glass bulb of about 2 litres capacity when it is dried completely before being introduced. I have pumped in the air by means of a bicycle pump through glass tubes with calcium chloride and phosphorus pentoxide. The bulb is arranged as shown in Fig. 4 (1). The reservoir (2) which holds a little over 50 cc. can be connected with the analysis apparatus through the tube (3).

When a control analysis of the standard air shows a deviation which is outside the limits of accidental error (above  $\pm 0.001$  % for the nitrogen) the experimental analyses made on the same day should be corrected accordingly.

Part of the expense of publication of this paper has been defrayed from a grant for which the Biochemical Society is indebted to the Royal Society.

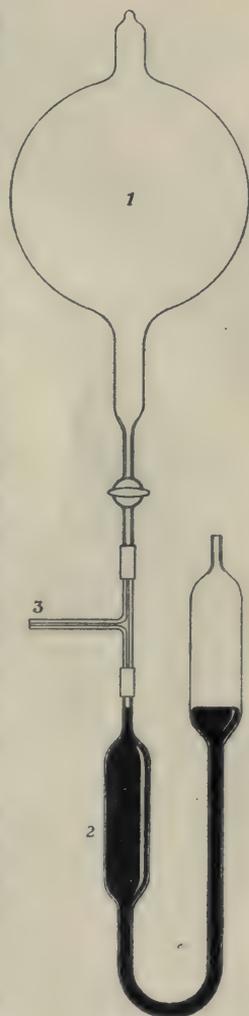


Fig. 4.

#### REFERENCES.

- Benedict (1912). "The composition of the atmosphere," *Carnegie Publication*, No. 166, Washington.
- Carpenter (1915). *Carnegie Publication*, No. 216, Washington.
- Haldane (1912). *Methods of air analysis*, London.
- Krogh, A. (1920). *Biochem. J.*, **14**.
- A. and M. (1913). *Medd. om Grönland*, **51**.
- Sondén and Tigerstedt (1895). *Skand. Arch. Physiol.*, **6**.

## XXIX. THE CALIBRATION, ACCURACY AND USE OF GAS METERS.

BY AUGUST KROGH.

*From the Laboratory of Zoophysiology, University of Copenhagen.*

*(Received August 26th, 1919.)*

GAS METERS are widely employed in physiological work for measuring continuous or interrupted currents of air, but they are not always employed correctly and their accuracy is generally underestimated. To this state of affairs it has doubtless contributed that a quantitative examination and calibration, especially of large gas meters, could not be made in the laboratories employing them but had to be left to the manufacturers.

When Professor Lindhard and I installed a Jaquet respiration chamber in the laboratory we were forced by circumstances to put in a meter for producing and measuring the ventilating current of air which was not, in the opinion of the firm, Dansk Maalerfabrik, by whom it had been made, large enough for the purpose, since it had to be used at a maximum rate of 180 revolutions per hour, while wet meters, especially when large, are not considered as accurate beyond 100 revolutions per hour. We had therefore to arrange calibrations at those rates at which we proposed to work it. The apparatus constructed for this purpose has proved very suitable for the examination and calibration of meters generally and some of the results hereby obtained may possibly be of service to other physiologists employing meters.

### *The Calibration Spirometer.*

The calibration spirometer (Fig. 1) is an adaptation of a similar instrument employed by the Dansk Maalerfabrik, the chief improvement being that I have arranged an automatic record of the volume per revolution of the meter calibrated. The drum (1) which is as nearly as possible cylindrical is suspended by a band of soft steel and counterbalanced by the adjustable weight (2). It moves up and down with a minimum of friction. When the drum is in its highest position the weight can be held by an electromagnet (not shown in the figure) from which it can be released at any desired moment by breaking the current. Along the steel band a fine brass wire is arranged carrying a number of lead weights (3). These can be displaced along the wire and a complete equilibrium in all positions of the drum can thus be secured. When the weight (2) is reduced, any desired pressure up to 20 mm. water can be produced in

the spirometer. The revolving fan (4) which can be driven from outside by a motor is used for mixing purposes. In calibration work with air it is revolved for a few minutes when the spirometer has been filled, to secure a uniform temperature and perfect saturation of the air with water vapour.

The drum is painted inside and outside with a hydrophobe composition so that its volume will not be altered by water adsorbed to its wall. This detail is very important. When left in contact with water, ordinary paint will take up a considerable volume of moisture which cannot drain off. According to experiments a painted tinplate continued to gain in weight while immersed in water during a couple of months, ultimately to the extent of 2.5 g. per 100 sq. cm. Such an increase would in my spirometer diminish by 3.5 cc. the effective volume per cm.

The spirometer drum has been calibrated by measuring a large number of internal diameters (133) evenly distributed over the whole length and circumference of the drum. These measurements have given as the average diameter  $450.135 \pm 0.09$  mm. or a volume of 1591.4 cc. per cm. height. When the drum is used in the spirometer a lowering of 1 cm. will drive out a little more air than the corresponding volume of the drum because the surface of the water in the reservoir will be raised when 1 cm. of the wall of the drum is lowered into it. The volume of 1 cm. of the wall of the drum is 9.4 cc. The rise in the water level will also, of course, correspond to 9.4 cc., but since only 19.0 % of the water surface is inside the drum the extra volume corresponding to 1 cm. will be  $\frac{19}{100} \times 9.4 = 1.8$  cc. and the effective volume of 1 cm. therefore 1593.2 cc. A further correction for the volume of the hydro-

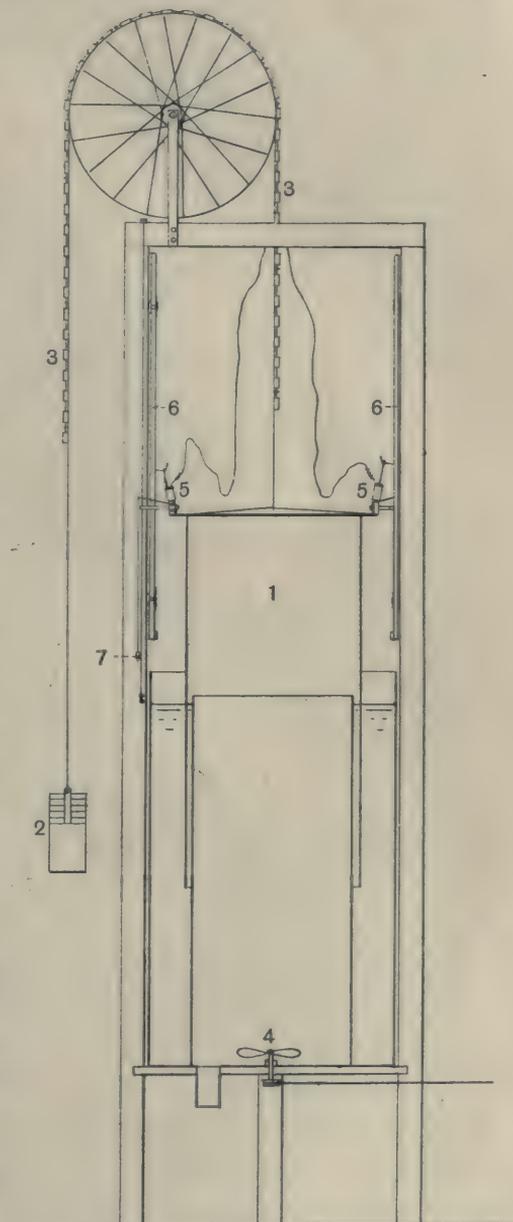


Fig. 1.

phobe paint and for water adhering to the inside reduces the volume to 1593.0 cc. This latter correction is of course a little arbitrary. When the drum has been rapidly raised the water must be allowed to drain off for about five minutes before a practically constant volume is attained. The water adhering to it at first may amount to a couple of cc. per cm. or more.

The spirometer is arranged to record electrically the revolutions of the meter which is being calibrated. Two electromagnets (5) are placed on the top of the spirometer drum. When the drum is lowered each of them will write a vertical line on a strip of paper stretched out on a detachable board (6). The meter is arranged to close a circuit through the magnets once during each revolution and the magnets will make corresponding notches (7, Fig. 2) on the lines drawn. When the distances between these notches are afterwards measured in cm. the volume per revolution is found by multiplication with 1593.0. As the measurements of diameters have shown that the sectional area of the spirometer drum is not quite uniform all measurements of vertical distances are made from the lowest position of the drum as a base line and a small table has been worked out giving the corrections which must be added to the distances as directly read off. The total working height of the drum is 95 cm. and the largest correction which must be applied between 51 and 68 cm. is  $-0.5$  mm.

Details of the arrangement of a recording magnet are shown in Fig. 2. The magnet is removed from the strip of paper while the drum is being raised and can be laid on by pulling the thread (8). Eight separate records can be made on a strip of paper 5 cm. broad.

#### *Wet gas meters.*

Wet gas meters consist essentially of a measuring drum which can revolve with a minimum of friction in horizontal bearings. The drum is mounted in a box filled with water to a constant level. Meters for commercial purposes are provided with a system of cog-wheels driven by the drum and indicating on dials the gas volumes passing through either in litres or in cubic feet. Meters for scientific purposes (Bohr meters of Dansk Maalerfabrik) have only an electric contact arrangement for counting the revolutions and a subdivision of the volume per revolution generally in 100 parts. The experimental meters are more accurate, partly because they are not influenced by the variable resistance offered by the cog-wheels but also because the volume of one revolution is a constant quantity when the meter is properly handled while the gas volume delivered during a fraction of a revolution is not strictly proportional to the angle through which the drum has been turned. The error may amount in a well-made meter to 1-2 % of the volume of a revolution. In a Bohr meter the subdivisions of the revolution can be calibrated by means of a gas pipette delivering a constant volume, suitably  $\frac{1}{10}$  or  $\frac{1}{20}$  of the volume of the meter.

Experimental meters are provided with a levelling arrangement which

insures that the axis of the drum shall be horizontal. When they are correctly mounted the volume per revolution depends (1) upon the water level and (2) upon the rate of revolution. Bohr meters have a line marked on the front indicating the proper level of the water. The error which can be committed when the water level is carefully adjusted to this line may according to my experience produce errors of  $\pm 0.1\%$  on the volume of the revolution.

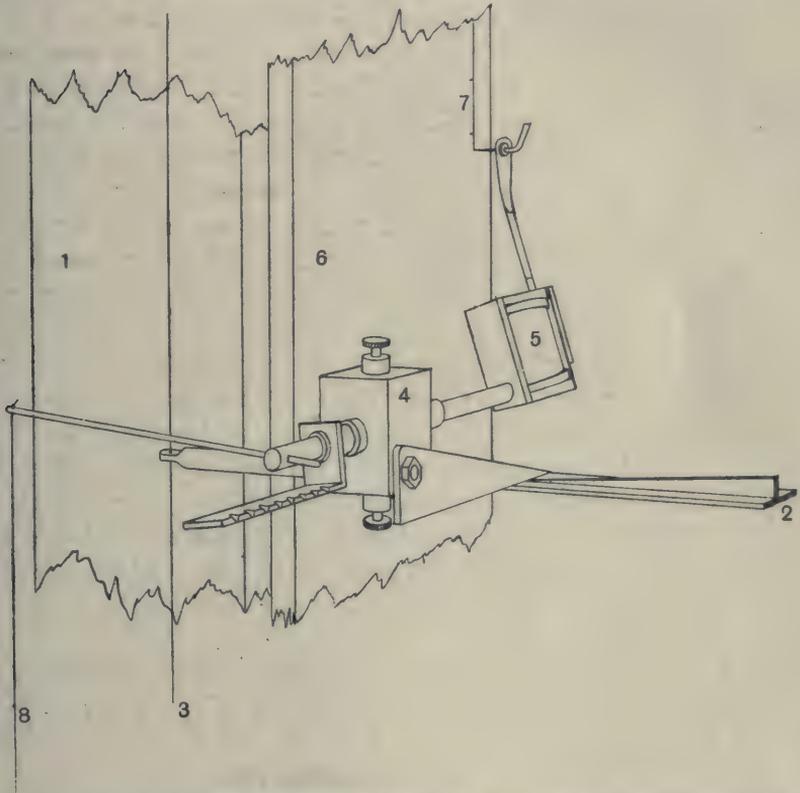


Fig. 2. 1, one of the uprights of the spirometer stand. 2, part of the iron beam across the top of the spirometer drum. 3, steel wire along which the drum is sliding. 4, crosshead with guide for the rod carrying the magnet 5. 6, the detachable board and 7, part of the record with notches corresponding to the revolutions of a meter. 8, the thread for laying on the magnet.

Another arrangement is to have an overflow tube which can be opened and allow any surplus of water to flow off down to the constant level. This arrangement is extremely accurate if sufficient time is allowed to drain off the whole of the excess of water. In experiments with a meter of 14.50 l. per revolution I found that the addition of 100 cc. water, which raised the water level about 1.5 mm., diminished the volume per revolution to 14.40 l. or by 0.7%. When the surplus of water was allowed to drain off during 12 hours 96 cc. were recovered and the attainable accuracy of the adjustment is therefore of the order  $\pm 0.03\%$ .

The volume per revolution of a properly adjusted wet meter is constant within less than 0.1 % at all rates below 1 revolution per minute and for small meters (10 l. per revolution or less) even at all rates below 100 per hour, but when the limit is exceeded there is a definite relation between the rate and the volume. This point has been specially investigated in the case of the 50 candle (4 cb. feet) meter employed for our Jaquet apparatus. This meter is driven by a motor and pumps air out of the respiration chamber. A constant water level is maintained in it irrespective of the rate by a slow current of water (1-2 l. per hour) passing constantly into it and out through the overflow tube. For calibration purposes it is connected by 60 mm. tubing<sup>1</sup> with the spirometer. A water seal which can be rapidly opened and closed is arranged on the tubing at such a distance from the meter that the resistance in the tubing between the seal and the meter corresponds as nearly as possible to the resistance offered by the chamber and tubing in respiration experiments. When the seal is opened the meter draws air from the atmosphere and when it is closed the meter is connected with the spirometer alone. The spirometer drum is loaded so that it will drive out air through the same opening at a rate similar to that at which the meter is pumping. The meter is kept working at a constant rate for a sufficient period to allow the perfect adjustment of the water level, the attainment of which is shown by the inflow and outflow of water being equal. The spirometer drum is raised and held in the highest position, and at a suitable moment shortly before the meter will close the electric circuit the spirometer drum is released and the water seal closed. When the drum approaches the lowest position the water seal is again opened. The meter is so large that only one revolution can be recorded during the sinking of the spirometer drum.

We have made a number of determinations at varying rates of the meter and found that at all rates below 1 revolution per minute the volume of air transported per revolution is constant, while at rates exceeding 1 revolution per minute the volume decreases with increasing rate.

The following numerical results have been obtained:

1 rev. in seconds	142	112	96	94	78	60	45
Vol. per rev. l.	113.98	114.02	114.07	113.96	114.00	114.00	113.55
1 rev. in seconds	33	31	29	24.5	22.5	20.6	18.6
Vol. per rev. l.	113.39	113.20	113.48	113.15	112.78	112.26	112.18

The determinations were made in the course of several days and slow and rapid speeds alternated irregularly. By graphic interpolation a table has been constructed from which the volume per revolution can be taken for all practicable rates of the meter.

If the water level of a meter is adjusted while the meter is at rest and the quantity of water remains constant while the rate is changed the volume per

<sup>1</sup> The tubing must be very wide to minimise the resistance which the meter has to overcome. The negative pressure in the drum of the meter should never exceed 5 mm. water. In the present case it is at the most rapid rate used about 2 mm. water.

revolution increases with increasing speed. A 14.5 l. meter was calibrated once by repeated fillings from a 1 l. glass bulb [described and figured by Krogh, 1915] and found to be 14.50 l. The rate in this case was extremely slow. Over a year later the meter was calibrated by means of the spirometer which was loaded so as to drive the air through at a rate of 1 revolution in 39 seconds. The pressure in the spirometer sufficient to produce this rate was 3.6 mm. water. The corrected readings of the record gave

Right mm.	Mean diff. mm.	Left mm.
875.6	—	876.9
784.0	91.55	785.4
692.8	91.2	694.2
601.9	91.0	603.1
510.7	91.15	512.0
419.7	91.15	420.7
328.8	91.0	329.6
237.9	90.8	238.9
147.0	90.9	148.0
56.2	90.9	57.0

The dispersion<sup>1</sup> of the single results is  $\delta = 0.22 \text{ mm.} = 35 \text{ cc.}$  and the average is  $91.07 \pm 0.07 \text{ mm.} = 14.508 \pm 0.018 \text{ l.}$  measured in the spirometer at a pressure of 3.6 mm. water above that in the meter. The correction for excess pressure will increase the result by 5 cc. to  $14.513 \pm 0.018 \text{ l.}$  When the rate is increased to 2 revolutions per m. the volume per revolution rises to 14.54 l. and at 3.4 revolutions it is 14.63 l. The corresponding pressures are 7 mm. and 9.5 mm. water.

When a meter is used at a constant rate the constant level arrangement with a current of water will generally be found to be the most suitable as no errors can arise from evaporation, but when the rate is inconstant or even discontinuous the constant quantity is the only possibility.

In respiration experiments, when a meter is used to measure the air expired from the lungs of man or an animal, the maximum rate during expiration can be taken to be approximately three times the total rate and care should be taken that the fastest rate does not exceed the capacity of the meter.

#### *Dry gas meters.*

Dry gas meters consist of two bellows which are alternately filled and emptied by the current of air passing through. They require no filling and will work equally well in any position. As the fractions of one revolution are often very incorrectly shown by a dry meter they should never for experimental purposes be divided in litres as commercial dry meters are but simply have a device for counting the revolutions.

<sup>1</sup> The term "dispersion" has been introduced by Charlier to take the place of "standard deviation."

A dry 10 candle meter (volume per revolution about 8 l.) arranged with one dial measuring 10 l. and divided in tenths of a litre was tested by passing air through it from the one litre bulb. The successive readings gave

Reading 1 l.	7.0	7.6	8.4	9.35	0.25	1.5	2.75	3.75	4.8	5.55	6.25	7.25
Diff. = 1 l.		0.6	0.8	0.95	0.9	1.25	1.25	1.0	1.05	0.75	0.7	1.0

The readings corresponding to 1 l. vary in this case between 0.6 and 1.25 l. but repeated tests starting from the same point gave very different results. When the meter was tested by the calibration spirometer it was found that successive revolutions of the hand indicating 10 l. per revolution showed similar variations, but since the volume of the drum was a submultiple of the volume corresponding to 50 l. the results were repeated with astounding regularity for each 50 l. The true volumes corresponding to successive readings of 10 l. were

0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100
10.32	9.85	9.83	9.99	10.62	10.32	9.85	9.83	9.99	10.62

The volume corresponding to a reading of 100 l. is 101.2.

A 5 candle meter arranged to count the true revolutions and subdivided into  $\frac{1}{100}$  parts of a revolution gave similar though smaller variations when tested by means of the 1 l. glass bulb but in this case a repeated test gave approximately the same errors at corresponding divisions. When tested by means of the calibration spirometer the volume per revolution proved constant so long as the rate remained constant.

The indications of a dry meter depend generally to a certain extent upon the rate, but the effect of the rate may differ in different meters. For the meters mentioned above I have found

10 candle meter		5 candle meter			
100 l. in	300 seconds	100-9 l.	1 rev. in 45 seconds	L. per revolution	Pressure mm. water
	150	101.1	22	5.10	3
	145	101.2	13	5.08	6
	110	101.8		5.04	15

The volume per revolution of a dry meter will not remain unchanged indefinitely, because the material from which the bellows are made will deteriorate. Within a year the change appears to be very slight but this of course depends upon the initial quality and age of the material. After nine months the calibration of the new 10 candle meter mentioned above has changed from 100.9 to 101.3 l. per 100 l. indicated.

#### SUMMARY.

A large recording spirometer for calibrating gas meters is described.

Wet gas meters properly handled are instruments of precision. Motor-driven wet meters acting as pumps and with a constant water level maintained by a slow current of water are accurate to less than 0.1 % at all rates below

1 revolution per minute. When they are calibrated for more rapid rates they can be used with almost the same accuracy at rates up to 3 revolutions per minute. The volume per revolution decreases with increasing rate.

In wet meters with a constant quantity of water the volume per revolution increases with increasing rate but can be determined with equal accuracy.

The subdivisions of the revolution of an experimental wet meter (Bohr meter) usually show slight errors which can be allowed for after calibration.

Dry gas meters are on the whole less accurate than wet, and when they are arranged to show the volumes directly in litres their indications are often very inaccurate and cannot be calibrated. They should be arranged to count revolutions.

Gas volumes representing fractions of a complete revolution are generally very inaccurately indicated but the volume corresponding to a whole revolution is a practically constant quantity. Varying rates may cause variations in the volume registered but at practicable rates usually within 1 %. The calibration of a dry meter changes with age.

Part of the expense of publication of this paper has been defrayed from a grant for which the Biochemical Society is indebted to the Royal Society.

#### REFERENCE.

Krogh (1915). *Abderhalden's Handbuch Biochem. Arbeitsmet.*, 8, 537.

# XXX. THE RELATIVE VALUE OF FAT AND CARBOHYDRATE AS SOURCES OF MUSCULAR ENERGY.

WITH APPENDICES ON THE CORRELATION BETWEEN STANDARD METABOLISM AND THE RESPIRATORY QUOTIENT DURING REST AND WORK.

BY AUGUST KROGH AND JOHANNES LINDHARD,

WITH THE COLLABORATION OF

GÖRAN LILJESTRAND AND KNUD GAD ANDRESEN.

*From the Laboratory of Zoophysiology, Copenhagen University.*

*(Received August 26th, 1919.)*

## CONTENTS.

	PAGE
Introduction and choice of method . . . . .	290
Description of apparatus and method . . . . .	294
Sources of error and accuracy . . . . .	300
Preliminary series of experiments . . . . .	304
Series I. General Table of Experiments . . . . .	306
Second series of experiments . . . . .	314
Third series of experiments . . . . .	322
Fourth series of experiments . . . . .	326
General results of the experiments . . . . .	339
The results of earlier investigations . . . . .	342
The theoretical significance of the waste of energy from fat . . . . .	345
APPENDIX I. Statistical treatment of the determinations of standard metabolism . . . . .	346
APPENDIX II. The correlation between the respiratory quotient and the standard metabolism . . . . .	350
APPENDIX III. The change in respiratory quotient taking place on the transition from rest to muscular work . . . . .	354
APPENDIX IV. The variation of the respiratory quotient and metabolism during one hour of constant work . . . . .	357
SUMMARY . . . . .	361
REFERENCES . . . . .	363

## INTRODUCTION.

A NUMBER of researches on isolated muscles, made most of them during the last decade by the Cambridge School of physiologists, have revived the old problem about the immediate source of muscular energy, in so far as their main results cannot be reconciled to the commonly accepted view, that the

muscle is able to make use indiscriminately and with the same coefficient of utilisation of different sources of energy. According to this view, which is based mainly on the researches of Zuntz and his school, the muscular machine can transform a definite fraction of the energy liberated by the combustion of any available substance into mechanical work—provided the substance can be oxidised in the organism. According to the Cambridge school the muscular contraction itself does not depend at all upon an oxidative reaction but upon the splitting up of a definite unknown molecule, resulting in the formation of lactic acid, while other definite processes, involving the recombination of lactic acid into the molecule directly concerned in the contraction, are necessary to restore the contractility.

The researches on which the supposed isodynamic value for muscular work of very different oxidisable substances are based have, without exception, been made on the organism as a whole, and the general principle has been to feed a subject during a certain period upon a diet consisting chiefly of a single foodstuff (fat, protein or carbohydrate), to let the subject perform a definite amount of work and to study the relation between the work performed and the corresponding metabolism. When a number of such periods, which differed only in the character of the chief substance catabolised, were compared the conclusion was arrived at by Zuntz [1911] and his collaborators that the coefficient of utilisation is practically independent of the substance catabolised<sup>1</sup>.

From the researches of Fletcher, Hopkins, A. V. Hill and their collaborators [see Fletcher and Hopkins, 1917] it must be inferred on the other hand that, when the muscular machine requires certain reactions to take place between definite substances which must be closely allied to carbohydrates, it is almost inconceivable that substances such as fats can be utilised without a transformation involving loss of energy, and one is led to expect therefore that the coefficient of utilisation should be lower for other substances than for carbohydrates.

We do not propose to enter upon a discussion of the Cambridge results, but the discrepancy between them and those of Zuntz has caused a growing feeling of uneasiness in our minds which has at last compelled us to take up the problem and to see whether Zuntz's conclusion will stand the test of a renewed experimental investigation.

The general plan of our proposed research was briefly as follows. A human subject should live for a certain number of days on a definite diet, containing a minimum of protein and a very decided preponderance of either fat or carbohydrate. During this period, or part of it, he should come to the laboratory in the morning before taking any food and with as little muscular effort as

<sup>1</sup> It should be remembered, however, that the researches made in Zuntz's laboratory were not primarily intended to demonstrate the equal value of the different foodstuffs, but to examine whether the 30 % difference in value between fat and carbohydrate postulated by the hypothesis of Chauveau could be found or not.

possible. After a suitable period of complete rest, during which the standard metabolism could be measured, he should work on a bicycle ergometer for a certain length of time, which we fixed provisionally at two hours, and the rate at which work was performed should be kept constant throughout for the same subject. During the work a number of determinations of the respiratory exchange should be made. After a suitable number of days the diet should be altered and the determinations repeated.

The experimental difficulties involved in such an investigation are very considerable, and it was evident from the first that a definite solution of our problem could only be hoped for when the accuracy of all the determinations could be raised to a high standard. The reliability must depend (1) on the technical accuracy of the determinations themselves, (2) on the constancy of the external conditions to which the subject was exposed and (3) on the constancy of reaction on the part of the subject. The greatest difficulty is presented by the subject. During a total experimental period of three weeks or a month effects of training and probably other changes are likely to occur, and it was impossible to tell beforehand how these could best be avoided or their influence minimised. We arranged therefore to make several series of experiments and to use the results of the first to guide us in the planning of the following. It is evident that a condition for doing this is that we should be able to recognise which variations in the results must be due to the variations in the reaction of the subject and which to errors in the determinations, and it was imperative therefore that the technical accuracy of the determinations should be as great as possible.

We had to choose between determining the pulmonary gas exchange by measurements and analyses of the expired air and determining the total gas exchange in a chamber. Although the methods for measuring the pulmonary gas exchange can give very accurate results and have been used in almost all former researches bearing upon our problem we thought it better to discard them because they would involve that the subject should wear a respiration mask (or a mouthpiece) during a period of two hours, which would become rather disagreeable and might influence the metabolism in unaccountable ways<sup>1</sup>.

When the determinations should be made by means of a respiration chamber we had to choose between the closed circuit (Regnault) and the air current (Jaquet) type of apparatus. The dangers and drawbacks of large Regnault apparatus have been pointed out by Krogh [1915, 2] and from the interesting control experiments reported by Carpenter [1915] it is clear,

<sup>1</sup> In the excellent series of experiments on muscular work on an ergometer made by Benedict and Cathcart [1913] the subject was connected with the mouthpiece for a number of short periods only which were distributed over the time of riding the ergometer. This device appears to us to involve serious dangers. For a certain time after the putting on of a mouthpiece and noseclip the respiration is apt to be distinctly abnormal and though the absorption of oxygen is as a rule scarcely affected by such changes the CO<sub>2</sub> output may become abnormal by washing out, when the ventilation is increased, and storing up when it is diminished.

moreover, that the accurate determination of respiratory quotients in a closed circuit apparatus is—to say the least—very difficult to attain.

Carpenter has carried out extensive comparisons between respiration apparatus of various types. The comparisons were made between instruments of two types at a time by employing them for alternate determinations of the respiratory exchange of the same subject. Usually about six alternate determinations were made on each experimental day, and care was taken to change the order in which the instruments were used from day to day. This method of experimenting is extremely suitable to bring out both the uniformity of results (accidental variations) obtained with each type and its absolute reliability (systematic error). The accidental variations have been given by Carpenter in "probability curves" but the systematic differences between the types have not been discussed. It is obvious, however, that when the averages of long series of such determinations differ by more than double the mean error of each average the difference cannot be purely accidental, but must be due to some inherent peculiarity in one or both of the apparatus.

From the probability curves given by Carpenter it can be deduced that the mean error on the average of each separate series of determinations of the respiratory quotient is of the order  $\pm 0.005$  and nearly the same for the different types of apparatus except the "Spirometer Unit" and the "Bed Calorimeter" which have slightly larger errors.

Table I.

Apparatus	Number of exp.	CO <sub>2</sub> cc./min.	O <sub>2</sub> cc./min.	R.Q.	R.Q. reduced
1	2	3	4	5	6
T.E. Unit	142	185	227	0.815	
Bed Cal.	95	190	223	0.85	0.865
T.E. Unit	31	197	231	0.855	
Sp. Unit	25	198	233	0.85 $\pm$ .005	0.83
T.E. Unit	36	190	224	0.85	
Zuntz-Gep.	35	186	227	0.82	0.80
Sp. Unit	74	182	219	0.83	
Zuntz-Gep.	58	176	220	0.80 $\pm$ .005	0.80
T.E. Unit	44	165	193	0.855	
Tissot	37	167	194	0.86	0.835
Sp. Unit	65	190	233	0.815	
Tissot	52	192	242	0.795	0.81
Sp. Unit	48	189	231	0.82	
Douglas	45	178	224	0.795	0.805

In Table I we have put together the average results of the various comparisons, compiled from Carpenter's book. The figures in column 5 show that while the "Tension Equaliser Unit" and the "Spirometer Unit" give the same average quotients, the differences found in the other comparisons are too large to be accidental.

In column 6 we have arranged a direct comparison between all the types of apparatus investigated by reducing the respiratory quotients found in the various series by means of the same apparatus (the "Spirometer Unit") to a uniform value put arbitrarily at 0.83 and it is seen that the "Bed Calorimeter," which is a large closed circuit apparatus, gives a much higher average quotient, while the Zuntz-Geppert and Douglas apparatus, in which the quotient depends exclusively on the results of a gas analysis, give only 0.80. (In the comparisons with the Tissot apparatus very special errors come into play which it is not deemed necessary to discuss.)

The respiratory quotient determined by gas analysis of the expired (and inspired) air correctly performed cannot have any *systematic* error, and it follows that the respiratory quotients determined by means of the closed circuit "Unit" apparatus are on an average 0.03 too high, while those found by means of the respiration calorimeter are 0.065 too high.

In a Jaquet apparatus on the other hand the accuracy of the respiratory quotient depends exclusively on the gas analysis. There is therefore no danger of systematic errors in the quotient while the limits of the accidental errors can always be controlled. A special gas analysis apparatus capable of analysing the respiratory gases to 0.001 % has been constructed for this and similar researches and described by Krogh [1920, 1].

#### DESCRIPTION OF APPARATUS AND METHOD.

Our Jaquet apparatus which has been constructed on the lines laid down by Grafe [1910] is shown in Fig. 1. The chamber consists of a framework of angle iron to which sheets of galvanised iron have been riveted and made airtight by soldering. The form and dimensions have been chosen with a view to accommodating the bicycle ergometer and the subject riding the machine. The floor is made from a single sheet of galvanised iron with the edges bent downwards into a U shaped rectangular groove (1, Fig. 1) which is filled with water. The chamber itself dips into the same water seal when closed during an experiment. As shown in the figure (at 2) one end can be lifted to let in the subject and put in apparatus. At the other end just above the groove where the movements are very slight a number of small tubes (3) are arranged to introduce wires etc. for the working of the ergometer, mixing fans, signals etc.

Near the top of the chamber are two wider tubes (4 and 5). Through a mouthpiece or mask, respiration valves and flexible tubing the subject can be connected with one of these (4) and through it with a dry meter placed outside the chamber. The air for inspiration has in such experiments been taken from the chamber and again returned to the chamber from the meter through the tube (5). This arrangement has been used during some introductory experiments to compare the pulmonary gas exchange with the total.

The chamber is ventilated with outside air from the street through a 60 mm. tube (6) which is connected up at the beginning of an experiment. At the other end of the chamber, a similar tube (7), which is likewise put in place when the chamber has been let down, connects it with the gas meter. The inlet tube simply opens into the chamber, but the outlet tube draws air simultaneously from three points distant about 50 cm. from each other (8).

During experiments the air in the chamber is mixed continuously by two revolving fans (9 and 10) each of which will move about 30 cb. meters per minute. The blast produced by these fans is rather disagreeable at first but during the work, when the temperature and moisture in the chamber rise, it is felt by the subject as very refreshing. Arrangements for controlling the temperature and moisture have not been put in though they would undoubtedly constitute a considerable improvement. As it is, work experiments can only be made during the cooler season of the year.

The chamber has been tested for tightness by closing it up and connecting it with a spirometer loaded so as to produce a negative pressure of about

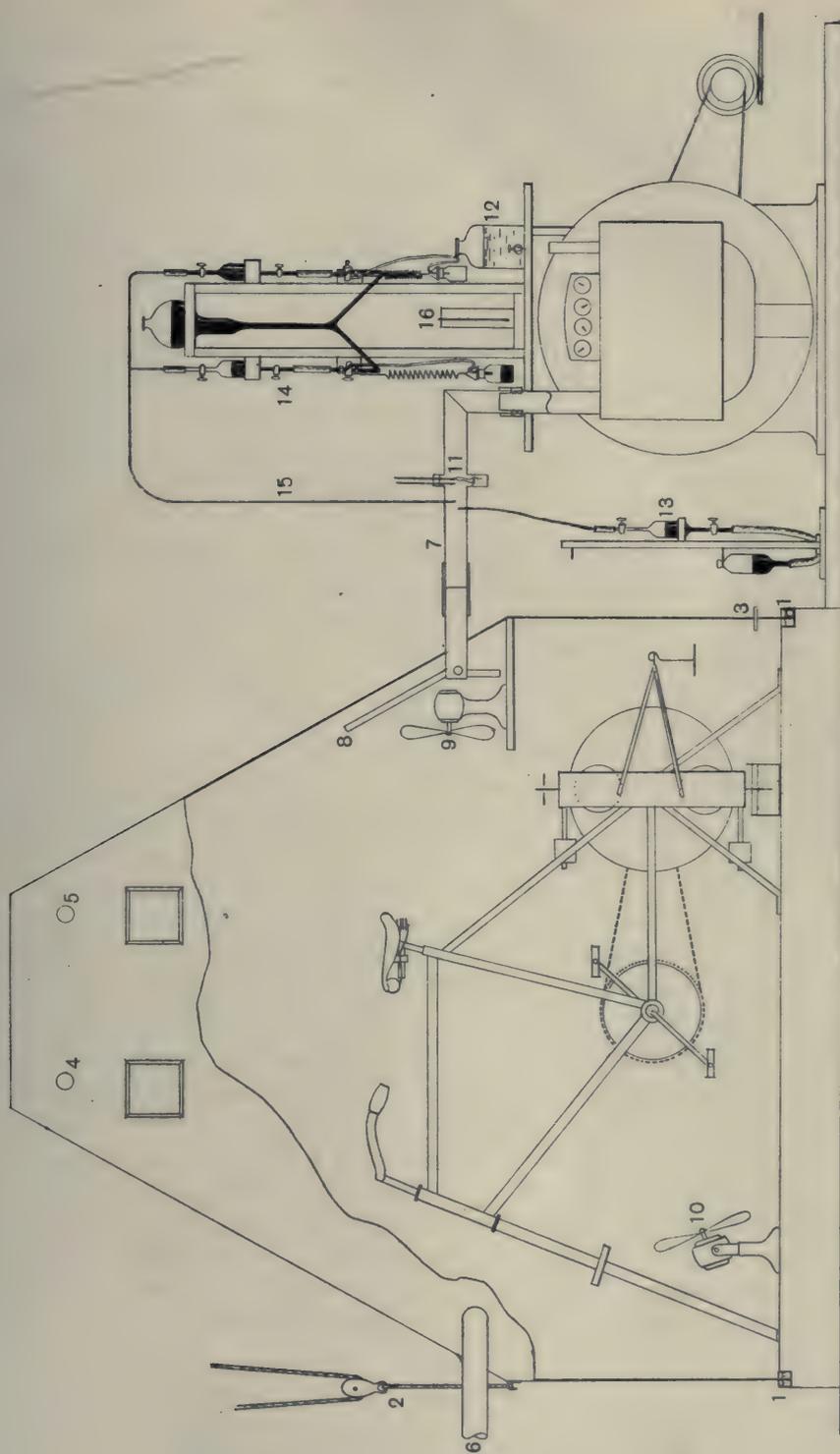


Fig. 1.

20 mm. water. No sign of leakage has been detected by such tests. During experiments the negative pressure inside is only about  $\frac{1}{2}$  mm. water.

The volume of the chamber has been calculated from numerous measurements of the inside dimensions to 2267 l. This determination was tested by gas analysis in the following way: by means of a 1 litre glass bulb (described by Krogh [1915, 1]) 15·000 l. hydrogen were put into the chamber and thoroughly mixed with the inside air from which 15 l. had been previously withdrawn. From analyses made with the new gas analysis apparatus the volume of the chamber was found to be 2276 l. The volume of the empty chamber is taken therefore to be 2270 l.

In the tubing between the chamber and the meter dry and wet bulb thermometers (11) are arranged and we have satisfied ourselves by repeated comparisons with instruments inside the chamber that these thermometers show accurately within a few tenths of  $1^\circ$  the average temperature and moisture of the air in the chamber—at the rates of ventilation and mixing employed by us.

The gas meter, which produces and measures the air current through the chamber, is a 50 candle wet meter with a drum of 4 cb. feet. It is driven by a small motor ( $\frac{1}{10}$  horse power). Through electrical resistances and suitable transmissions the revolutions can be regulated between about 1 in 3 minutes and 3 per minute. When set at a certain speed the motor is controlled by a regulator which will keep the rate constant, generally within  $\frac{1}{2}$  % and always within 1 %.

A constant water level is maintained in the meter by a slow current of water passing through it. Water is admitted from the 3 l. bottle (12) and flows off through an overflow tube. The rate at which water is admitted is about  $\frac{3}{4}$  l. per hour and care has always been taken not to begin any measurements before the inflow and outflow of water were approximately equal.

As we had to use the meter at the rather rapid rate of nearly 2 revolutions per minute special care has been bestowed upon its calibration at various rates. The results are given in the separate paper by Krogh [1920, 2] and here it is sufficient to mention that at all the rates used the volume per revolution has been determined and can be kept constant within 0·1 %.

*Sampling of the air.* The atmospheric air entering the chamber has been sampled during each experiment. Sometimes we have taken two separate samples during short periods at the beginning and end of the experiment, but as the differences found between these were always extremely slight we have later taken one sample of 120 cc. in portions of about 20 cc. at more or less regular intervals during the course of the experiment. Details about the composition and constancy of the atmospheric air as taken from a Copenhagen street are given by Krogh [1919].

The outgoing air has been sampled in two ways. At the beginning and end of each experimental period, which is normally of 20 minutes duration, we have drawn a sample by hand in a sampling vessel of about 100 cc.

capacity (13). The taking of such an "intermediate sample" occupies about 1 minute. During the course of each experimental period an "average sample" has been drawn automatically by means of the apparatus shown at (14) which was employed in Greenland by A. and M. Krogh [1913] for a similar purpose. The mercury from the sampling vessel flows off into a bottle suspended by a spring which is so adjusted as to maintain constant within 1 cm. the vertical distance between the surface of the mercury in the sampling vessel and the point of the tube through which it flows out. The volume of air taken into the sampling vessel per minute is therefore practically constant, and as the volume transported by the meter is constant also the sample will represent accurately the average composition of the air even though this should vary considerably. During experiments with constant work the variations are extremely slight. The tubing connecting the sampling vessels with the pipe (7) is of 1 mm. bore. The volume of the tube (15) is about 1.2 cc. and as about 6 cc. are drawn per minute the sample is therefore 12 seconds behind the meter.

*Analysis and calculation of experiments.* The air samples have been analysed for  $\text{CO}_2$  and  $\text{O}_2$  in the special gas analysis apparatus constructed for the purpose and described in a separate paper [Krogh, 1920, 1] where the methods of calculating the increase in  $\text{CO}_2$  percentage and the  $\text{O}_2$  deficit in the outgoing air as well as the correction on the oxygen deficit, which is necessary when the ingoing and outgoing volumes of air are not identical, have also been given in detail and illustrated by an example. The atmospheric air has in all experiments been analysed in duplicate, and in all the experiments made in 1917 the average samples were likewise analysed in duplicate. As the double analyses practically never differed more than 0.001 % we have in the later series usually analysed only a single one of the average samples in duplicate.

*Calculation of the gas exchange.* We have in all cases taken experimental periods of the length of a whole number of revolutions of the meter (usually 38). By multiplication of the volume per revolution with the number and reduction to  $0^\circ$ , 760 mm. and dryness from the temperature of the meter (shown by the thermometer (16) placed in the outgoing current of air) and the barometric pressure, we obtain the reduced ventilation during the period, which is multiplied by the  $\text{CO}_2$  increase and the corrected  $\text{O}_2$  deficit found in the corresponding average sample.

As the volume of air which has passed through the chamber during a period is only about double the volume of the chamber itself we cannot take the composition of air in the chamber as unaltered but have to subtract the  $\text{CO}_2$  increase and  $\text{O}_2$  deficit in the chamber at the beginning of the period and add the corresponding figures for the end of the period. The volume of the chamber (— the ergometer, subject etc.) is therefore calculated for the beginning and end of each period and reduced to  $0^\circ$ , 760 mm. and dryness from the readings of the dry and wet bulb thermometers (11) and the barometric

pressure. The water vapour tension corresponding to the dry and wet bulb readings is taken by interpolation from a psychometric table in Landolt-Börnstein's tables.

From the corrected gas exchange for the experimental period the gas exchange per minute is calculated by division with the length of the period and the respiratory quotient is made out. Finally the total heat production per minute is calculated from the oxygen intake and the respiratory quotient by means of Zuntz's table [1901] disregarding the protein metabolism.

A detailed example of the calculation is given below. The working out of the formulas on which the calculation of respiration experiments with intermediate samples is based is given in detail in the paper by A. and M. Krogh [1913] quoted above.

*Rest experiments.* In order to calculate the coefficients of efficiency in the muscular work experiments the standard metabolism of the subject must be determined and subtracted from the metabolism during the work periods. It would have been desirable to make these determinations also in a chamber, but for technical reasons this was impossible and we have therefore made them by means of the technique usually employed by us involving the use of a mouthpiece and respiration valves.

The subject who had taken no breakfast sat down in an easy chair on arriving in the laboratory and remained absolutely at rest for 20 minutes. Thereupon the mouthpiece and noseclip were put on, and the subject breathed for about 10 minutes through the valves and expired through a mixing vessel of about 3 l. capacity and a wet meter of 14.50 l. per revolution. During this period the duration of 1 revolution of the meter was observed repeatedly and when it had become approximately constant the determinations were begun. Each experimental period lasted for approximately 2 revolutions of the meter and was begun and finished at the end of an expiration. Two consecutive determinations were made each day. During each a sample of the expired air from the mixing vessel was taken into a 40 cc. sampling vessel of the type usually employed by us [Krogh, 1915, 1]. The sample was drawn by hand, about 2 cc. at a time. One sample of the air inspired from the room was taken in small portions during the two experimental periods. The analyses were reliable to about 0.01 %.

The general routine of making and calculating the experiments is best shown by an example taken from the protocol.

April 19, 1918. The subject O. H. arrived and sat down at 9 o'clock. Mouthpiece etc. at 9.22. Barometer 751.5 mm.

As soon as possible after the determinations of the standard metabolism the subject entered the respiration chamber and began riding the ergometer at 9.45. During the first nine minutes the chamber is not ventilated, to allow the inside air to attain the composition which will remain nearly constant during the whole of the experiment. The subject rides for about half-an-hour before the determinations begin, and during this time the general accommoda-

Rest experiment begun	Duration	Vol. expired l.	Temp. of meter	Samples	Atm. sample
0.31	4' 27.3"	28.95	17.0°	1	3
	4' 42.0"	29.20	17.0°	2	

*Analyses:*

cc.	%	Expired %	Inspired	Corr.	Diff.		
3. 9.988	0.008	0.08 CO <sub>2</sub>	1. 9.996	0.395	3.95	0.08	3.87
9.980	2.087	20.90 O <sub>2</sub>	9.601	1.670	16.71	20.90 + 0.08	4.27
7.893		20.98	7.931		20.66		
					20.98		
					0.265 × 0.32 = 0.08		
			2. 9.990	0.391	3.91	0.08	3.82
			9.599	1.666	16.68	20.90 + 0.10	4.32
			7.933		20.59		
					20.98		
					0.265 × 0.39 = 0.10		

*Calculation:*

Duration m.	Ventilation per m. L. reduced	CO <sub>2</sub> increase %	O <sub>2</sub> deficit %	R.Q.	Per minute cc. CO <sub>2</sub>	cc. O <sub>2</sub>	Calories
1 4.455	5.81	3.87	4.27	0.905	225	248	1.23
2 4.70	5.67	3.82	4.32	0.885	216	245	1.215

tion of the subject to the work, of the temperature in the chamber, of the water level of the meter etc. will take place.

Exp. begun	Duration	Reading of meter	Ventilation revolutions	Temperature of chamber		Temp. of meter	Samples	
				dry	moist		Inter-mediate	Average
10.16		9003		18.0°	14.4°	13.2°	1	
	20' 44.5"		38					A
10.37		9041		19.5°	17.0°	13.2°	2	
	20' 43.0"		38					B
10.58		9079		20.0°	18.4°	13.3°	3	
	20' 45.3"		38					C
11.18		9117		20.2°	19.0°	13.4°	4	

Barometer 752 mm. Volume of meter per revolution 113.9 l.

Volume of chamber corrected 2170 l.

The analyses are left out as sufficient examples are given in the preceding paper. The CO<sub>2</sub> increases and O<sub>2</sub> deficits found are given in the calculation.

*Calculation:*

Sample	Barometer - vapour tension mm.	Temp.	Volume reduced l.	CO <sub>2</sub> increase hundredths	O <sub>2</sub> deficit of 1 %	CO <sub>2</sub> increase litre	O <sub>2</sub> deficit litre
1	742	18.0°	1987	56.15	63.75	11.16	12.67
A	740.5	13.2°	4019	56.95	64.7	22.90	26.00
2	739	19.5°	1968	56.75	64.65	11.17	12.73
B	740.5	13.25°	4019	56.9	64.5	22.87	25.92
3	737.5	20.0°	1961	56.95	64.55	11.17	12.66
C	740.5	13.35°	4019	56.8	64.85	22.83	26.07
4	736.5	20.2°	1956	56.9	65.45	11.14	12.81

Period	Duration m.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	Per minute		
		eliminated l.	absorbed l.		CO <sub>2</sub> cc.	O <sub>2</sub> cc.	Cal.
A	20.74	22.91	26.06	0.880	1105	1256	6.22
B	20.72	22.87	25.85	0.884	1104	1248	6.18
C	20.755	22.80	26.22	0.870	1099	1264	6.24

## SOURCES OF ERROR AND ACCURACY.

The possible sources of error in the respiration experiments are few in number and not *a priori* likely to be serious. The ventilation recorded by the meter can be taken as correct to about 0.1 %, but the reduced volume of the chamber may possibly be erroneous to the extent of 0.2 %, if the recorded temperature and moisture differ from the true averages by  $\frac{1}{2}^{\circ}$  and  $\frac{1}{2}$  mm. vapour pressure. Such an error will influence the final result to the extent of 0.1 %.

Errors in the analyses may amount to 0.001 % and since the CO<sub>2</sub> percentage is generally about 0.5 % this will correspond to an error on the gas exchange of 0.2 %. If the CO<sub>2</sub> increase should be found, say, 0.001 % too low and the O<sub>2</sub> deficit 0.001 % too high the resulting error on the respiratory quotient would be 0.004.

*A priori* the possibility cannot be excluded that the samples analysed do not represent accurately the average composition of the air. This may be the case especially with the intermediate samples if the air in the chamber is not absolutely mixed.

To test this point we have made a small number of control experiments. In these we have added CO<sub>2</sub> from a cylinder to the chamber through a 1 litre meter. The CO<sub>2</sub> was washed with permanganate and the water in the meter

## Control experiment 1.

Time	Duration seconds	CO <sub>2</sub> %
10.45	52	.5085
	57	.5085
	70	.508
	59	.5015
	70	.511
	9	.509
	31	.5085
	67	.5095
	7	.508
	10.55	65
Average		0.5082
Dispersion $\sigma = \pm$		0.0025

## Control experiment 2.

Time	Duration seconds	CO <sub>2</sub> %
10.38	33	.509
	60	.5105
	10	.510
	90	.513
	7	.5105
	70	.510
	15	.5075
	92	.5095
	16	.5035
	70	.5095
10.51	60	.510
	61	.514
	60	.5115
	13	.510
	55	.5125
11.00	55	.5035
Average		0.5097
Dispersion $\sigma = \pm$		0.0028

was thoroughly saturated with the gas before any experiments were made. During the experiments CO<sub>2</sub> was added at an approximately constant rate of 1 l. per minute while the chamber was being ventilated at the rate used in actual work experiments. It is obvious that the CO<sub>2</sub> from a constant slow current of the pure gas is not so likely to become uniformly mixed with the air of the chamber as the expired air from a human subject, which contains only about 5 % CO<sub>2</sub>.

The first control experiments consisted simply in taking intermediate samples as rapidly as possible and comparing their CO<sub>2</sub> percentage, which should remain constant.

The dispersions of the single samples do not differ significantly in both series, viz. about 0.5 % of the quantity of CO<sub>2</sub>. If the CO<sub>2</sub> percentage in one of the intermediate samples is, say, 1 % too high the CO<sub>2</sub> production in the preceding 20 minutes period will be found 0.5 % too high, since the volume of the chamber is about half the ventilation during 20 minutes, while in the following period it will be 0.5 % too low.

We have made two complete control experiments consisting of five 15 minute periods during which CO<sub>2</sub> was added at an approximately constant rate. The results were as follows:

## Control experiment 3.

No.	Duration m.	CO <sub>2</sub> found l.	CO <sub>2</sub> added l.	CO <sub>2</sub> per minute		Difference %
				found cc.	added cc.	
1	15-395	14.21	14.33	922	930	-0.9
2	15-42	14.20	14.29	920	926	-0.7
3	15-37	14.28	14.32	928	931	-0.3
4	15-39	14.32	14.31	930	929	+0.1
5	15-355	14.54	14.26	946	928	+1.9
		<u>71.55</u>	<u>71.51</u>			

## Control experiment 4.

1	16-54	15.29	15.43	924	932	-0.9
2	15-44	14.41	14.37	933	930	+0.3
3	15-41	14.24	14.37	924	932	-0.9
4	15-44	14.27	14.34	924	928	-0.7
5	15-44	14.26	14.39	924	931	-0.8
		<u>72.17</u>	<u>72.90</u>			

According to these control experiments we have every reason to expect that the error in the respiration experiments on a working subject in which the conditions for complete mixing of the air are distinctly more favourable than in the controls will practically never exceed 1 %.

In order finally to test the accuracy with which the respiratory quotient can be determined we have made a control experiment on a human subject, doing the normal amount of work on the ergometer, while the chamber was ventilated as usual, by taking a continuous series of "intermediate samples." We cannot of course expect that the gas exchange of the subject will remain

constant during the whole period covered by the samples, but as the air in the chamber is only very gradually renewed the possible changes in the composition of the air and the respiratory quotient must obviously be very gradual. Since the air expired from the subject must show the same respiratory quotient whether it be mixed with a small or a large quantity of pure air, the oscillations observed in the respiratory quotient must be due chiefly to errors in the gas analysis, while oscillations in the  $\text{CO}_2$  increase coincident with similar oscillations in the  $\text{O}_2$  deficit must be due to incomplete mixing. The results were as follows:

## Control experiment 5.

Time	Duration seconds	$\text{CO}_2$ increase ‰	$\text{O}_2$ deficit ‰	R. Q.	
10.16	61	65.75	70.8	0.929	
	69	65.1	70.4	0.925	
	68	66.0	71.1	0.928	
	91	65.95	71.3	0.925	
	62	65.65	70.85	0.928	
	47	66.0	70.85	0.932	
	75	66.2	71.3	0.929	
	83	66.4	71.65	0.927	
	10.28	70	66.7	71.8	0.930

Average 0.928

Dispersion  $\sigma = \pm 0.002$ 

The variations in the respiratory quotient correspond closely to the unavoidable errors in the gas analysis, but the percentages of  $\text{CO}_2$  and  $\text{O}_2$  show somewhat larger variations especially in the second and fifth sample indicating deficiencies in the mixing amounting sometimes to  $\frac{1}{2}$  or even 1 % of the  $\text{CO}_2$  increase or  $\text{O}_2$  deficit.

In the calculation of the energy metabolism from the results of the respiration experiments we have like Benedict and Cathcart [1913] used the method introduced by Zuntz and Schumburg [1901]. We have not used the original table but the latest given by Zuntz and Loewy [1913]. This table which shows the caloric value of oxygen at quotients between 0.71 and 1.00 is partly reproduced on p. 310. It differs slightly from the earlier tables. The use of this table presupposes that anabolic processes do not take place, and it refers only to the catabolism of fats and carbohydrates, assuming that the oxygen and carbon dioxide corresponding to the protein metabolism are subtracted beforehand. Like Benedict and Cathcart, Loewy [1911] and others we have made our calculations on the basis that fats and carbohydrates are exclusively catabolised. It will be necessary to estimate the error resulting from this simplification. Almost all the experiments have been made on low protein diets, but it has not been practicable to collect the urine and determine the protein metabolism.

We can assume a protein metabolism of 75 g. per day as the maximum during our experiments. This corresponds to 12 g. nitrogen or 0.5 g. per hour.

0.5 g. nitrogen per hour requires per minute 49.4 cc. oxygen and produces 0.222 Calorie, while the same quantity of oxygen would when used to burn a mixture of fat and carbohydrate produce 0.242 Calorie. The maximum error committed by disregarding the protein metabolism is therefore 0.02 Calorie or 0.3–0.4 % of the metabolism during work.

The accuracy of the technique adopted for the respiration experiments during rest has been studied and discussed by Liljestrand [1916]. The error on a 5 minute experiment may amount to about 5 % or 10 cc. CO<sub>2</sub> or O<sub>2</sub> per minute. We have made a technical error in taking the samples of expired air from the mixing vessel synchronously with the periods of ventilation. As the volume of the lungs + tubing + mixing vessel is about 6 l. a change in ventilation will not correspond to a simultaneous change in the composition of the air from which the sample is drawn, but this change will on an average take place 6 respired liters or 1 minute later. The taking of samples should therefore have been begun 1 minute after the beginning of each period and continued for 1 minute after the end of it. With an absolutely uniform respiration no error can arise from this source, but the respiration during rest is seldom absolutely uniform. The large number of double determinations made by us furnish material for a statistical study of the accuracy which will be referred to below (Appendix I, p. 346).

The muscular work was in all our experiments performed on the bicycle ergometer constructed by Krogh [1913]. The instrument was provided with automatic control of the current [Krogh, 1915, 3]. A load of 10 g. is sufficient to bring about a contact actuating the adjusting motor, and the maximum error on the determination of the load is therefore 10 g. or 1 % of the load usually employed. When work is being performed it can be observed that the adjusting motor is generally actuated for a moment about every minute, now increasing now diminishing the load. This is due to the unavoidable small irregularities in the pedalling, but these oscillations must in our opinion counterbalance each other so that the final error on the effective load will be much below 1 %.

The rate of pedalling was controlled by a metronome which could be observed by the subject through one of the windows in the chamber. We soon found that it was difficult to control the rate by sight and installed an electric bell actuated by the metronome inside the chamber. After a minute or two a certain phase in the movements of the legs is synchronous with the signal, and the rate is maintained with a minimum of mental effort.

We found that the metronome employed by us would not work with absolute constancy and in most experiments the subject has therefore employed 10 minutes during each of the experimental periods in controlling the metronome by counting and comparing with a watch. This has the further advantage of relieving somewhat the monotony of the work. The maximum variation observed in the rate of the metronome is 0.4 %.

In preliminary tests with each subject the load and rate of pedalling were

adjusted to the muscular strength and the convenience of the subject. Some preferred a rate of 60 revolutions per minute, but most of the experiments were done at the rate of about 50.

The subject always began to work just after entering the chamber and worked for half-an-hour before the experiments began. We have repeatedly made determinations of 10-20 minutes duration during this introductory period, but we have found that the results were distinctly less regular than they became later and these determinations have been left out of account in the working out of the results.

We have made several separate series of experiments and after each series we have reviewed the results secured and the technique employed to find out whether by suitable changes in the arrangement we might hope to avoid some of the irregularities encountered. As this is, in our opinion, one of the most important points in the research we propose to report the experiments in the order in which they were made, to set forth the reasons for the changes in routine adopted and discuss their results.

#### PRELIMINARY SERIES OF EXPERIMENTS.

Our first plan was worked out in collaboration with Dr G. Liljestrand of Stockholm who took part also in this series of experiments. We would make determinations on four subjects and would act alternately as subjects ourselves. On each subject we would make a preliminary experiment which should not be included in the series. After a suitable interval the subject should take a diet consisting either chiefly of carbohydrates or chiefly of fats. In the first experiments (on J. L. and G. L.) no precautions were taken regarding protein, but later it was provided that the experimental diets should contain a minimum of protein. The diet should be taken for two consecutive days and on the morning of the third day an experiment should be made before the subject took any food. During the third day the diet should be continued and a second experiment should be made on the morning of the fourth day. Each experiment should consist of 2 hours' work with 3 half-hour periods in which the metabolism should be determined. Thereupon the subject should have a rest of several days before taking the alternative diet. We hoped by taking two days' diet before the experiments to obtain very high or respectively very low quotients. It was arranged further that in the fat periods the subjects should take a certain amount of exercise to get rid of as much glycogen as possible.

Two of the subjects should begin with the carbohydrate diet and two with the fat diet, and we hoped in this way to neutralise the possible influence of training.

The technique was during these preliminary experiments not so elaborate and precise as it became later. The automatic device on the ergometer was not regularly controlled and the metronome was assumed to work at a constant rate when once set and was controlled only at long intervals. As the rate was

found to have changed distinctly we had to obtain the rates in the experiments done during the interval by interpolation. The motor driving the gas meter was not provided with a regulator and its rate varied about 3 %. The revolutions of the meter were counted by an electrical device which proved not to be absolutely reliable. The length of the experimental periods was regulated by the rate of outflow of the mercury from the sampling vessels which differed somewhat in size; they varied therefore between 22 and 32 minutes. In a few cases, which will be duly noted, these imperfections may have caused errors in the determinations but usually their influence is imperceptible.

Determinations of the metabolism during rest were not made regularly on the first two subjects (J. L. and G. L.) but the standard metabolism must be deduced from 2-3 experiments.

Experiments were made on the following subjects:

J. L. Age 46 years. Weight 67 kg. Height 171 cm. Practised in bicycling but not specially trained.

G. L. Age 31 years. Weight 51 kg. Height 166 cm. Had very little practice in bicycling.

A. K. Age 42 years. Weight 67 kg. Height 176 cm. Practised in bicycling but not trained.

R. E. Age 25 years. Weight 64 kg. Height 170 cm. In moderate training as a bicyclist.

From the notes made by the subjects the following points are selected:

J. L. Fat diet, Jan. 13th to 15th (inclusive). Work on the ergometer on the 15th and 16th. The diet consisted chiefly of pork, eggs and meat with cabbage and butter. Some claret was taken at meals. The subject took plenty of food and had no digestive trouble.

Exercise: a 2-3 hours' walk on the 13th and 14th. Very tired afterwards. Indisposition with fever and headache began on the 14th and continued throughout the period. Body temperatures: 15th morning, 37.5°, night, 38.2°; 16th, 36.7°, 37.4°; 17th, night, 39.2°; 18th, 38.0°, 37.0°; 19th, 36.0°, 37.0°. Pulse rate: 15th, 77; 17th, 90 and 19th, 58 which latter figure is normal for the subject.

Work on the ergometer on the 15th. Moderate perspiration, tired afterwards. On the 16th, profuse perspiration, work performed with great difficulty. Extremely tired during the afternoon.

Carbohydrate diet, Jan. 30th to Feb. 1st. The diet consisted chiefly of porridge, bread, cakes, sugar, honey, marmalade, green vegetables.

No particular exercise was taken.

Work on the ergometer on Feb. 1st and 2nd, performed with ease.

G. L. Carbohydrate diet, Jan. 16th to 18th, same as for J. L. A meal of cakes was taken late in the evening on the 17th and 18th. No exercise.

Work on the ergometer 18th and 19th performed without difficulty.

Fat diet, Jan. 23rd to 25th, same as for J. L.

Exercise: about two hours' walk on the 23rd and 24th.

Table II.

## SERIES I. GENERAL TABLE OF EXPERIMENTS.

Date and subject	Rest				Work					Techn. work Cal. per min.
	Vent. l. per min. 0°, 760 mm.	O <sub>2</sub> per min. cc.	r.q.	Cal. per min.	Length of per. <sup>1</sup>	O <sub>2</sub> per min. ° cc.	r.q.	Cal. per min.	Revolut. per min.	
J. L.				c			b	a		
15. i.					32	1220	0-772	5-92		
					30	1256	0-756	6-07	60-2 <sup>2</sup>	0-918
					29	1316	0-760	6-46		
16. i.					27	1256	0-767	6-08		
					24	1247 <sup>3</sup>	0-771	6-05	60-3 <sup>2</sup>	0-920
					22	1265	0-791	6-16		
1. ii.	4-03	218	0-77	1-055	26	1156	0-846	5-68		
	4-11	216	0-81	1-055	25	1151	0-873	5-68	61-3 <sup>2</sup>	0-935
					26	1163	0-868	5-74		
2. ii.	3-99	203	0-81	0-995	28	1187	0-846	5-84		
	3-92	205	0-80	1-00	26	1194	0-843	5-87	61-5 <sup>2</sup>	0-937
					28	1186	0-836	5-82		
G. L.										
18. i.					25	860	0-906	4-28		
					29	898	0-885	4-45	60-5 <sup>2</sup>	0-645
					28	902	0-867	4-45		
19. i.					32	944	0-927	4-72		
					28	936	0-913	4-66	60-7 <sup>2</sup>	0-647
					29	903	0-900	4-48		
25. i.	5-17	228	0-765	1-10	31	957	0-766	4-63		
	5-29	229	0-765	1-11	24	970	0-777	4-71	61-0 <sup>2</sup>	0-650
					26	1014	0-751	4-90		
26. i.	4-80	216	0-765	1-045	28	989 <sup>4</sup>	0-762	4-79		
	4-89	210	0-80	1-025	29	995	0-757	4-81	61-1 <sup>2</sup>	0-651
					28	1009	0-762	4-88		
26. ii.	5-32	212	0-80	1-035	28	931	0-863	4-59		
	4-43	196	0-79	0-955	30	971	0-857	4-78	62-3	0-663
					23	954	0-833	4-68		
A. K.										
12. ii.	6-50	209	0-875	1-035	27	1053	0-896	5-22		
	6-60	213	0-87	1-05	26	1070	0-867	5-28	61-7 <sup>2</sup>	0-940
					28	1087 <sup>5</sup>	0-861 <sup>5</sup>	5-36 <sup>5</sup>		
13. ii.	6-51	215	0-88	1-065	27	1081	0-909	5-38		
	6-68	206	0-94	1-03	27	1090	0-893	5-41	61-8	0-941
					29 <sup>6</sup>	1132	0-883	5-60		

<sup>1</sup> The length of the period has been given in whole minutes.

<sup>2</sup> Revolutions obtained by interpolation from countings of metronome on the 13th Jan. and 13th Feb. and following days.

<sup>3</sup> Recorded revolutions of meter 46, corrected according to the rate in the first and third period to 47. (See also notes 4 and 6.)

<sup>4</sup> Counter on meter failed. Number of revolutions calculated from ventilation in second and third period.

<sup>5</sup> The last "intermediate" sample of air was lost. As the composition of the air in the preceding samples was approximately constant the composition was assumed in accordance with these.

<sup>6</sup> Counter on meter was seen to fail just before the reading.

Table II *continued.*

Date and subject	Rest				Work					Techn. work Cal. per min.
	Vent. l. per min. $\sigma$ , 760 mm.	O <sub>2</sub> per min. cc.	R.Q.	Cal. per min. <i>c</i>	Length of per. <sup>1</sup>	O <sub>2</sub> per min. cc.	R.Q.	Cal. per min. <i>b</i>	Revolut. per min. <i>a</i>	
A. K.										
19. ii.	6.96	254	0.725	1.22	28	1094 <sup>2</sup>	0.781	5.31 <sup>2</sup>		
	6.14	215	0.75	1.04	28	1134	0.774	5.50	61.9	0.941
					28	1135	0.755	5.49		
20. ii.	6.88	241	0.70	1.15	26	1221	0.757	5.90		
	6.91	246	0.73	1.185	26	1238	0.762	5.99	62.0	0.945
					26	1255	0.757	6.07		
1. iii.	6.18	225	0.83	1.10	22	1068	0.891	5.30		
	6.20	222	0.885	1.10	30	1122	0.870	5.54	62.4	0.950
					22	1145	0.846	5.63		
R. E.										
20. iii.	5.59	234	0.85	1.15	31	1092	0.854	5.38		
	5.44	233	0.83	1.14	30	1096	0.836	5.38	49.8	0.986
					32	1112	0.817	5.44		
25. iii.					29	1210	0.747	5.84	49.8	0.986
	6.09	290	0.73	1.395	30	1308	0.742	6.30	?	?
	5.61	276	0.71	1.32	29	1406	0.751	6.79	(57.2) <sup>3</sup>	(1.13)
26. iii.	5.48	258	0.745	1.245	28	1194	0.742	5.76		
	5.48	264	0.76	1.275	27	1221	0.730	5.87	49.8	0.986
					29	1250	0.738	6.02		
6. iv.	6.40	269	0.93	1.34	29	1054	0.891	5.23		
	6.05	263	0.90	1.305	26	1052	0.895	5.22	49.9	0.988
					30	1056	0.879	5.23		
7. iv.	5.71	250	0.93	1.25	30	1064	0.932	5.32		
	5.91	253	0.95	1.27	25	1086	0.918	5.41	49.9	0.988
					31	1140 <sup>4</sup>	0.897	5.66 <sup>4</sup>		

<sup>1</sup> See note 1 on preceding page.

<sup>2</sup> See note 4 on preceding page.

<sup>3</sup> Several countings showed that the subject was not keeping time with the metronome.

<sup>4</sup> Duration of last period slightly uncertain. The figure for the metabolism possibly 1 % too high.

Felt very tired on the 24th.

Work on the ergometer 25th and 26th performed with considerable difficulty. After the work the subject was very tired.

A. K. Carbohydrate diet, Feb. 10th to 12th. Food: potatoes, flour, bread, cakes, marmalade, sugar. A meal of cakes and marmalade with a little alcohol taken late (about 11 p.m.) on the 11th and 12th. No special exercise.

Work on the ergometer 12th and 13th performed with ease. No perspiration.

Fat diet, Feb. 17th to 19th. Very fat bacon, cream, butter, eggs, and cabbage. Plenty of food taken on the 17th and 18th. Less on the 19th. Some diarrhoea on the 18th.

Exercise: 3½ hours' walk (about 18 km.) on the 18th. Felt tired afterwards.

Work on the ergometer 19th performed with some difficulty. Rather

tired afterwards. On the 20th work carried through with extreme difficulty. Very tired for several hours afterwards.

R. E. Fat diet, March 22nd to 25th. Food: fat bacon, butter, cream, cabbage. Three meals a day. Felt hungry between meals

Exercise: 1 hour's walk and a little gymnastics each day.

Work on the ergometer 25th and 26th performed without difficulty.

Carbohydrate diet, April 4th to 6th. Food: bread, cakes, apples, potatoes and green peas. No special exercise.

Had very little sleep between the 5th and the 6th.

Work on the ergometer on the 6th. Felt tired and sleepy. Work appeared difficult. 7th, work easier but apparently not easier than on the fat diet.

An inspection of the columns R.Q. and Cal. in the general table shows an unmistakable tendency for the amount of energy, expended to perform a practically constant amount of muscular work, to vary inversely as the quotient, which means that the energy value of fat and carbohydrate for work is not the same. The value of fat is smaller than that of carbohydrate.

The difference in value for muscular work of fat and carbohydrate can be expressed quantitatively either as a difference in percentage muscular efficiency or as a difference in the number of Calories expended to perform 1 Calorie of external work. Representing by  $a$  the output of external work in Calories per minute, by  $b$  the total amount of energy expended per minute, while doing the work, and by  $c$  the energy output per minute during rest we have the percentage "gross efficiency"  $\frac{100a}{b}$  and the corresponding "net efficiency"  $\frac{100a}{b-c}$ , while the corresponding expenditure of Calories per unit work are gross  $\frac{b}{a}$  and net  $\frac{b-c}{a}$ . There is a general agreement that valid comparisons can only be obtained by using "net" values, but certain differences of opinion exist about the most correct definitions for  $a$  and  $c$ .

Lindhard [1915] has shown in detail that the amount of work recorded by a bicycle ergometer is considerably lower than the total amount actually performed in riding. The real efficiency is therefore greater than the apparent or "technical" efficiency obtained from the amount of work directly recorded. Since, however, we have to deal with differences only it does not much matter for our purposes whether the efficiencies calculated by us are true or only technical efficiencies, provided we are justified in assuming that there is a constant or approximately constant relation between the technical and the true efficiency. It has been shown by Lindhard [1915] that the relation between the true and the technical efficiency varies with the rate of pedalling and also to a certain extent with the load, but we have every reason to believe that when both the load and the rate are kept constant throughout each series of determinations the use of technical efficiencies for comparison purposes will give correct results.

A further point of difficulty and controversy in calculating efficiencies or expenditure of Calories per unit work is the choice of a proper value for  $c$ ,

the "base line" of the work. Benedict and Cathcart [1913] have made calculations of efficiencies from several different base lines, viz. complete rest, rest in a sitting posture on the ergometer, riding on an ergometer without load and riding on an ergometer which was revolved by a motor while the legs of the subject followed the motion passively. Lindhard [1915] has criticised these base lines and shown that complete rest and rest in the position in which the work is performed furnish the most reliable base lines. In our case we must expect that any difference between the value of carbohydrates and fats will exercise its influence equally upon all kinds of muscular work and also on the work of maintaining a certain posture on the ergometer, and it follows therefore that we must take complete rest as our logical base line.

The question remains as to whether the rest experiments of each day should be used for combination with the work experiment of the same day or whether a large number of rest experiments should be averaged and the average standard metabolism used as base line for all the work experiments. Lindhard [1915] has pointed out that if the variation in the rest experiments is purely accidental there is no reason to suppose that the same accidental factors (*e.g.* slight movements of the subject, small technical errors in the determination) will influence the work experiments of the same day in the same direction, and it will therefore be safer and give more correct results to average all the available determinations made during rest. In our experiments it is possible, though *a priori* not very probable, that the variations in diet may have an influence upon the resting metabolism. If that should be the case rest experiments on a certain diet should be used as base line for work experiments on the same diet.

In the above tables the rest experiments are too few in number to decide whether the diet has any influence or not upon the standard metabolism, though there is in the experiments on A. K. and R. E. some indications of a slightly increased metabolism when the quotient is very low. We have therefore averaged the rest experiments done on each subject and used the averages as base lines  $c$  in the calculations mentioned above.

When we began working out the results of this research we calculated "net efficiencies"  $\frac{100a}{b-c}$  in accordance with the principles indicated above, but we found later that the reciprocal values  $\frac{b-c}{a}$  (expenditure of energy per unit work) were better suited for the comparisons between the value of fat and carbohydrate. What we want to find is the difference in value between fat and carbohydrate or the waste of energy resulting from the combustion of fats. Putting  $b - c = d$  we have, when fats alone are the source of energy during muscular work, the expenditure of energy per unit work  $e_F = \frac{d_F}{a}$  and for carbohydrates  $e_C = \frac{d_C}{a}$ . The difference between these figures  $e_F - e_C = w$  represents the waste of energy from fat and  $\frac{100w}{e_F}$  the percentage waste of energy.

As we are unable to obtain experimentally a catabolism exclusively of fat (R.Q. = 0.71) or exclusively of carbohydrate (R.Q. = 1.00) and may obtain all possible quotients between these limits it becomes necessary to make out the quantitative relation between the respiratory quotient and the relative amounts of fat and carbohydrate catabolised. Table III, which is calculated from the corresponding table of Zuntz, shows for quotients varying from 0.71 to 1.00 the relative amounts of energy derived from carbohydrate and fat respectively.

Table III.

R.Q.	Energy value of 1 l. O <sub>2</sub> Cal.	Cal. from carbohydrate	Cal. from fat	Per cent. energy from fat	$\frac{100(1 - \text{R.Q.})}{1.00 - 0.71}$
0.71	4.795	0	4.795	100	100
0.75	4.829	0.647	4.182	86.6	86.3
0.80	4.875	1.530	3.345	68.6	69.0
0.85	4.921	2.411	2.510	51.0	51.8
0.90	4.967	3.295	1.672	33.65	34.5
0.95	5.012	4.175	0.837	16.7	17.2
1.00	5.058	5.058	0	0	0

The calculation shows that the percentage amount of energy derived from fat is approximately a straight line function of the respiratory quotient and can be expressed by the formula  $\frac{100(1 - \text{R.Q.})}{1.00 - 0.71}$ .

If, therefore, we take as our theoretical basis the hypothesis that the energy output per unit work can be expressed as the sum of a certain amount of energy derived from carbohydrate, which can be utilised "directly," and another amount derived from fat, which can only be utilised after a "conversion" involving the loss of a definite fraction of the energy, we can deduce as consequences of this hypothesis (1) that the curve expressing the relation between the energy output per unit work  $\frac{d}{a} = e$  as ordinate and the respiratory quotient as abscissa should be a straight line and (2) that the percentage waste of energy from fat should be the same for different subjects.

The percentage waste of energy from fat  $\frac{100(e_F - e_C)}{e_F}$  can be found from any two points of the curve by computation and graphically by extrapolation of the curve to the quotients 0.71 and 1.00. When we have the energy per unit work at the quotient  $q_1$  to be  $e_1$  and at the quotient  $q_2$  to be  $e_2$  we get

$$e_F - e_C = \frac{e_1 - e_2}{q_2 - q_1} 0.29 \text{ and } e_F = e_1 + \frac{e_1 - e_2}{q_2 - q_1} (q_1 - 0.71).$$

In Table IV the determinations on each subject are arranged in series according to the quotients. By means of the dates and numbers given each period can be referred back to the general table. In the figures 2-5 the results have been plotted with the quotients as abscissae and the expenditure of energy per unit work  $e$  as ordinates. The first periods of each experiment have been denoted by  $\times$ , the second by  $\bullet$  and the third by  $\circ$ . It is seen that the third periods deviate in certain cases and notably at low quotients from

Table IV.

Date and number	R.Q. work	Work Cal.	Rest Cal.	Difference	Cal. per unit of work	Technical work Cal.	Deviation from curve
J. L.		<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>a</i>	<i>δ</i>
15. i. 2	.756	6.07	1.03	5.04	5.49	0.918	
15. i. 3	.760	6.46		5.43	5.91	0.918	
16. i. 1	.767	6.08		5.05	5.49	0.920	
16. i. 2	.771	6.05 <sup>1</sup>		5.02	5.46	0.920	
15. i. 1	.772	5.92		4.89	5.33	0.918	
16. i. 3	.791	6.16		5.13	5.58	0.920	
2. ii. 3	.836	5.82		4.79	5.12	0.937	
2. ii. 2	.843	5.87		4.84	5.17	0.937	
1. ii. 1	.846	5.68		4.65	4.97	0.935	
2. ii. 1	.846	5.84		4.81	5.14	0.937	
1. ii. 3	.868	5.74		4.71	5.04	0.935	
1. ii. 2	.873	5.68		4.65	4.97	0.935	
G. L.							
25. i. 3	.751	4.90	1.04	3.86	5.94	0.650	+0.2
26. i. 2	.757	4.81		3.77	5.79	0.651	+0.07
26. i. 1	.762	4.79		3.75	5.76	0.651	+0.05
26. i. 3	.762	4.88		3.84	5.90	0.651	+0.19
25. i. 1	.766	4.63		3.59	5.52	0.650	-0.18
25. i. 2	.777	4.71		3.67	5.64	0.650	-0.03
26. ii. 3	.833	4.68		3.64	5.49	0.663	-0.03
26. ii. 2	.857	4.78		3.74	5.64	0.663	+0.18
26. ii. 1	.863	4.59		3.55	5.36	0.663	-0.08
18. i. 3	.867	4.45		3.41	5.29	0.645	-0.15
18. i. 2	.885	4.45		3.41	5.29	0.645	-0.10
19. i. 3	.900	4.48		3.44	5.32	0.647	-0.03
18. i. 1	.906	4.28		3.24	5.02	0.645	-0.32
19. i. 2	.913	4.66		3.62	5.60	0.647	+0.28
19. i. 1	.927	4.72		3.68	5.69	0.647	+0.41
						Sum	+0.47
A. K.							
19. ii. 3	.755	5.49	1.10	4.39	4.67	0.941	-0.25
20. ii. 1	.757	5.90		4.80	5.08	0.945	+0.16
20. ii. 3	.757	6.07		4.97	5.26	0.945	+0.34
20. ii. 2	.762	5.99		4.89	5.18	0.945	+0.28
19. ii. 2	.774	5.50		4.40	4.68	0.941	-0.19
19. ii. 1	.781	5.31 <sup>1</sup>		4.21	4.48	0.941	-0.37
1. iii. 3	.846	5.63		4.53	4.77	0.950	+0.09
12. ii. 3	.861	5.36		4.26	4.53	0.940	-0.11
12. ii. 2	.867	5.28		4.18	4.45	0.940	-0.18
1. iii. 2	.870	5.54		4.44	4.68	0.950	+0.06
13. ii. 3	.883	5.60		4.50	4.78	0.941	+0.19
1. iii. 1	.891	5.30		4.20	4.42	0.950	-0.14
13. ii. 2	.893	5.41		4.31	4.58	0.941	+0.02
12. ii. 1	.896	5.22		4.12	4.38	0.940	-0.17
13. ii. 1	.909	5.38		4.28	4.55	0.941	+0.03
						Sum	-0.24
R. E.							
26. iii. 2	.730	5.87	1.27	4.60	4.67	0.986	+0.02
26. iii. 3	.738	6.02		4.75	4.82	0.986	+0.20
26. iii. 1	.742	5.76		4.49	4.55	0.986	-0.06
25. iii. 1	.747	5.84		4.57	4.63	0.986	+0.04
20. iii. 3	.817	5.44		4.17	4.23	0.986	-0.12
20. iii. 2	.836	5.38		4.11	4.17	0.986	-0.11
20. iii. 1	.854	5.38		4.11	4.17	0.986	-0.05
6. iv. 3	.879	5.23		3.96	4.01	0.988	-0.13
6. iv. 1	.891	5.23		3.96	4.01	0.988	-0.09
6. iv. 2	.895	5.22		3.95	4.00	0.988	-0.08
7. iv. 3	.897	5.66		4.39	4.44	0.988	+0.36
7. iv. 2	.918	5.41		4.14	4.19	0.988	+0.19
7. iv. 1	.932	5.32		4.05	4.10	0.988	+0.15
						Sum	+0.32

<sup>1</sup> Slightly uncertain.

the rest and give higher values for  $e$ . This must be ascribed to the fatigue which was repeatedly very pronounced.

It is obvious that the experiments are too few and too discordant to allow of a quantitative estimate of the energy waste from fat. It is possible to draw

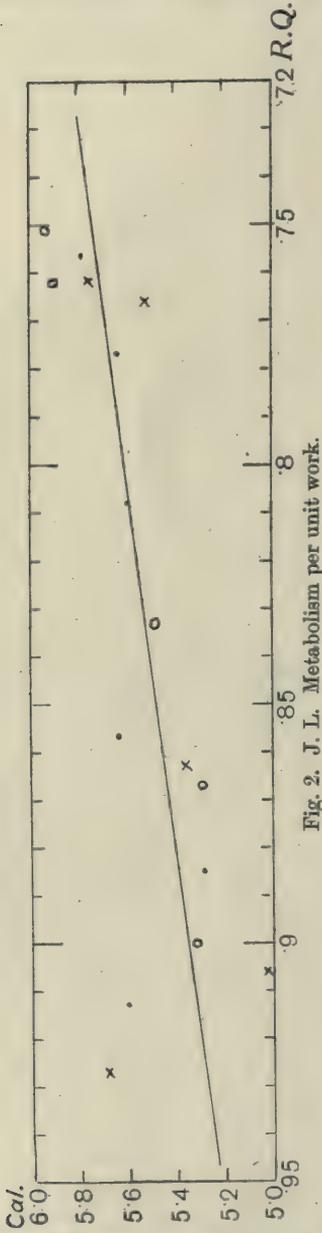


Fig. 2. J. L. Metabolism per unit work.

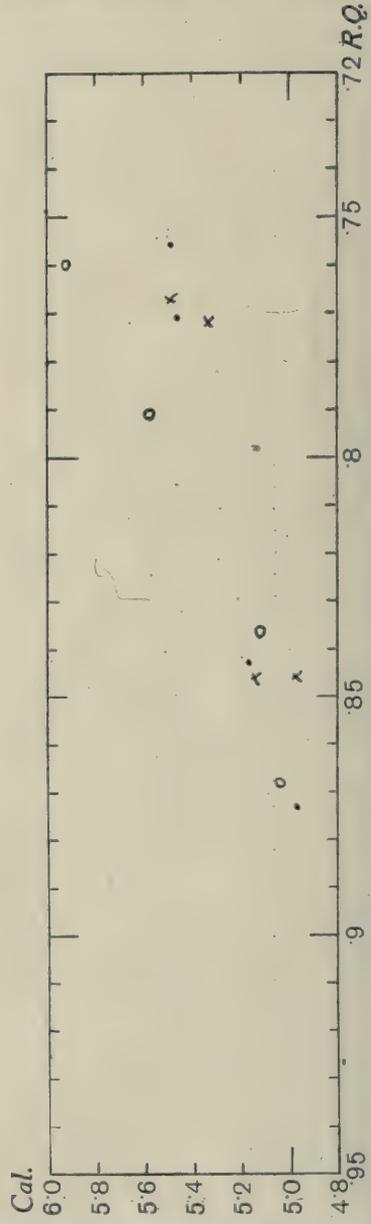


Fig. 3. G. L. Metabolism per unit work.  
 x First experimental period, • second, o third period.

rather different curves which will suit the determinations at least as well as the straight lines which we have drawn. In the case of J. L. we have not drawn any curve. On account of his illness during the fat period we think it better to disregard the experiments made on this subject.

We have measured the vertical distance (in Cal. per unit work) of each point in Figs. 2-5 from the curve to which it belongs and these distances have been given in the last column of Table IV as the deviation from the curve ( $\delta$ ). If the curves were correctly drawn and all the determinations were

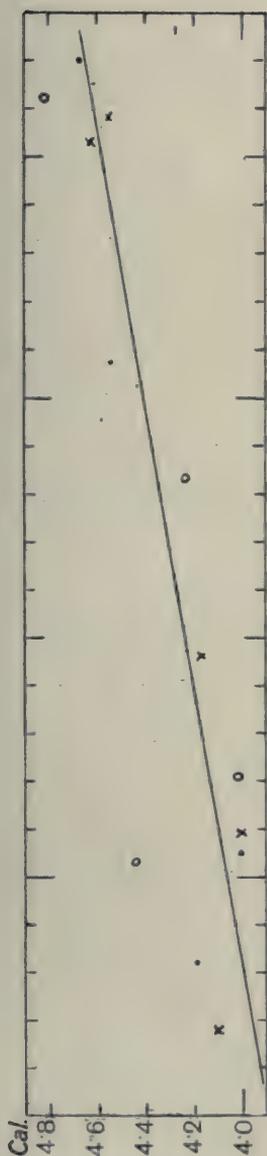


Fig. 4. A. K. Metabolism per unit work.

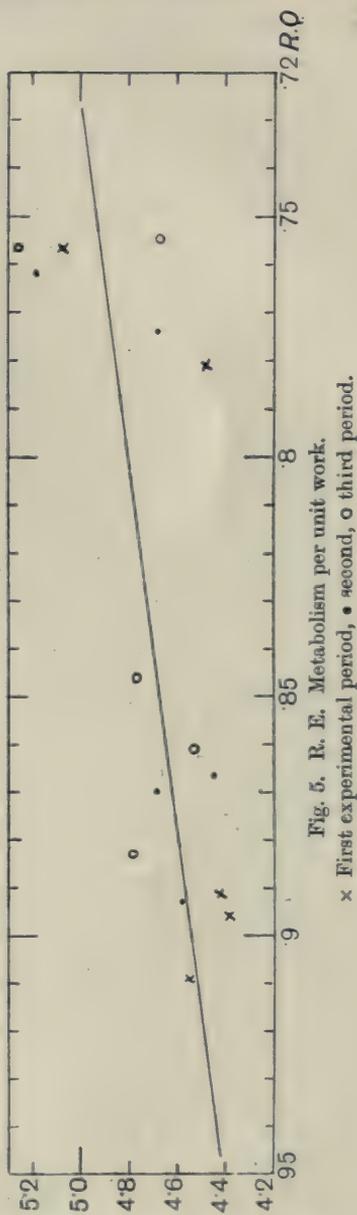


Fig. 5. R. E. Metabolism per unit work.  
 x First experimental period, • second, o third period.

of equal weight the algebraic sum of these deviations should be 0. From the deviations given the dispersion ( $\sigma$ ) or "standard deviation" has been calculated for each curve according to the formula  $\sigma = \sqrt{\frac{\sum \delta^2}{n-1}}$  and we have further

calculated the dispersion in per cent. of the average metabolism per unit work  $e_{0.85}$ . We find

Table V.

	G. L.	A. K.	R. E.
$n$	15	15	13
$e_{0.85}$ Cal.	5.48	4.17	4.24
$\sigma$ Cal.	0.20	0.21	0.155
$\sigma$ %	3.6	4.4	3.7

When the waste of energy is calculated from the straight lines drawn we obtain the following results:

Table VI.

	G. L.	A. K.	R. E.
$e_F$ Cal.	5.84	5.04	4.72
$e_C$ Cal.	5.09	4.28	3.72
Waste from fat Cal.	0.75	0.76	1.00
Waste %	12.9	15.1	21.2
Efficiency on carbohydrate $\frac{100}{e_C}$	19.6	23.4	26.8

The low efficiency of the subject G. L. is probably to be explained by his want of training as a bicycle rider.

The results are obviously of very limited value quantitatively and it should be specially pointed out that the exercise taken during the fat periods and not during the carbohydrate periods may constitute a systematic error.

#### SECOND SERIES OF EXPERIMENTS.

As a result of the preliminary series of experiments several improvements were introduced into the technique and routine. The purely technical improvements have been alluded to above. As we ourselves were evidently not suitable as subjects, partly because our other duties made it impossible for us to lead an absolutely uniform life during the experimental period, we resolved to secure the assistance of an intelligent student who should be, as far as possible, a trained athlete both generally and as a bicyclist, hoping thereby to minimise the influence of training. We would further shorten the dietary periods and make a larger number of determinations also at intermediate quotients to obtain data for determining the form of the efficiency curve. The length of the experimental periods should be somewhat reduced to minimise the influence of fatigue.

We were very fortunate in securing the services as subject of Mr Olaf Hansen a student of languages and gymnastics who possessed all the qualifications desired.

His age was 23 years, weight 80 kg. and height 179 cm. He is a trained athlete and was in good form at the time but lately he had practised bicycling to a very limited extent only. He was carefully instructed with regard to the experimental routine, and the most suitable load on the machine and rate of pedalling were found by trials. No hint whatever was given of the

subjective results expected from the different diets, but the subject was asked to make notes both of the food eaten and of the influence of the work upon his well-being and physical fitness. From these notes, which gave very full information, we have made the following extract.

From May 6th (1917) to 11th mixed diet, poor in protein. Went for a walk of 7 km. on the 6th. Otherwise no exercise. Work on the ergometer 7th, 8th, 10th and 11th. Not the least tired after each day's work. Weight on the 9th, 80 kg.

Carbohydrate diet 12th to 18th. Three meals a day on the experiment days, otherwise four. Last meal in the evening between 7 and 10 o'clock. Food chiefly porridge, bread, potatoes and other vegetables, sugar, honey, marmalade and fruit. Exercise: walk of 7 km. on the 13th, cycling about 17 km. on the 17th. This tour was taken in the afternoon after work on the ergometer in the morning. No fatigue whatever was felt. Work on the ergometer 14th, 15th, 16th and 17th performed with very slight perspiration and not in the least fatiguing. The work was felt to be easier than in the preceding week and on the 14th the subject asked if the load had not been diminished. Weight on the 16th, 79.5 kg.

Fat diet 19th to 24th. On the experiment days a cup of tea without sugar or milk was taken in the morning and large meals at 12 noon and 7 night. Cocoa and cream in the afternoon at 3. Food: ham, eggs, bacon, butter, cream and green vegetables. Exercise: cycling about 38 km. on the 20th in slow time, 25 km. on the 23rd in the evening; felt tired in the legs especially during first half of the tour. No experiments on these days. Cycling about 9 km. on the 24th, in the evening after an experiment. Felt very tired during the whole week and could not do the usual amount of mental work. Work on the ergometer 21st and 22nd. Perspiration profuse, extremely tired during the latter part of the day's work and afterwards and had to rest during most of the afternoons. Took some bread in the evening of the 22nd and the morning of the 23rd. Work again on the 24th and 25th. Profuse perspiration but not quite so tired as on the preceding days. Weight on the 25th, 76.5 kg. The technical work on the ergometer was 1.12 Cal. per minute throughout.

A simple inspection of the general table shows:

1. Somewhat irregular variations of the standard metabolism with a distinct tendency to become higher during the last days of the period when the quotient was very low.

2. The correlation between the quotient and the total metabolism during work is marked when the changes from day to day are considered, but it is unmistakable at the same time that the metabolism at a constant quotient becomes gradually lower during the period. We take this lowering to be due to training.

In order to find the most reliable numerical expression for the function studied we have examined and combined the experimental results in several ways.

Table VII.

## GENERAL TABLE OF EXPERIMENTS.

Date	Rest				Length of period	O <sub>2</sub> per min. cc.	Work			
	Ventilation l. per min.	O <sub>2</sub> per min. cc.	r. q.	Cal. per min.			r. q.	Cal. per min.	Revolut. per min.	Tech. work Cal. per min.
O.H. I				<i>c</i>				<i>b</i>		<i>a</i>
1917	5-93	261	0-82	1-275	30	1399	0-836	6-86	49-4	1-123
8. v.	5-93	258	0-82	1-265	26	1393	0-829	6-83		
					29	1405	0-818	6-87		
10. v.	6-05	290	0-82	1-42	31	1395	0-814	6-82	49-4	1-123
					29	1395	0-803	6-81		
					32	1395	0-810	6-82		
11. v.	5-97	303	0-79	1-475	24	1365	0-830	6-69	49-4	1-123
					25	1347	0-835	6-61		
					23	1375	0-814	6-72		
14. v.	6-34	276	0-92	1-375	24	1353	0-864	6-68	49-4	1-123
					24	1336	0-870	6-60		
					23	1320	0-876	6-53		
15. v.	6-24	291	0-945	1-455	24	1303	0-929	6-50	49-4	1-123
					24	1312	0-922	6-54		
					23	1306	0-919	6-51		
17. v.	6-16	280	0-92	1-395	24	1276	0-904	6-34	49-4	1-123
					25	1301	0-896	6-45		
					23	1314	0-881	6-50		
18. v.	5-95	276	0-93	1-38	23	1337	0-839	6-56	49-4	1-123
					24	1366	0-817	6-68		
					24	1375	0-799	6-70		
21. v.	5-77	300	0-71	1-435	23	1419	0-731	6-82	49-3	1-120
					24	1427	0-715	6-85		
					22	1434	0-714	6-88		
22. v.	5-94	291	0-71	1-395	23	1398	0-717	6-71	49-3	1-120
					25	1396	0-709	6-70		
					23	1413	0-719	6-78		
24. v.	6-22	324	0-71	1-55	22	1363	0-735	6-56	49-4	1-123
					25	1387	0-734	6-63		
					23	1325 <sup>1</sup>	0-765 <sup>1</sup>	6-42		
25. v.	5-61	292	0-69	1-395	22	1384	0-744	6-68	49-4	1-123
					26	1387	0-731	6-68		
					22	1385	0-746	6-68		

<sup>1</sup> The exceptionally large increase in quotient and decrease in metabolism must arouse suspicion but no reason can be found for doubting the technical accuracy of the results.

The determinations of the resting metabolism have been plotted in Fig. 6 as ordinates with the dates as abscissae. The curve drawn shows that the average standard metabolism remained practically constant except during the last few days when the quotients were very low. We observed in the earlier experiments on A. K. and R. E. that with a low quotient the standard metabolism shows a tendency to rise and we may add that this is borne out also by the later series of determinations given below. We have therefore no

hesitation in concluding that the standard metabolism remained constant during the whole period except for the variations correlated with the variations in quotient. The determinations have accordingly been plotted a second time (Fig. 7) with the quotient as abscissa and a curve drawn to represent them.

We have now the material to combine the standard metabolism  $c$  with the metabolism during work  $b$  in three different ways. We can either:

1. Use the determination of  $c$  for each particular day in combination with the determinations of  $b$  for the same day.
2. Use the average value for  $c$  from all the determinations in combination with each  $b$ .

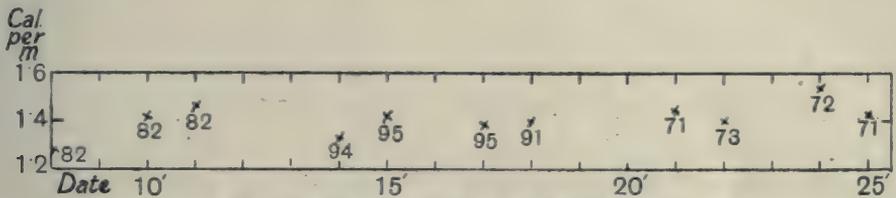


Fig. 6. O. H. Standard metabolism. Variations from day to day.  
Figures: respiratory quotients.

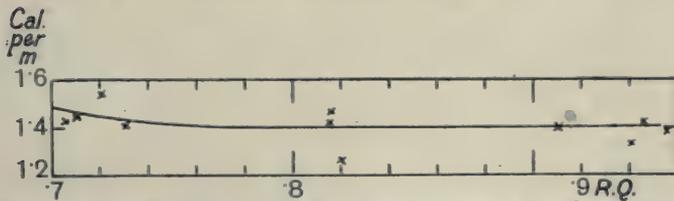


Fig. 7. O. H. Standard metabolism. Variation with respiratory quotient.

3. Use for combination with each  $b$  a value for  $c$  selected from the curve Fig. 7, on the principle that the respiratory quotient during rest is the same as that during work. Though this latter assumption might seem to be somewhat arbitrary an inspection of Table VII shows unmistakably that there is a very close connection between the quotient during rest and the quotient during work just afterwards, the difference being seldom more than a few per cent.<sup>1</sup>

The alternatives 1 and 2 have been tested in a preliminary calculation of the efficiency at varying quotients the results of which are given in the curves, Fig. 8<sup>2</sup>. When the standard metabolism  $c$  found on a particular day depended mainly on some cause which would act also during the work on the same day the combination of the values obtained each day ought to give the most concordant results, but if the variations in the  $c$  values are purely accidental and have nothing to do with the results obtained in the corresponding work

<sup>1</sup> This point will be discussed in detail below. Appendix III, p. 354.

<sup>2</sup> In these curves corrections have been introduced for the effect of training according to the principles given below, p. 320.

experiments (which have likewise their own accidental variations) the elimination of the accidental variations of the  $c$  values ought to diminish the variability of the final results.

It is seen at a glance that the individual determinations of efficiency show considerably larger deviations from the curve when the  $c$  values for each day are utilised than when they are replaced by the average value. A numerical expression for the difference is obtained when the dispersions of the two sets of values are calculated. For Curve I we find a dispersion  $\sigma = 0.38$  and for Curve II  $\sigma = 0.31$ .

As it might be thought that the correction for the effect of training applied to the curves, Fig. 8, might influence the result of the comparison we have made a similar comparison between the uncorrected values for the expenditure of energy per unit work, using in one case the  $c$  values for each day and in the other the  $c$  values taken from the curve, Fig. 7, according to the third alternative. The results of this comparison are shown in Fig. 9. It is obvious that the  $c$  values taken from the curve make the final results much more uniform than those obtained day by day, and in our opinion this furnishes conclusive evidence in favour of discarding the single determinations and using either the simple average of a number of determinations or a graph in which the accidental variations have been smoothed out<sup>1</sup>. In the final calculations we have also rejected the second alternative (an average  $c$ ), because it would increase the apparent metabolism per unit work at low quotients, and the later experiments show that there is no reason to doubt the reality of the observed increase in resting metabolism at low quotients.

The curves, Fig. 9, show an increase in  $e$  (energy per unit work) when the quotient falls from 0.93 to 0.8, but a slight decrease on the further fall from 0.8 to 0.71. This, as well as certain other irregularities, is due in the main to the fact that the metabolism, when determined at an approximately constant quotient, is evidently decreasing during the 18 days occupied by the series of experiments. In order to examine this decrease we have arranged Table VIII giving for each experimental day the respiratory quotient for the three determinations during work, taken together, the corresponding Calories per minute ( $b$ ), the metabolism during rest  $c$  as found from the curve, Fig. 7, by means of the quotient in column 1, the difference  $b-c$ , the amount of technical work  $a$  and the metabolism per unit work  $e$ . The figures in this latter column have been plotted in Fig. 10 as ordinates with the dates as abscissae. All the results corresponding to quotients between 0.78 and 0.88 have been marked by a  $\times$  and the others by a small  $\bullet$ , and it has been attempted to draw a curve representing the variation of the metabolism with time at a quotient of about 0.83. This curve is called the "training curve" and is taken to represent the change in metabolism per unit work depending, as we

<sup>1</sup> We have also in the later series of experiments made similar comparisons between average and individual  $c$  values with the same result as that illustrated above, but we do not think it necessary to reproduce them.

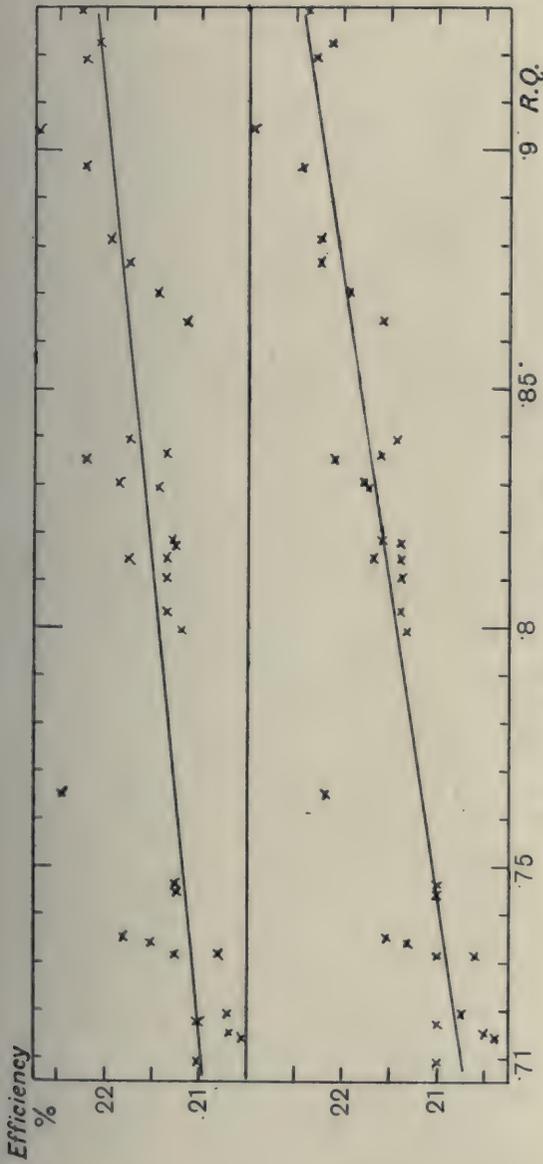


Fig. 8. O. H. Efficiency of muscular work. Lower curve: average value for standard metabolism. Upper curve: day to day values for standard metabolism.

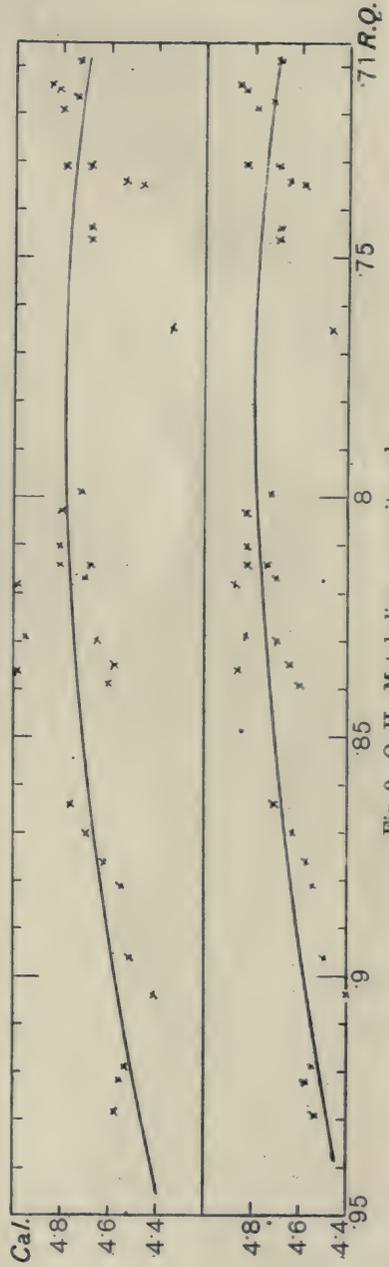


Fig. 9. O. H. Metabolism per unit work. Lower curve: values for standard metabolism from curve, Fig. 7. Upper curve: day to day values for standard metabolism.

think, upon the increasing training of the subject. Unfortunately we have no determinations at suitable quotients after the 18th and for the last week we have had therefore to extrapolate the training curve as best we might. We have thought it safest to assume a very slight influence of the training after that date.

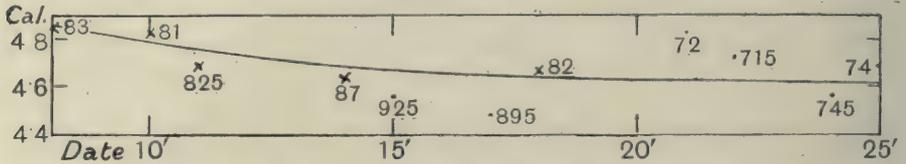


Fig. 10. O. H. Metabolism per unit work. Influence of training.

At the end of the experimental period we assume that the metabolism per unit work at a quotient of 0.83 would have decreased to 4.62 Cal. as shown by the curve, and by finding from the curve the corresponding figure for each experimental day and dividing 4.62 by the same we have obtained a series of figures, increasing from 0.950 on the 8th to 1.000 on the 22nd to 25th, given in the last column of Table VIII as the "correction for training." In the final Table IX the effect of the training has been eliminated by multiplying each  $e$  value by the correction coefficient for the day.

Table VIII.

Date 1917	R.Q.	Average Cal. during work <i>b</i>	Cal. at rest from curve <i>c</i>	Difference <i>d</i>	Technical work <i>a</i>	Cal. per unit of work <i>e</i>	Training correction (from Fig. 10)
8. v.	.83	6.85	1.40	5.45	1.123	4.85	.950
10. v.	.81	6.82	1.40	5.42	1.123	4.83	.967
11. v.	.825	6.67	1.40	5.27	1.123	4.69	.973
14. v.	.87	6.60	1.40	5.20	1.123	4.63	.985
15. v.	.925	6.52	1.40	5.12	1.123	4.56	.989
17. v.	.895	6.43	1.40	5.03	1.123	4.48	.993
18. v.	.82	6.65	1.40	5.25	1.123	4.67	.996
21. v.	.72	6.85	1.44	5.41	1.120	4.83	.998
22. v.	.715	6.73	1.45	5.28	1.120	4.72	1.000
24. v.	.745	6.54	1.41	5.13	1.123	4.57	1.000
25. v.	.74	6.68	1.41	5.27	1.123	4.69	1.000

We are fully aware that objections can be raised against this method of attempting an elimination of the training effect. We must admit of course that the training curve as drawn is more or less arbitrary, but we have convinced ourselves that even if the training curve is drawn differently from the one we have adopted in any way compatible with the actual experiments on which it must be based, the resulting effect on the final curve will be very slight.

It must be conceded further that the effect of training is not necessarily the same at all quotients. This point will be touched upon below, p. 334.

Table IX.

Date and number	R.Q.	Cal. work	Cal. obs.	Rest from curve	Difference		Calories per unit work			Deviation from curve $\delta$	
					$b$	$c_I$	$c_{II}$	$d_I$	$d_{II}$		$e_I$
May 1917											
22	2	.709	6.70	1.40	1.45	5.30	5.25	4.73	4.69	4.69	-.10
21	3	.714	6.88	1.45	1.43	5.43	5.45	4.85	4.865	4.855	+.075
21	2	.715	6.85	1.45	1.43	5.40	5.42	4.82	4.84	4.83	+.05
22	1	.717	6.71	1.40	1.42	5.31	5.29	4.74	4.72	4.72	-.055
22	3	.719	6.78	1.40	1.42	5.38	5.36	4.80	4.79	4.79	+.02
21	1	.731	6.82	1.45	1.40	5.37	5.42	4.79	4.84	4.83	+.08
25	2	.731	6.68	1.43	1.40	5.25	5.28	4.68	4.70	4.70	-.05
24	2	.734	6.63	1.54	1.40	5.09	5.23	4.54	4.66	4.66	-.085
24	1	.735	6.56	1.54	1.40	5.02	5.16	4.47	4.60	4.60	-.14
25	1	.744	6.68	1.43	1.40	5.25	5.28	4.68	4.70	4.70	-.03
25	3	.746	6.68	1.43	1.40	5.25	5.28	4.68	4.70	4.70	-.025
24	3	.765	6.42	1.54	1.40	4.88	5.02	4.34	4.47	4.47	-.225
18	3	.799	6.70	1.40	1.40	5.30	5.30	4.72	4.72	4.70	+.06
10	2	.803	6.81	1.42	1.40	5.39	5.41	4.80	4.82	4.66	+.02
10	3	.810	6.82	1.42	1.40	5.40	5.42	4.81	4.825	4.665	+.04
10	1	.814	6.82	1.42	1.40	5.40	5.42	4.81	4.825	4.665	+.05
11	3	.814	6.72	1.47	1.40	5.25	5.32	4.68	4.74	4.61	$\pm$ .00
18	2	.817	6.68	1.40	1.40	5.28	5.28	4.70	4.70	4.68	+.07
8	3	.818	6.87	1.27	1.40	5.60	5.47	4.99	4.87	4.625	+.015
8	2	.829	6.83	1.27	1.40	5.56	5.43	4.95	4.84	4.60	+.01
11	1	.830	6.69	1.47	1.40	5.22	5.29	4.65	4.71	4.58	-.01
11	2	.835	6.61	1.47	1.40	5.14	5.21	4.53	4.64	4.515	-.065
8	1	.836	6.86	1.27	1.40	5.59	5.46	4.98	4.86	4.615	+.035
18	1	.839	6.56	1.40	1.40	5.16	5.16	4.60	4.60	4.58	$\pm$ .00
14	1	.864	6.68	1.34	1.40	5.34	5.28	4.76	4.705	4.63	+.095
14	2	.870	6.60	1.34	1.40	5.26	5.20	4.69	4.63	4.56	+.04
14	3	.876	6.53	1.34	1.40	5.19	5.13	4.62	4.57	4.50	-.015
17	3	.881	6.50	1.39	1.40	5.11	5.10	4.55	4.54	4.51	+.005
17	2	.896	6.45	1.39	1.40	5.06	5.05	4.51	4.50	4.47	-.01
17	1	.904	6.34	1.39	1.40	4.95	4.94	4.41	4.40	4.37	-.10
15	3	.919	6.51	1.43	1.40	5.08	5.11	4.53	4.55	4.50	+.055
15	2	.922	6.54	1.43	1.40	5.11	5.14	4.55	4.58	4.53	+.09
15	1	.929	6.50	1.43	1.40	5.13	5.10	4.57	4.54	4.49	+.06
											Sum
											-.035

In Table IX all the determinations have been arranged in the order of increasing quotients and in the last column but one the  $e$  values are corrected for training by means of the figures given in Table VIII. These corrected  $e$  values have been plotted in Fig. 11, which shows very distinctly the regular variation of the expenditure of energy per unit work with the respiratory quotient. It should be noted that the determinations number 3 which are marked  $\circ$  are on the whole slightly above the curve probably on account of fatigue. The determination R.Q. = 0.765 (No. 3, May 24th) forms a very striking exception to this rule and deviates from all the rest in that the third period shows a marked rise in quotient and a great fall in metabolism. As pointed out above (Table VII, note 1) there is no reason to doubt the technical accuracy of this determination.

In the last column of Table IX are given the deviations of the determinations from the curve. Their number is 33 and the dispersion works out as  $\sigma = 0.072$  Cal. or 1.55 % of the average metabolism  $e_{0.85} = 4.56$  Cal., a very striking improvement on the results of the preliminary series.

The straight line drawn corresponds to an  $e_F = 4.79$  Cal. and  $e_C = 4.32$  Cal. The waste of energy from fat is therefore  $w = 0.47$  Cal. or 9.8 %.

### THIRD SERIES OF EXPERIMENTS.

The second series of determinations would have been quite satisfactory if the curve of training and the corrections based upon this curve had not been a little uncertain, and we resolved therefore to change the routine so as to insure the best possible data from which to construct a curve of training and at the same time to make the influence of the training as uniform as possible throughout the series. This end we hoped to attain by making determinations each day on the same subject during a period of three weeks and letting the subject undergo repeated changes of diet from carbohydrate to fat and *vice versa* beginning and ending the series on a mixed diet and effecting the changes through the interpolation of one day's mixed diet.

The experiments of this series were carried out in January and February 1918 on Mr A. Möller Nielsen (age 24 years, height 174.5 cm.) a student of languages and gymnastics who kindly undertook to act as a subject and carried out his part of the work very carefully. From his notes which were not so elaborate as those of O. H. we give the following essential points.

Jan. 28th to 30th, ordinary mixed diet, poor in protein. Work on the ergometer from the 29th. Weight on the 29th, 63 kg. No exercise. Carbohydrate diet 31st to Feb. 4th. Mixed diet on the 5th. Went for a short walk (2 km.) in the evening. Fat diet 6th to 9th. A short walk every evening. Felt tired in the evening on the 6th. Mixed diet on the 10th and 11th. Carbohydrate diet 12th and 13th. Fat diet 14th to 16th. Diarrhoea in the afternoon on the 14th. Very tired in the evening. Severe headache on the 15th. Slightly unwell on the 16th. Mixed diet 17th to 19th. Weight on the 19th, 69.8 kg.

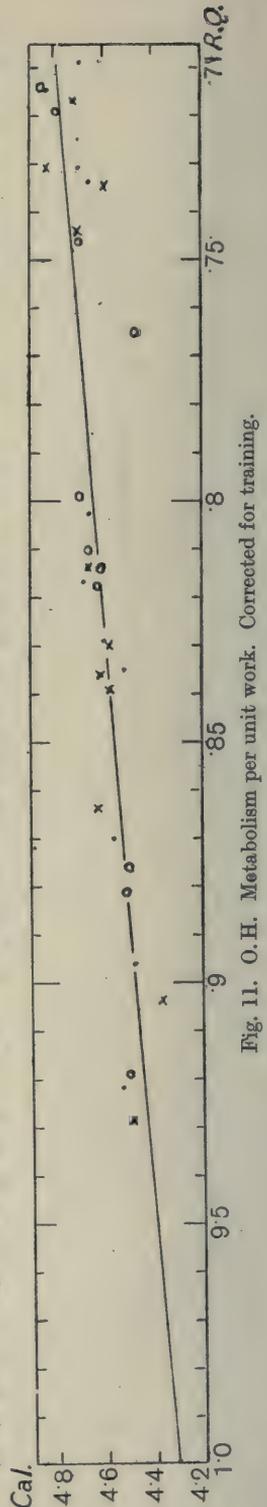


Fig. 11. O. H. Metabolism per unit work. Corrected for training.

Table X.

## GENERAL TABLE OF EXPERIMENTS.

Date	Rest				Work					
	Ventilation l. per min.	O <sub>2</sub> per min. cc.	r.q.	Cal. c	Length	O <sub>2</sub> per min. cc.	r.q.	Cal. b	Revolutions per min.	Techn. work Cal. a
A. M. N.										
1918	6-79	236	.845	1-16	Normal period of work. Training experiment					
29. i.	7-30	226	.855	1-11						
					25	1234	.848	6-07		
30. i.	6-15	231	.82	1-13	21	1080	.873	5-335		
	6-38	212	.84	1-04	22	1075	.862	5-30	49-3	.910
					22	1102	.855	5-43		
31. i.	6-20	221	.87	1-09	20	1050	.890	5-205		
	5-97	213	.87	1-05	20	1057	.888	5-24	49-3	.910
					20	1074	.883	5-32		
1. ii.	6-41	242	.85	1-19	21	1075	.850	5-29		
	6-20	244	.86	1-20	21	1090	.846	5-36	49-3	.910
					21	1113	.846	5-475		
2. ii.	6-00	245	.90	1-215	21	1049	.886	5-20		
	5-60	235	.88	1-165	21	1067	.870	5-27	49-2	.9085
					21	1080	.872	5-335		
3. ii.	6-17	238	.895	1-18	21	1141	.910	5-68		
	6-11	242	.89	1-195	21	1135	.896	5-635	49-2	(1-05 <sup>1</sup> )
					21	1145	.892	5-68		
4. ii.	6-20	235	.98	1-185	Work as usual but no determinations on account of trouble with ergometer regulator and gas analysis apparatus					
	6-55	242	.99	1-22						
5. ii.	5-61	234	.93	1-175	21	1049	.882	5-19		
	6-01	227	1-01	1-15	21	1039	.907	5-165	49-2	.908
					21	1065	.876	5-265		
6. ii.	5-80	231	.88	1-14	21	1034	.876	5-11		
	5-83	231	.88	1-14	21	1029	.876	5-09	49-2	.908
					21	1054	.868	5-205		
7. ii.	5-00	231	.83	1-13	21	1057	.787	5-14		
	5-07	223	.83	1-095	21	1080	.782	5-25	49-2	.9075
					21	1063	.805	5-19		
8. ii.	5-29	231	.79	1-125	21	1106	.770	5-36		
	5-33	239	.77	1-16	21	1112	.761	5-38	49-2	.9075
					21	(1112) <sup>2</sup>	.764 <sup>2</sup>	5-38		
9. ii.	5-17	230	.775	1-115	21	(1060) <sup>2</sup>	(.768) <sup>2</sup>	(5-125) <sup>2</sup>		
	4-81	224	.78	1-085	21	1110	.764	5-375	49-5	.9135
					21	1109	.760	5-365		
10. ii.	5-28	233	.75	1-125	21	1087	.770	5-27		
	5-28	223	.78	1-08	21	1093	.763	5-295	49-5	.9135
					20	1113	.761	5-385		

<sup>1</sup> Regulator on ergometer did not work. The load was determined after the experiment.<sup>2</sup> Oxygen determination in last average sample lost. Percentage determined by interpolation between remaining samples which were practically constant.<sup>3</sup> Oxygen determination in first intermediate sample not quite reliable.

Table X *continued.*

Date	Rest				Work					
	Ventilation l. per min.	O <sub>2</sub> per min. cc.	R.Q.	Cal.	Length	O <sub>2</sub> per min. cc.	R.Q.	Cal.	Revolutions per min.	Techn. work Cal.
A. M. N.				<i>c</i>				<i>b</i>		<i>a</i>
11. ii.	5-05	212	·85	1·045	21	1043	·829	5·11		
	5-10	206	·87	1·02	21	1045	·830	5·125	49·5	·9135
					20	1066	·825	5·22		
12. ii.	5-46	217	·94	1·085	20	1000	·889	4·96		
	5-13	214	·90	1·06	21	1000	·884	4·95	49·5	·9135
					21	1026	·876	5·07		
13. ii.	6-16	216	·97	1·085	21	988	·930	4·935		
	6-19	219	·99	1·105	21	995	·925	4·965	49·5	·9135
					20	1020	·928	5·09		
14. ii.	6-23	214	1·06	1·095	21	(1030) <sup>1</sup>	(·926) <sup>1</sup>	(5·14) <sup>1</sup>		
	6-00	212	1·03	1·075	20	1023	·924	5·10	49·5	·9135
					20	1030	·940	5·15		
15. ii.	5-30	216	·83	1·06	21	1016	·841	4·99		
	4-81	205	·84	1·005	21	1031	·838	5·06	49·5	·9135
					21	1040	·842	5·11		
16. ii.	5-16	221	·79	1·075	21	1063	·793	5·16		
	4-94	212	·81	1·035	21	1089	·779	5·275	49·4	·912
					21	1052	·802	5·13		
17. ii.	5-10	214	·81	1·045						
	5-38	213	·79	1·035	70	1120	·719	5·375	49·4	·912
18. ii.	5-56	208	·97	1·045	22	(998) <sup>2</sup>	(·843) <sup>2</sup>	(4·91) <sup>2</sup>		
	5-35	204	·92	1·02	21	1011	·829	4·96	49·4	·9115
					21	1033	·833	5·065		
19. ii.	4-98	206	·86	1·015	21	1007	·844	4·95		
	5-10	209	·85	1·03	21	1020	·846	5·015	49·4	·9115
					21	1022	·840	5·02		

<sup>1</sup> The oxygen determination in the first average sample is probably erroneous. The first intermediate sample gave an O<sub>2</sub> deficit of 0·493 % the second gave 0·4935, while the intervening average sample gave 0·5085 %. The probable values would be oxygen per min. 1004 cc., R.Q. 0·95, calories 5·025.

<sup>2</sup> First average sample lost. Values obtained by interpolation between intermediate samples.

During each day's work on the ergometer this subject noticed no distinct difference between the effects of the different diets and never became very tired. The technical work per minute was 0·91 Cal.

The determinations of the standard metabolism show a very marked decline during the whole of the experimental period, a decline which is brought out very clearly when the results are arranged graphically with the dates as abscissae (Fig. 12). The three first determinations give low values, but otherwise there is a steady and extremely regular decrease from first to last. The low values observed on the three first days are probably due to a fall in the body temperature. When the subject suppressed all movements, as he did very carefully throughout, he complained of feeling cold. In all the later determinations he was carefully wrapped up and felt warm. The decrease in standard metabolism, taking place at all events from Feb. 1st to 19th, is

remarkable, and the more so as the subject gained in weight from 63 to 70 kg. As the diet during the experimental period was poor in protein it is conceivable that the decrease may be due to the elimination of a nitrogen reserve from the body as in the recent experiments of Benedict and his collaborators [1918], but we have no observations to support such a hypothesis.

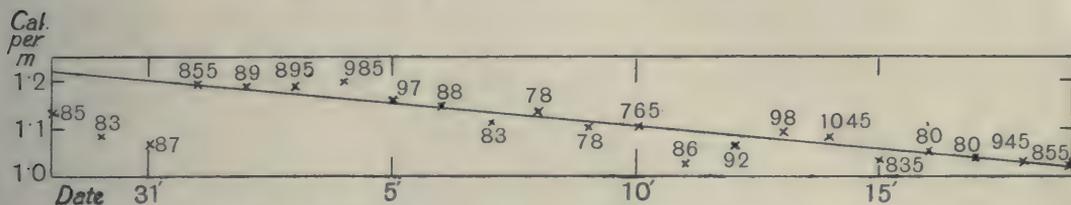


Fig. 12. A. M. N. Standard metabolism. Variation from day to day.

As the quotient during rest has in all experiments been above 0.77 there is very little influence of the quotient upon the resting metabolism to be detected, and we have therefore used the values for standard metabolism deduced from the straight line curve, Fig. 12, in the subsequent treatment of the material.

Table XI.

Date	R.Q. work	Average Cal. during work	Cal. at rest from curve	Difference	Technical work	Cal. per unit work	Training correction (from Fig. 13)
1918		<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>e</i>	
30. i.	.865	5.355	1.21	4.145	.910	4.555	.952
31. i.	.885	5.255	1.20	4.055	.910	4.46	.957
1. ii.	.85	5.375	1.19	4.185	.910	4.60	.962
2. ii.	.875	5.27	1.18	4.09	.9085	4.50	.969
3. ii.	.90	5.665	1.17	4.495	(1.05) <sup>1</sup>	(4.35) <sup>1</sup>	.973
5. ii.	.89	5.205	1.15	4.055	.908	4.47	.982
6. ii.	.875	5.135	1.14	3.995	.908	4.40	.985
7. ii.	.79	5.19	1.13	4.06	.9075	4.47	.989
8. ii.	.765	5.37	1.12	4.25	.9075	4.685	.991
9. ii.	.76	5.36	1.115	4.245	.9135	4.65	.993
10. ii.	.765	5.32	1.105	4.215	.9135	4.615	.995
11. ii.	.83	5.15	1.095	4.055	.9135	4.44	.997
12. ii.	.885	4.995	1.085	3.91	.9135	4.28	.999
13. ii.	.93	5.00	1.075	3.925	.9135	4.30	1.000
14. ii.	.93	5.13	1.07	4.06	.9135	4.445	1.000
15. ii.	.84	5.055	1.06	3.995	.9135	4.375	1.000
16. ii.	.79	5.19	1.05	4.14	.912	4.54	1.001
17. ii.	.72	5.375	1.04	4.335	.912	4.755	1.002
18. ii.	.83	5.00	1.03	3.97	.9115	4.355	1.002
19. ii.	.845	4.995	1.02	3.975	.9115	4.36	1.002

<sup>1</sup> Doubtful.

In Table XI the determinations during work done on each day have been averaged and the metabolism per unit work calculated for each separate day taken as a whole, and in Fig. 13 these results have been plotted with the dates as abscissae. By means of the values showing quotients between 0.82

and 0.89 a training curve has been constructed. The distribution of the determinations on which this curve is based shows that its possible errors must be comparatively slight. Owing to the distribution of the fat and carbohydrate periods the difference between the efficiency of these diets for work comes out about as distinctly and practically the same quantitatively with no correction whatever for training. See Figs. 14 and 15.

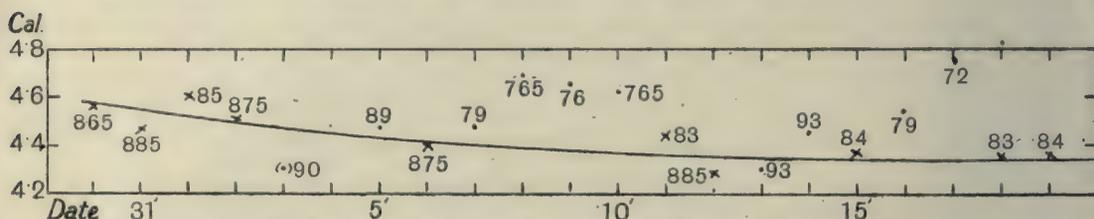


Fig. 13. A. M. N. Metabolism per unit work. Influence of training.

In the final Tables XII–XIII the determinations are arranged as usual according to their quotients, and as an inspection of the general Table X shows that the metabolism in the third experimental periods is generally a little higher than in periods 1 and 2, the third periods have been sorted out and arranged in the separate Table XIII. The results have been plotted and curves drawn to represent them in Figs. 14 and 15. In drawing the curves we have *not* attempted to represent all the determinations as nearly as possible. That would obviously mean curves which were not straight lines but slightly convex towards the abscissa, but such curves would be incompatible with our theoretical conception.

We can safely maintain, however, that the lines, drawn straight to be in accordance with theory, are perfectly compatible with the determinations, since the dispersion of these is only  $\sigma = 0.074$  Cal. or 1.7 % for the curve representing periods 1 and 2 and  $\sigma = 0.080$  Cal. or 1.8 % for the curve representing period 3.

It should be pointed out that the comparatively large deviation of a few determinations *upwards* from the curve as at R.Q. = 0.924 in Fig. 14 and at 0.940 in Fig. 15 is a thing which must be expected in experiments of this kind. A slight indisposition on the part of the subject will be enough to account for them.

The straight line, Fig. 14, representing periods 1 and 2 corresponds to an  $e_F = 4.70$  Cal. and  $e_C = 4.02$ . The waste of energy from fat is therefore 0.68 Cal. or 14.5 %. The line in Fig. 15 representing the third experimental periods corresponds to  $e_F = 4.74$  Cal. and  $e_C = 4.11$  Cal. or a waste of energy from fat of 0.63 Cal. = 13.3 %.

#### FOURTH SERIES OF EXPERIMENTS.

Our fourth and final series of determinations was again made on the subject O. H. on whom we had obtained in 1917 the remarkably constant results given above pp. 314–322. Mr H. had been working hard for his

degree during the preceding term and his general physical training was therefore below his usual standard. During a fortnight preceding the experiments he undertook to make a bicycle ride of about 30 km. every day. We hoped thereby to induce such an amount of bicycle training that the training effect of the experiments themselves, which had been so pronounced in the two preceding series, would be greatly diminished. As will be seen below this hope was in the main disappointed. In all other respects the routine was the same as that adopted in the third series.

Table XII.

Date and number Jan. and Feb. 1918	R.Q. work	Work Cal. <i>b</i>	Rest Cal. <i>c</i>	Differ- ence <i>d</i>	Cal. per unit work		Deviation from curve <i>δ</i>
					<i>e</i>	<i>e</i> corrected	
8 2	·761	5·38	1·12	4·26	4·69	4·645	+·065
10 2	·763	5·295	1·105	4·19	4·59	4·57	±·00
9 2	·764	5·375	1·115	4·26	4·665	4·635	+·065
9 1	(·768)	(5·125)	1·115	(4·01)	(4·39)	4·36) <sup>1</sup>	
8 1	·770	5·36	1·12	4·24	4·67	4·625	+·065
10. 1	·770	5·27	1·105	4·165	4·56	4·54	−·02
16 2	·779	5·275	1·05	4·225	4·63	4·635	+·095
7 2	·782	5·25	1·13	4·12	4·54	4·49	−·04
7 1	·787	5·14	1·13	4·01	4·42	4·37	−·15
16 1	·793	5·16	1·05	4·11	4·51	4·515	+·01
11 1	·829	5·11	1·095	4·015	4·395	4·38	−·04
18 2	·829	4·96	1·03	3·93	4·31	4·32	−·10
11 2	·830	5·125	1·095	4·03	4·41	4·40	−·02
15 2	·838	5·06	1·06	4·00	4·38	4·38	−·02
15 1	·841	4·99	1·06	3·93	4·305	4·305	−·085
18 1	·843	(4·91)	1·03	(3·88)	(4·26)	4·27) <sup>2</sup>	
19 1	·844	4·95	1·02	3·93	4·31	4·32	−·06
1 2	·846	5·36	1·19	4·17	4·585	4·41	+·03
19 2	·846	5·015	1·02	3·995	4·385	4·395	+·15
1 1	·850	5·29	1·19	4·10	4·51	4·34	−·03
30 2	·862	5·30	1·21	4·09	4·495	4·28	−·06
2 2	·870	5·27	1·18	4·09	4·50	4·36	+·04
30 1	·873	5·335	1·21	4·125	4·535	4·32	±·00
6 1	·876	5·11	1·14	3·97	4·375	4·31	±·00
6 2	·876	5·09	1·14	3·95	4·35	4·285	−·025
5 1	·882	5·19	1·15	4·04	4·45	4·37	+·075
12 2	·884	4·95	1·085	3·865	4·23	4·225	−·065
2 1	·886	5·20	1·18	4·02	4·425	4·29	+·005
31 2	·888	5·24	1·20	4·04	4·44	4·25	−·03
12 1	·889	4·96	1·085	3·875	4·24	4·235	−·045
31 1	·890	5·205	1·20	4·005	4·40	4·21	−·07
3 2	·896	5·635	1·17	4·46	(4·3)	4·2) <sup>3</sup>	
5 2	·907	5·165	1·15	4·015	4·42	4·34	+·10
3 1	·910	5·68	1·17	4·51	(4·3)	4·2) <sup>3</sup>	
14 2	·924	5·10	1·07	4·03	4·41	4·41	+·21
13 2	·925	4·965	1·075	3·89	4·26	4·26	+·065
14 1	(·926)	5·14	1·07	4·07	(4·455)	4·455) <sup>4</sup>	
13 1	·930	4·935	1·075	3·86	4·225	4·225	+·04
Sum							+·185

<sup>1</sup> Slightly uncertain.<sup>2</sup> Slightly uncertain.<sup>3</sup> Doubtful.<sup>4</sup> Doubtful.

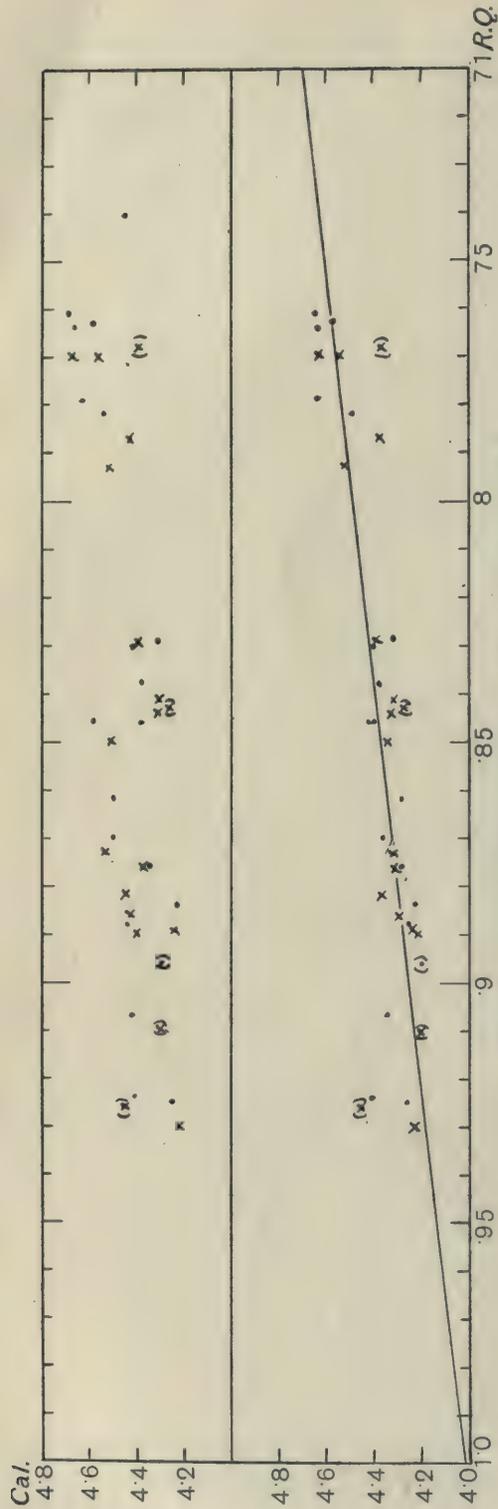


Fig. 14. A. M. N. Metabolism per unit work. Periods 1 and 2.

Lower section: values corrected for training. Upper section: values not corrected for training.

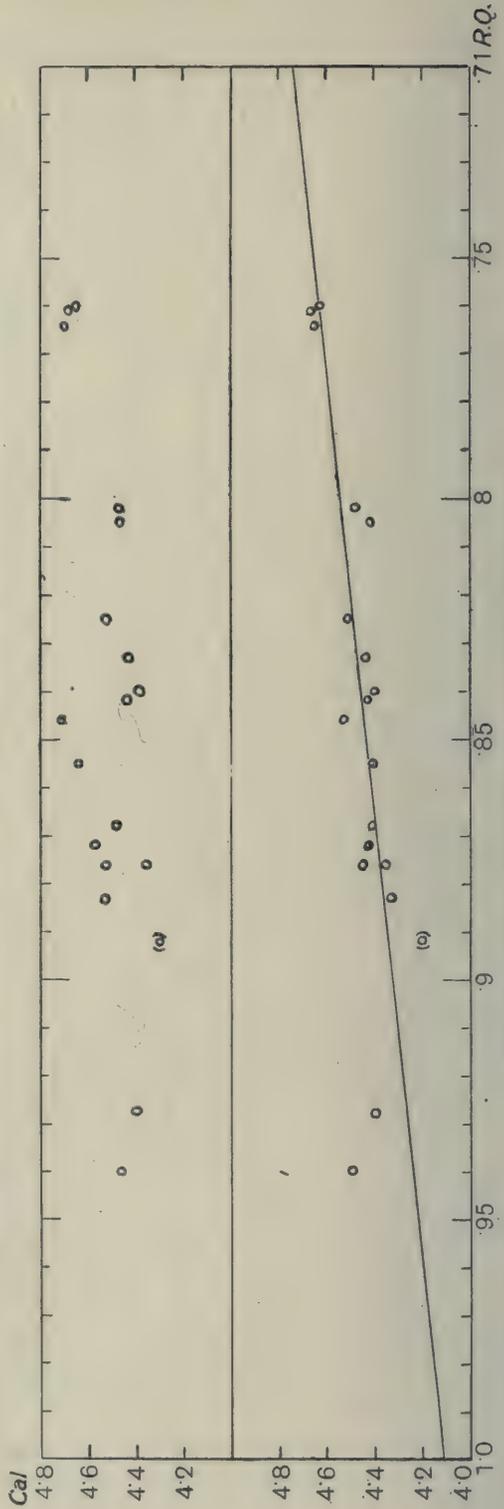


Fig. 15. A. M. N. Metabolism per unit work. Third period.

Lower section: values corrected for training. Upper section: values not corrected for training.

Table XIII.

9	3	·760	5·365	1·115	4·25	4·655	4·625	-·01
10	3	·761	5·385	1·105	4·28	4·685	4·665	+·03
8	3	·764	5·38	1·12	4·26	4·695	4·65	+·03
16	3	·802	5·13	1·05	4·08	4·475	4·48	-·06
7	3	·805	5·19	1·13	4·06	4·47	4·42	-·11
11	3	·825	5·22	1·095	4·125	4·52	4·51	+·02
18	3	·833	5·065	1·03	4·035	4·43	4·44	-·03
19	3	·840	5·02	1·02	4·00	4·39	4·40	-·055
15	3	·842	5·11	1·06	4·05	4·435	4·435	-·015
1	3	·846	5·475	1·19	4·285	4·71	4·53	+·085
30	3	·855	5·43	1·21	4·22	4·64	4·41	-·01
6	3	·868	5·205	1·14	4·065	4·48	4·41	+·015
2	3	·872	5·335	1·18	4·155	4·575	4·43	+·04
5	3	·876	5·265	1·15	4·115	4·53	4·45	+·07
12	3	·876	5·07	1·085	3·985	4·365	4·36	-·015
31	3	·883	5·32	1·20	4·12	4·53	4·335	-·025
3	3	·892	5·68	1·17	4·51	(4·3	4·2) <sup>1</sup>	
13	3	·928	5·09	1·075	4·015	4·40	4·40	+·135
14	3	·940	5·15	1·07	4·08	4·47	4·47	+·23
								Sum +·145

<sup>1</sup> Doubtful.

From the notes of the subject we give the following extract:

When the experiments began on April 2nd the general athletic training was distinctly lower than in 1917 but the special training for bicycling was better. The fatigue *during* work on the ergometer did not become very pronounced, but in the afternoon the subject was almost constantly tired and had to take rest on a couch for 1½ to 2 hours. The difference between the fatigue on fat days and carbohydrate days was less pronounced than in 1917 but on several occasions very distinct, nevertheless. Work was performed on the ergometer each day throughout the period and no other exercise was taken except in a few instances specified below.

Mixed diet from 2nd to 5th. Somewhat tired in the afternoon and evening. Weight on the 4th, 84·3 kg.

Fat diet, 6th to 8th. Not distinctly fatigued during the riding, but repeatedly suspected the metronome to be too fast, though as a matter of fact it was rather slower than the normal rate of pedalling of the subject. Somewhat tired during each afternoon and evening.

Mixed diet on the 9th. Weight, 81·5 kg.

Carbohydrate diet, 10th to 13th. On the 11th less tired than usual, took no rest in the afternoon. 12th tired for a short period during the work, tired in the afternoon with feeling of "pressure" in the abdomen. No objective symptoms of digestive trouble. 13th, symptoms and fatigue less pronounced. Weight, 83·5 kg. 14th, felt well during the work. No abdominal "pressure." Very slight fatigue afterwards.

Mixed diet, 14th to 17th. 15th, 18th, again rather tired in the afternoon.

Weight on the 15th, 83·1 kg., on the 18th 82·2 kg.

Table XIV.

## GENERAL TABLE OF EXPERIMENTS.

Date	Rest				Work					
	Ventilation l. per min.	O <sub>2</sub> per min. cc.	R. Q.	Cal.	Length of per.	O <sub>2</sub> per min. cc.	R. Q.	Cal.	Revolutions per min.	Techn. work Cal.
O. H. II				c				b		a
1918	5-71	263	.79	1.28	21	1304	.855	6.42		
3. iv.	(6-50)	260	.86) <sup>1</sup>	1.28	21	1317 <sup>2</sup>	.843	6.475	49.3	1.122
					21	1316 <sup>2</sup>	.845	6.47		
4. iv.	5-95	275	.815	1.345	21	1347	.840	6.62		
	5-32	242	.80	1.18	21	1334	.839	6.555	49.5	1.127
					21	1327	.831	6.508		
5. iv.	5-63	269	.84	1.32	20	1290	.858	6.36		
	5-39	266	.815	1.30	20	1284	.846	6.315	49.4	1.125
					20	1321	.826	6.47		
6. iv.	5-52	263	.785	1.28	20	1300	.819	6.36		
	5-505	256	.795	1.25	20	1275	.839	6.26	49.3	1.122
					20	1300	.830	6.375		
7. iv.	5-58	288	.75	1.30	20	1339	.768	6.49		
					20	1345	.766	6.52	49.4	1.125
					20	1355	.763	6.56		
8. iv.	5-13	282	.695	1.35	21	1381	.728	6.64		
	5-67	285	.725	1.38	21	1361	.740	6.56	49.5	1.128
					21	1386	.732	6.67		
9. iv.	5-38	314	.645	(1.42) <sup>3</sup>	21	1333	.751	6.44		
	5-20	292	.66		21	1366	.737	6.58	49.5	1.128
					21	1348	.748	6.51		
10. iv.	5-75	254	.83	1.245	21	1333	.797	6.50		
	5-64	252	.84	1.24	21	1320	.804	6.44	49.5	1.130
					21	1347	.804	6.57		
11. iv.	6-23	248	.985	1.25	21	1270	.904	6.315		
	6-38	252	.995	1.275	21	1271	.903	6.32	49.5	1.128
					21	1280	.902	6.36		
12. iv.	6-47	255	1.00	1.29	21	1244	.944	6.225		
	6-33	260	.97	1.31	21	1231	.945	6.16	49.4	1.126
					21	1261	.917	6.28		
13. iv.	6-49	248	.985	1.25	21	1227	.961	6.16		
	6-63	266	.94	1.33	21	1217	.967	6.12	49.4	1.127
					21	1250	.944	6.26		
14. iv.	6-55	244	1.01	1.25	21	1211	.974	6.10		
	7-05	274	1.00	1.385	21	1255	.938	6.275	49.5	1.128
					21	1236	.940	6.185		
15. iv.	6-36	260	.845	1.275	21	1221	.904	6.07		
	6-19	263	.855	1.295	26	1235	.895	6.13	49.4	1.126
					21	1250	.882	6.185		

<sup>1</sup> The high quotient certainly due to over-ventilation.<sup>2</sup> Oxygen determination in intermediate sample 3 obviously erroneous. Value obtained by interpolation.<sup>3</sup> Caloric value of oxygen by extrapolation in Zuntz's table.

Table XIV *continued.*

Date	Rest				Work					
	Ventilation l per min.	O <sub>2</sub> per min. cc.	r. q.	Cal.	Length of per.	O <sub>2</sub> per min. cc.	r. q.	Cal.	Revolutions per min.	Techn. work Cal.
O.H. II				<i>c</i>				<i>b</i>		<i>a</i>
1918	5-78	256	-83	1-255	21	1254	-862	6-18		
16. iv.	5-80	262	-805	1-275	21	1268	-851	6-24	49-5	1-129
					21	1269	-849	6-24		
17. iv.	5-90	260	-79	1-265	21	1282	-816	6-27		
	5-96	277	-80	1-35	21	1289	-811	6-30	49-4	1-126
					21	1338	-791	6-51		
18. iv.	5-52	261	-77	1-265	21	1285	-808	6-275 <sup>1</sup>		
	5-81	261	-79	1-27	21	1287	-808	6-28	49-5	1-127
					21	1310	-800	6-39		
19. iv.	5-81	248	-905	1-23	21	1256	-880	6-22		
	5-67	245	-885	1-215	21	1248	-884	6-18	49-4	1-126
					21	1264	-870	6-24		
20. iv.	6-54	266	-94	1-33	21	(1183)	-966	5-945) <sup>2</sup>		
	6-43	261	-95	1-31	21	1225	-935	6-12	49-4	1-127
					21	1236	-937	6-18		
21. iv.	6-43	248	-98	1-25	21	1209	-950	6-055		
	6-52	261	-98	1-315	21	1204	-939	6-02	49-4	1-124
					21	1192	-970	6-00		
22. iv.	6-15	201	1-13		21	1192	-951	5-98		
	6-81	262	-97	1-32	21	1192	-935	5-96	49-4	1-126
					21	1210	-949	6-06		
23. iv.	5-32	213	-84	1-045	21	1255	-796	6-11		
	5-64	223	-87	1-10	21	1276	-788	6-21	49-5	1-128
					21	1295	-778	6-29		
24. iv.	6-46	275	-73	1-32	21					
	5-91	264	-725	1-27	21	(1332)	-752	6-44) <sup>3</sup>	49-3	1-124
					21	1334	-760	6-455		
25. iv.	6-59	258	-855	1-27	21	1252	-832	6-14		
	6-35	244	-88	1-205	21	1260 <sup>4</sup>	-822	6-17	49-4	1-124
					21	1264 <sup>4</sup>	-829	6-20		
26. iv.	6-03	254	-875	1-255	21	1249	-838	6-13		
	5-59	245	-825	1-20	20	1255	-846	6-17	49-4	1-124
					21	1247	-848	6-135		
27. iv.	5-84	240	-86	1-185	21	1227	-890	6-08		
	6-05	254	-87	1-255	18	1212	-907	6-03	49-3	1-124
					21	1233	-892	6-11		
									Average: 1-126	

<sup>1</sup> All the average samples lost. Experiments calculated from intermediate samples which varied little and regularly.

<sup>2</sup> Oxygen determination in first average sample somewhat improbable: O<sub>2</sub> deficit in sample 1 0-645 %, sample A 0-6175 %, sample 2 0-625 %.

<sup>3</sup> Intermediate samples 1 and 2 lost. 2 obtained by interpolation.

<sup>4</sup> Intermediate sample 3 lost. Value obtained by interpolation.

Carbohydrate diet, 18th to 21st. Distinctly less tired than before but more so than on the corresponding diet in 1917. On the 21st went for a walk of 10 km. (Had not felt any inclination to take exercise during the preceding days of the experimental period.) Weight on the 22nd, 83.1 kg.

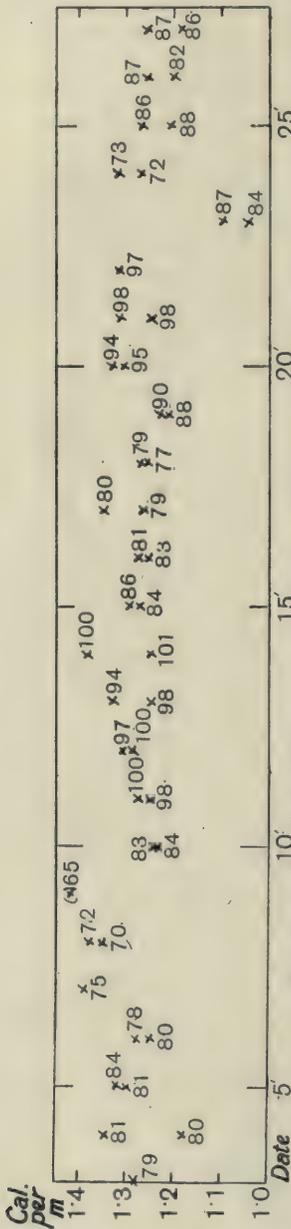


Fig. 16. O. H., 1918. Standard metabolism. Variations from day to day.

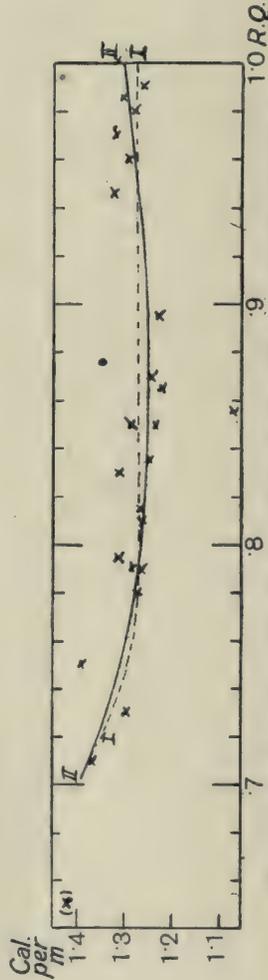


Fig. 17. O. H., 1918. Standard metabolism. Variations with respiratory quotient.

Fat diet, 22nd to 23rd. Very tired in the evening of the 22nd. Tired during the work on the 23rd. Very tired and hungry in the afternoon and evening. Work on the 24th very difficult to perform though somewhat easier during the last half hour. Weight on the 24th, 81.3 kg.

Mixed diet, 24th to 26th. Distinctly less tired after and especially during the work on the 25th to 27th. Weight on the 27th, 82.8 kg.

In Fig. 16 the single experiments made during rest have been plotted with the dates as abscissae and it is seen that while the standard metabolism does not vary with the time the determinations showing a low quotient are distinctly higher than the rest. In Fig. 17 the results (averages for each day) have therefore been plotted again with the respiratory quotient as abscissa. The standard metabolism appears to be distinctly lower at intermediate quotients than it is at low or very high ones. The full line curve drawn represents, we think, fairly accurately all the results except the single one at 0.855. As the increase in metabolism at high quotients might possibly be due to accidental errors we have drawn also the dotted line curve shown, and all the succeeding calculations of the metabolism per unit work have been carried out with both sets of values for the resting metabolism. A discussion of the influence of the quotient upon the standard metabolism will be given below in Appendix II dealing with all the determinations made on the various subjects.

Table XV.

Date	R.Q. work	Average Cal. during work <i>b</i>	Cal. at rest from		Difference		Cal. per unit work		Training correction (from Fig. 18)
			Curve I	Curve II	<i>d</i> <sub>I</sub>	<i>d</i> <sub>II</sub>	<i>e</i> <sub>I</sub>	<i>e</i> <sub>II</sub>	
			<i>c</i> <sub>I</sub>	<i>c</i> <sub>II</sub>					
3. iv.	.85	6.455	1.27	1.25	5.185	5.205	4.605	4.62	.937
4. iv.	.835	6.56	1.27	1.25	5.29	5.31	4.70	4.715	.941
5. iv.	.845	6.38	1.27	1.25	5.11	5.13	5.54	4.56	.945
6. iv.	.83	6.33	1.27	1.25	5.06	5.08	4.495	4.515	.949
7. iv.	.765	6.52	1.27	1.29	5.25	5.23	4.66	4.645	.952
8. iv.	.735	6.62	1.31	1.33	5.31	5.29	4.72	4.70	.955
9. iv.	.745	6.51	1.295	1.31	5.215	5.20	4.63	4.62	.958
10. iv.	.80	6.50	1.27	1.26	5.23	5.24	4.645	4.655	.962
11. iv.	.905	6.33	1.27	1.26	5.06	5.07	4.495	4.505	.964
12. iv.	.935	6.22	1.27	1.27	4.95	4.95	4.395	4.395	.968
13. iv.	.955	6.18	1.27	1.275	4.91	4.905	4.36	4.36	.971
14. iv.	.95	6.19	1.27	1.27	4.92	4.92	4.37	4.37	.973
15. iv.	.895	6.13	1.27	1.255	4.86	4.875	4.32	4.33	.976
16. iv.	.855	6.22	1.27	1.25	4.95	4.97	4.395	4.415	.979
17. iv.	.805	6.36	1.27	1.26	5.09	5.10	4.52	4.53	.981
18. iv.	.805	6.315	1.27	1.26	5.045	5.055	4.48	4.49	.983
19. iv.	.88	6.21	1.27	1.25	4.94	4.96	4.39	4.405	.985
20. iv.	.935	6.15	1.27	1.265	4.88	4.885	4.335	4.34	.987
21. iv.	.95	6.025	1.27	1.27	4.755	4.755	4.225	4.225	.989
22. iv.	.945	6.00	1.27	1.27	4.73	4.73	4.20	4.20	.991
23. iv.	.79	6.20	1.27	1.27	4.93	4.93	4.38	4.38	.993
24. iv.	.76	6.455	1.28	1.295	5.175	5.16	4.595	4.585	.995
25. iv.	.83	6.17	1.27	1.25	4.90	4.92	4.35	4.37	.9965
26. iv.	.845	6.145	1.27	1.25	4.875	4.895	4.33	4.35	.998
27. iv.	.895	6.07	1.27	1.255	4.80	4.815	4.265	4.28	.999

In Table XV the determinations are averaged for each day and arranged for the construction of a training curve which is given in Fig. 18. As only the experiments showing quotients between 0.8 and 0.9 are utilised for the con-

struction of the curve the difference between the two sets of values for  $e$  will be the same throughout and we have therefore utilised the second set only for drawing the curve. A training curve corresponding to  $e_I$  would be parallel to the one drawn but lie 0.02 Cal. lower. It is evident that training curves representing the determinations with quotients from 0.73 to 0.8 or with quotients between 0.9 and 0.96 would be very nearly parallel to the curve drawn in Fig. 18, and it follows therefore that the training effect is substantially the same at different quotients—as it ought to be theoretically.

It is worthy of note that the training effect of the experiments is considerable and persistent in spite of the measures taken to reduce it (the fortnight's preliminary training). There is no approach to a constant level, and the only advantage we have gained is that we have avoided the steep initial part of the training curves, Figs. 10 and 13, which makes the correction of the first experiments somewhat uncertain.

In the final Tables XVI and XVII we have again sorted out the third periods which show a slightly higher metabolism than the first and second. We have made the calculations with the two sets of values for the standard metabolism  $c_I$  and  $c_{II}$  obtained from curves I and II, Fig. 17 and corrected them for training by means of the figures given in Table XV. Both sets of corrected values and one set of the uncorrected have been plotted in Figs. 19 and 20 and curves drawn to represent them.

The lower curves in Fig. 19 and Fig. 20, corresponding to the standard metabolism curve II, Fig. 17, which gave the most accurate representation of the rest experiments and showed an increase in metabolism both at very high and at low quotients, are practically straight lines while the best curves (middle curves Fig. 19 and Fig. 20) corresponding to the values  $e_I$  are slightly convex towards the abscissa. We believe that the straight line curves are the most reliable.

The curves representing the results  $e_{II}$  as uncorrected for training are likewise straight and show about the same waste of energy from fat as do the corrected curves. This is due of course to the experiments with high and low quotients being suitably distributed. Had the experiments with low quotients been all made first the apparent waste would have become greater, and it could have been almost abolished by making the experiments at

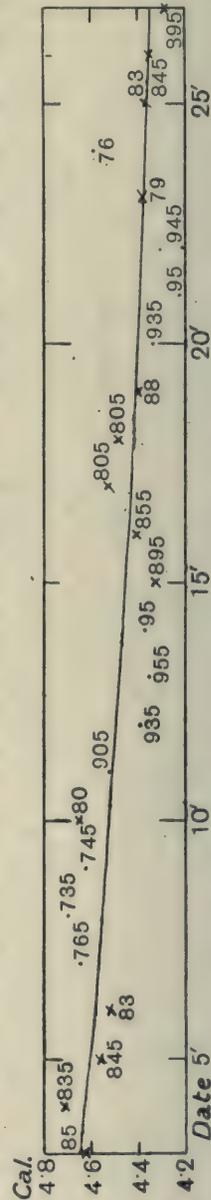


Fig. 18. O. H., 1918. Metabolism per unit work. Influence of training.



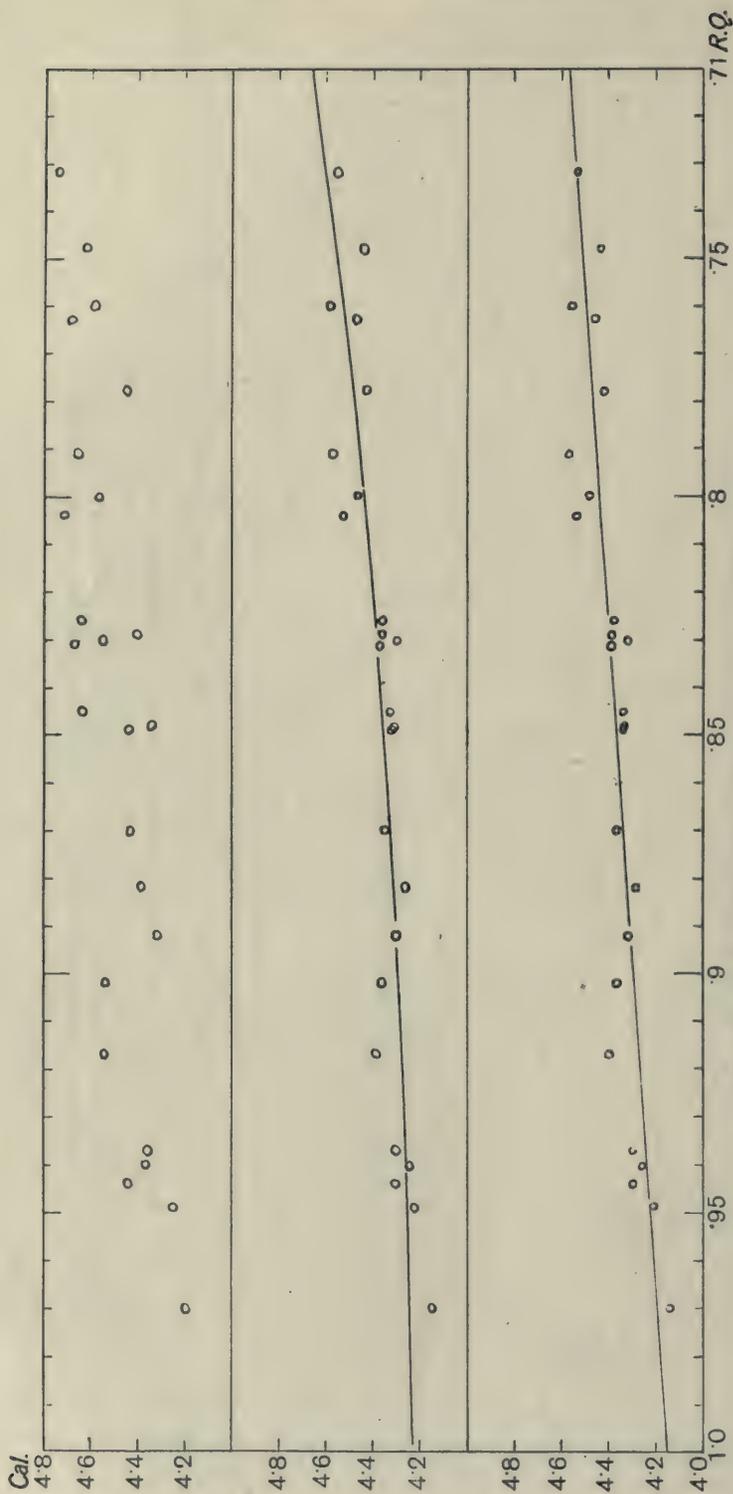


Fig. 20. O. H., 1918. Metabolism per unit work. Third periods.

Upper curve: not corrected for training. Standard metabolism from curve II, Fig. 17.

Middle curve: corrected for training. Standard metabolism from curve I, Fig. 17.

Lower curve: corrected for training. Standard metabolism from curve II, Fig. 17.

Table XVI.

Date and number April 1918	n. o. work	Work Cal. <i>b</i>	Rest <i>c<sub>I</sub></i>	Cal. <i>c<sub>II</sub></i>	Difference		Cal. per unit work				Deviation from curve ( <i>e<sub>II</sub></i> corr.) <i>δ</i>
					<i>d<sub>I</sub></i>	<i>d<sub>II</sub></i>	<i>e<sub>I</sub></i>	<i>e<sub>II</sub></i>	<i>e<sub>I</sub></i> corr.	<i>e<sub>II</sub></i> corr.	
8. 1	·728	6·64	1·32	1·34	5·32	5·30	4·725	4·71	4·51	4·50	+·01
9. 2	·737	6·58	1·305	1·325	5·275	5·255	4·685	4·67	4·49	4·475	±·00
8. 2	·740	6·56	1·30	1·32	5·26	5·24	4·67	4·655	4·46	4·45	-·03
9. 1	·751	6·44	1·29	1·305	5·15	5·135	4·575	4·56	4·38	4·37	-·09
24. 2	·752	6·44	1·29	1·305	5·15	5·135	4·575	4·56	4·55	4·54 <sup>1</sup>	+·08
7. 2	·766	6·52	1·27	1·29	5·25	5·23	4·66	4·645	4·44	4·42	-·02
7. 1	·768	6·49	1·27	1·29	5·22	5·20	4·64	4·62	4·42	4·40	-·04
23. 2	·788	6·21	1·27	1·27	4·94	4·94	4·39	4·39	4·36	4·36	-·045
23. 1	·796	6·11	1·27	1·265	4·84	4·845	4·30	4·305	4·27	4·275	-·125
10. 1	·797	6·50	1·27	1·265	5·23	5·235	4·645	4·65	4·47	4·47	+·075
10. 2	·804	6·44	1·27	1·26	5·17	5·18	4·59	4·60	4·42	4·43	+·04
18. 1	·808	6·275	1·27	1·255	5·005	5·02	4·445	4·46	4·37	4·38 <sup>2</sup>	±·00
18. 2	·808	6·28	1·27	1·255	5·01	5·025	4·45	4·465	4·37	4·39 <sup>2</sup>	+·01
17. 2	·811	6·30	1·27	1·255	5·03	5·045	4·47	4·48	4·39	4·40	+·02
17. 1	·816	6·27	1·27	1·25	5·00	5·02	4·44	4·46	4·36	4·38	+·01
6. 1	·819	6·36	1·27	1·25	5·09	5·11	4·52	4·54	4·29	4·31	-·055
25. 2	·822	6·17	1·27	1·25	4·90	4·92	4·355	4·37	4·34	4·36	±·00
25. 1	·832	6·14	1·27	1·25	4·87	4·89	4·325	4·345	4·31	4·33	-·01
26. 1	·838	6·13	1·27	1·25	4·86	4·88	4·32	4·335	4·31	4·33	-·005
4. 2	·839	6·555	1·27	1·25	5·285	5·305	4·695	4·71	4·42	4·43	+·09
6. 2	·839	6·26	1·27	1·25	4·99	5·01	4·435	4·45	4·21	4·22	-·12
4. 1	·840	6·62	1·27	1·25	5·35	5·37	4·75	4·77	4·47	4·49	+·155
3. 2	·843	6·475	1·27	1·25	5·205	5·225	4·625	4·64	4·33	4·345	+·015
5. 2	·846	6·315	1·27	1·25	5·045	5·065	4·48	4·50	4·23	4·25	-·075
26. 2	·846	6·17	1·27	1·25	4·90	4·92	4·355	4·37	4·35	4·36	+·035
16. 2	·851	6·24	1·27	1·25	4·97	4·99	4·415	4·435	4·32	4·34	+·02
3. 1	·855	6·42	1·27	1·25	5·15	5·17	4·575	4·59	4·29	4·30	-·01
5. 1	·858	6·36	1·27	1·25	5·09	5·11	4·52	4·54	4·27	4·29	-·02
16. 1	·862	6·18	1·27	1·25	4·91	4·93	4·36	4·38	4·27	4·29	-·01
19. 1	·880	6·22	1·27	1·25	4·95	4·97	4·40	4·415	4·33	4·35	+·07
19. 2	·884	6·18	1·27	1·25	4·91	4·93	4·36	4·38	4·29	4·31	+·04
27. 1	·890	6·08	1·27	1·255	4·81	4·825	4·27	4·285	4·265	4·28	+·015
15. 2	·895	6·13	1·27	1·255	4·86	4·875	4·315	4·33	4·21	4·23	-·03
11. 2	·903	6·32	1·27	1·255	5·05	5·065	4·485	4·50	4·32	4·34	+·095
11. 1	·904	6·315	1·27	1·255	5·045	5·06	4·48	4·495	4·32	4·33	+·09
15. 1	·904	6·07	1·27	1·255	4·80	4·815	4·265	4·28	4·16	4·18	-·06
27. 2	·907	6·03	1·27	1·26	4·76	4·77	4·23	4·24	4·225	4·235	±·00
20. 2	·935	6·12	1·27	1·265	4·85	4·855	4·31	4·315	4·255	4·26	+·06
22. 2	·935	5·96	1·27	1·265	4·69	4·695	4·17	4·17	4·13	4·13	-·07
14. 2	·938	6·275	1·27	1·27	5·005	5·005	4·45	4·45	4·33	4·33	+·135
21. 2	·939	6·02	1·27	1·27	4·75	4·75	4·22	4·22	4·17	4·17	-·02
12. 1	·944	6·225	1·27	1·27	4·955	4·955	4·40	4·40	4·26	4·26	+·075
12. 2	·945	6·16	1·27	1·27	4·89	4·89	4·345	4·345	4·205	4·205	+·025
21. 1	·950	6·055	1·27	1·275	4·785	4·78	4·25	4·245	4·20	4·20	+·025
22. 1	·951	5·98	1·27	1·275	4·71	4·705	4·185	4·18	4·15	4·14	-·035
13. 1	·961	6·16	1·27	1·28	4·89	4·88	4·345	4·335	4·22	4·21	+·05
20. 1	(·966	5·945)	1·27	1·28	(4·675	4·665	4·155	4·145	4·10	4·09) <sup>3</sup>	-·06
13. 2	·967	6·12	1·27	1·28	4·85	4·84	4·31	4·30	4·185	4·175	+·025
14. 1	·974	6·10	1·27	1·29	4·83	4·81	4·29	4·275	4·175	4·16	+·02

Sum +·355

<sup>1</sup> Slightly uncertain.<sup>2</sup> Slightly uncertain.<sup>3</sup> Determination rather doubtful.

high quotients first. The uncorrected curves are therefore of little value quantitatively.

Table XVII.

Date April 1918	R.Q. work	Work Cal. <i>b</i>	Rest <i>c<sub>I</sub></i>	Cal. <i>c<sub>II</sub></i>	Difference		Cal. per unit work				Deviation from curve ( <i>e<sub>II</sub></i> corr.) <i>δ</i>
					<i>d<sub>I</sub></i>	<i>d<sub>II</sub></i>	<i>e<sub>I</sub></i>	<i>e<sub>II</sub></i>	<i>e<sub>I</sub></i> corr.	<i>e<sub>II</sub></i> corr.	
8.	.732	6.67	1.315	1.33	5.355	5.34	4.76	4.745	4.55	4.53	-.01
9.	.748	6.51	1.29	1.31	5.22	5.20	4.635	4.62	4.44	4.43	-.08
24.	.760	6.455	1.28	1.295	5.175	5.16	4.60	4.585	4.58	4.56	+ .06
7.	.763	6.56	1.275	1.29	5.285	5.27	4.695	4.68	4.47	4.46	-.035
23.	.778	6.29	1.27	1.28	5.02	5.01	4.46	4.45	4.43	4.42	-.05
17.	.791	6.51	1.27	1.265	5.24	5.245	4.655	4.66	4.57	4.57	+ .115
18.	.800	6.39	1.27	1.26	(5.12	5.13)	4.55	4.56	4.47	4.48 <sup>1</sup>	+ .04
10.	.804	6.57	1.27	1.26	5.30	5.31	4.71	4.72	4.53	4.54	+ .105
5.	.826	6.47	1.27	1.25	5.20	5.22	4.62	4.635	4.365	4.38	-.02
25.	.829	6.20	1.27	1.25	4.93	4.95	4.38	4.40	4.36	4.385	-.01
6.	.830	6.375	1.27	1.25	5.105	5.125	4.535	4.55	4.30	4.32	-.075
4.	.831	6.505	1.27	1.25	5.235	5.255	4.65	4.67	4.37	4.39	± .00
3.	.845	6.47	1.27	1.25	5.20	5.22	4.62	4.635	4.33	4.34	-.035
26.	.848	6.135	1.27	1.25	4.865	4.885	4.32	4.34	4.31	4.33	-.04
16.	.849	6.24	1.27	1.25	4.97	4.99	4.415	4.435	4.32	4.34	-.03
19.	.870	6.24	1.27	1.25	4.97	4.99	4.415	4.435	4.35	4.37	+ .03
15.	.882	6.185	1.27	1.25	4.915	4.935	4.365	4.385	4.26	4.28	-.04
27.	.892	6.11	1.27	1.255	4.84	4.855	4.30	4.315	4.30	4.31	± .00
11.	.902	6.36	1.27	1.255	5.09	5.105	4.52	4.535	4.36	4.37	+ .075
12.	.917	6.28	1.27	1.26	5.11	5.12	4.54	4.55	4.39	4.40	+ .13
20.	.937	6.18	1.27	1.27	4.91	4.91	4.36	4.36	4.30	4.30	+ .06
14.	.940	6.185	1.27	1.27	4.915	4.915	4.365	4.365	4.25	4.25	+ .01
13.	.944	6.26	1.27	1.27	4.99	4.99	4.435	4.435	4.30	4.30	+ .07
22.	.949	6.06	1.27	1.275	4.79	4.785	4.255	4.25	4.22	4.21	-.01
21.	.970	6.00	1.27	1.285	4.73	4.715	4.20	4.19	4.15	4.14	-.05

Sum + .21

<sup>1</sup> Slightly uncertain.

The dispersion about the curve of the corrected  $e_{II}$  values of the first and second periods is  $\sigma = 0.06$  Cal. or 1.4 % of the average metabolism  $e_{0.85} = 4.32$  Cal. For the third periods the corresponding dispersion is likewise  $\sigma = 0.06$  Cal. or 1.35 % of  $e_{0.85} = 4.37$  Cal. As the unavoidable technical errors of the determinations are on an average about 1 %, it is obvious that the variations due to the subject are very slight indeed.

The straight line representing periods 1 and 2 corresponds to an  $e_F = 4.52$  Cal. and  $e_C = 4.10$  Cal. The waste of energy from fat is therefore 0.42 Cal. or 9.3 %. The straight line, Fig. 20, representing the third experimental periods corresponds to  $e_F = 4.57$  Cal. and  $e_C = 4.15$  Cal. or a waste of energy from fat of 0.42 Cal. = 9.2 %.

## THE GENERAL RESULTS OF THE FOUR SERIES OF EXPERIMENTS.

1. *The fatigue at different quotients.*

The subjects J. L., G. L., A. K. and O. H. observed distinct differences in the facility (or difficulty) with which the prescribed amount of work was performed, coincident with changes in diet and noted that on fat diets the fatigue became considerable and sometimes excessive. For several hours after the work on the ergometer these subjects were generally very tired when on a fat diet and much less tired or not tired at all when on carbohydrates. In the notes of O. H. indications are found of a perceptible difference also between work on a mixed diet and on a carbohydrate diet.

The subjects R. E. and A. M. N. failed to observe any appreciable difference between work on different diets. They never became very tired, either during or after the work. As the subjective estimate of the load upon an ergometer is liable to very considerable errors, especially when work done on different days has to be compared, the failure of the two subjects to notice the difference need not mean that such a difference was absent. On the other hand we think it rather probable that work at low quotients has not the same effect upon different individuals. The variations in fatigue may for instance be correlated with individual differences in the liability to acidosis. The clearing up of this point will require a special investigation in which the hydrogen ion concentration of the blood will have to be determined during and after the work.

2. *The waste of energy from fat.*

It is obvious that the objective difference in the economy of work at different quotients cannot be due to any incomplete oxidations at low quotients, since the difference is apparent at all quotients and is on the whole a straight line function of the quotient.

As mentioned above, p. 310, we took as our starting point the hypothesis that fats have to undergo a certain definite transformation involving loss of heat before they can be utilised for the production of muscular energy, and we deduced as necessary consequences of the hypothesis that the curves representing the relation between the expenditure of energy per unit work and the respiratory quotient should be straight lines and should show the same percentage waste of energy from fats in different individuals.

The first of these deductions has been verified by our experiments belonging to the second and fourth series. The best curve for the third series is very nearly a straight line, while in the preliminary series the experiments are too few and too discordant to decide anything quantitatively.

The three straight lines found for O. H. in the second and fourth series of determinations show practically the same waste of energy from fat, viz. 9.8, 9.3 and 9.2 % respectively, but the two straight lines representing the third series on A. M. N. give somewhat higher values, viz. 14.4 and 13.3 %.

We are unable to decide whether the difference between the result of the third series and the other two is real or due to the unavoidable errors with which our determinations of the waste of energy are infested. As the "best" curves for A. M. N. are not absolutely straight there is also the possibility that the metabolism at the lowest quotients has been slightly increased by some special influence. All the determinations (periods 1 and 2) made at quotients above 0.78 can be very accurately represented by a straight line giving  $e_F = 4.6$  Cal. and  $e_C = 4.1$  Cal. or  $w = 0.5$  Cal. = 10.9 %.

Table XVIII.

Subject and series	Calories per unit work		Difference		Number of exp.	Dispersion Cal.	Weight	Average efficiency		
	from fat	from carbohydrate	Cal.	%				100w $\frac{100w}{e_F}$	100w $\frac{100w}{e_F \sigma}$	200 $\frac{200}{e_F + e_C}$
	$e_F$	$e_C$	w	%						
J. L.	5.69	4.59	1.10	19.4	10					
G. L.	5.84	5.09	0.75	12.8	15	0.20	75	960	18.3	
A. K.	5.04	4.28	0.76	15.1	15	0.21	71	1072	21.6	
R. E.	4.72	3.72	1.00	21.2	13	0.155	84	1780	23.7	
M. N. Tb. XII	4.70	4.02	0.68	14.5	33	0.074	447	6485	23.0	
M. N. Tb. XIII	4.73	4.10	0.63	13.3	18	0.080	225	2993	22.7	
O. H. Tb. IX	4.79	4.32	0.47	9.8	33	0.072	459	4500	22.0	
O. H. Tb. XVI	4.52	4.10	0.42	9.3	49	0.060	818	7600	23.2	
O. H. Tb. XVII	4.57	4.15	0.42	9.2	24	0.060	400	3680	23.0	
							Sum 2579	29070		

In Table XVIII we have put together the results obtained in all the different series. There is no definite reason to exclude any of these except the first on J. L. (on account of his illness during the fat diet period) but it is evident that as determinations of the waste of energy from fat their value is very different, and a simple average of the figures in column  $\frac{100w}{e_F}$  would be very misleading. We have therefore endeavoured to assign a definite "weight" to each series which should represent its relative reliability, but we are aware that our method of doing so is somewhat arbitrary. We have given the series weight in proportion to the number of determinations in each and further in inverse ratio of the dispersion of the determinations. The approximate weights (nearest whole numbers) are given in column  $\frac{n}{\sigma}$ , and in column  $\frac{100w}{e_F \sigma}$  we have the products of the weights with the percentage waste of energy found in the series. When the sum of these figures is divided by the sum of the weights we have  $\frac{29070}{2579} = 11.25$  as the average percentage waste of energy from fat for the whole of our experimental material.

When the preliminary experiments are excluded and the waste of energy observed in the third series is reduced to 11 % by taking into account only those determinations showing quotients above 0.78, the remaining series can be given weights simply in proportion to their number of determinations since their standard deviations will be practically equal and the average works out as  $\frac{1456}{148} = 9.9$  %.

Table XIX.

Subject and series	Calories per unit work		Difference		Number of exp. <i>n</i>	$n \times \frac{100w}{e_F}$
	from fat	from carbo- hydrate	Cal.	%		
	$e_F$	$e_C$	<i>w</i>	$\frac{100w}{e_F}$		
M. N. Tb. XII	4.58	4.08	0.50	10.9	27	294
M. N. Tb. XIII	4.68	4.18	0.50	10.7	15	161
O. H. Tb. IX	4.79	4.32	0.47	9.8	33	324
O. H. Tb. XVI	4.52	4.10	0.42	9.3	49	456
O. H. Tb. XVII	4.57	4.15	0.42	9.2	24	221
					148	1456

3. *Possible systematic errors in the determination of the waste of energy from fat. The work of respiration.*

The total amount of work incidental to the production of 1 Cal. external work includes several items which are expended in the organism itself, as the increased circulation and respiration and the sweat secretion. It has been assumed in the calculation that this work is constant and independent of the respiratory quotient. This assumption cannot be proved in any case, and in the case of the respiration it can be shown to be incorrect. It is necessary therefore to examine this point somewhat closely and to see what influence the systematic error introduced may possibly have upon the result.

If at a quotient of 1.00 the amount of work performed by a subject corresponds to an oxygen absorption of 1070 cc. per minute, which represents fairly the conditions in most of our experiments, the oxygen absorption at a quotient of 0.71 will, according to our result, be 1210 cc. The corresponding CO<sub>2</sub> productions will be 1070 and 860 cc. respectively. The elimination of the surplus of CO<sub>2</sub> at the high quotient 1070 - 860 = 210 cc. will require an increase in ventilation, and on the assumption that the alveolar CO<sub>2</sub> percentage remained the same in both cases the increase could be calculated. Hasselbalch [1912] has shown however that on a carbohydrate diet the CO<sub>2</sub> percentage in the alveoli is somewhat increased, and the increase might possibly be sufficient to make the ventilation constant throughout the whole range of quotients. In any case it makes it impossible to calculate *a priori* the increase in ventilation.

From the work experiments of Frenzel and Reach [1901] (referred to in detail below) in which the ventilation of the lungs was measured at different quotients, Zuntz [1911] has calculated the increase in ventilation incidental to a definite increase in quotient. At an average quotient of 0.778 he finds the ventilation per minute and per Calorie of metabolism to be 4.875 l. while at a quotient of 0.894 it is 5.135 l. or a difference of 0.26 l. At the quotients 0.71 and 1.00 the difference per Calorie would amount to 0.62 l. and in our

experiments with a total metabolism between 6 and 5 Cal. the difference would be 3.5 l.<sup>1</sup>

Zuntz calculates that each litre ventilation corresponds to an oxygen consumption in the respiratory muscles of 5 cc. or 0.025 Cal., but the very careful determinations of the respiration work made by Liljestrand [1917] show that, when the respiration is free to adjust itself, it is much more economically performed, and the probable consumption of energy per litre ventilation is of the order 0.005 to 0.01 Cal. when the ventilation is, as in our experiments, about 25 to 30 l. per minute. The increased cost of eliminating the CO<sub>2</sub> when the quotient rises from 0.71 to 1.00 amounts therefore in our experiments to between 0.02 and 0.03 Cal., or certainly not more than 0.6 % of the total metabolism per Calorie external work. When corrected for the difference in work of breathing the waste of energy from fat is therefore increased by 0.4 to 0.6 %.

The cost of ventilating the lungs is not the same in different individuals, and it is obvious that other activities of the body incidental to the work, *e.g.* the secretion of sweat, need not be the same on carbohydrates as on fat and may vary from one individual to another. The final figure obtained by us for the waste of energy from fat may therefore be influenced by several systematic errors. Seeing that the difference in ventilation, which is very pronounced, causes only an error of about 0.5 % it is very unlikely, however, that the influence of other varying activities can be anything but very slight.

As mentioned on p. 298 we have used the table of caloric values for oxygen at different quotients given by Zuntz in 1913. This table differs somewhat from the table originally computed by Zuntz and Schumburg and shows a higher caloric value for oxygen at low quotients. The difference amounts to 1.7 %, and the waste of energy from fat computed from our experiments will therefore have to be reduced by 1.7 % (from 11.25 to 9.55 %) if the later table is replaced by the earlier. The difference is mainly due to differences in the assumed composition of fat, and it is obvious that so long as the constitution of the fat actually catabolised is unknown the possibility both of a small systematic error and of slight individual differences cannot be avoided.

#### THE RESULTS OF EARLIER INVESTIGATIONS.

We have compared the result obtained by us with the figures found in earlier investigations to find out how far the actual determinations made—apart from the conclusions drawn—agree or disagree with ours.

A few (5) experiments made on a dog were published in 1894 by Zuntz and Loeb [1894]. They show about the same average metabolism per unit work

<sup>1</sup> The increase in CO<sub>2</sub> production was found above to be 210 cc. or 6 % of the increase in ventilation. The increase in alveolar CO<sub>2</sub> percentage with increasing quotient must therefore have been very slight during the work.

at the different quotients, but the individual results at about the same quotient differ by about 12 % and no valid conclusions can therefore be drawn.

An extensive series of experiments was performed under the supervision of Zuntz by Newton Heineman [1901] on a labourer who worked each day during a period of several months on an "ergostat" which could be turned by hand. The exchange measured varies enormously from day to day—sometimes by 100 %. There is a distinct decrease throughout the period in the metabolism per unit work, from 4.7 Calories per Cal. technical work in February to 4.38 in May according to a calculation by Zuntz [1901]. The main result was that the average metabolism per unit work was lower on a fat diet (4.0 Cal.) than on a diet consisting chiefly of carbohydrates (4.4 Cal.), but Zuntz points out that the difference is probably due to the increasing training as the fat experiments were carried out later than the carbohydrate experiments. In any case the individual determinations are too discordant to allow any definite conclusion.

At about the same time Frentzel and Reach [1901] published another research carried out in the laboratory of Zuntz. These authors acted as subjects themselves, and the work was performed by walking upwards on an inclined treadmill. Each diet was maintained for a week at a time, during which 3–4 periods of work of about 10 minutes duration each were measured each day. In these series the dispersion of individual experiments was much smaller than in Heineman's series, amounting in the most uniform week to  $\sigma = 3.4$  % and in the least uniform to  $\sigma = 12.5$  %. The average for each week should therefore be fairly reliable, but unfortunately the two weeks for each subject on the same diet differ about as much from one another as from those on a different diet.

They obtained the following results:

Table XX.

				Average R.Q.	Cal. per kg. per m.	Average
Frentzel.	Fat diet	first week	31. v.–4. vi.	0.766	2.088	2.07
		second week	28. vi.–3. vii.	0.778	2.049	
	Carbohydrate diet	first week	9. vi.–12. vi.	0.896	1.932	1.98
		second week	5. vii.–10. vii.	0.880	2.031	
Reach.	Fat diet	first week	16. vi.–19. vi.	0.805	2.259	2.15
		second week	12. vii.–17. vii.	0.766	2.034	
	Carbohydrate diet	first week	22. vi.–26. vi.	0.899	2.202	2.10
		second week	19. vii.–24. vii.	0.901	2.005	

In both series the metabolism is lower on "carbohydrates" than on fat and when the corresponding differences are calculated for quotients of 0.71 and 1.00 respectively the percentage waste of energy from fat works out as 11 % in the case of Frentzel and 6 % for Reach. It must be conceded however that these results may be due to training, as the carbohydrate experiments

were done after the fat experiments, and in any case the results obtained on the same diet are too discordant to allow any very definite conclusions<sup>1</sup>.

Benedict and Cathcart [1913] have made some experiments on the influence of diet in their extensive study of the metabolism during muscular work. They made experiments on a professional bicyclist, who rode on the Atwater-Benedict bicycle ergometer, and determined the respiratory exchange by means of the Benedict "Universal Respiration Apparatus." In the experiments here considered two experimental periods were taken during each ride, and these have been averaged by the authors. Generally the rate of pedalling varied considerably from period to period, and in some cases the respiratory quotients and metabolism per unit work were also very different.

Benedict and Cathcart consider that "the evidence obtained is by no means conclusive" but they have put together such results as are suitable for comparison. In nine experiments the external muscular work was not far from 2 Cal., and in six of these the preceding diet was rich in carbohydrate while in three it was poor in carbohydrate. Comparing the average for the six first-named days with that for the three last they find the same metabolism per unit work (standard metabolism not deducted). When, however, the experiments are arranged according to the quotients actually determined during the work we obtain the following results:

Table XXI.

Date	E.Q.	Cal. per unit work. Gross
Feb. 15	0.95	5.17
Feb. 14	0.93	5.20
Jan. 25	0.92	5.38
Feb. 7	0.91	5.30
Feb. 16	0.88	5.12
Jan. 24	0.87	5.52
Feb. 8	0.86	5.40
Jan. 26	0.83	5.81
Feb. 9	0.82	5.23

It is seen from the plotting given in Fig. 21 that the results are too irregular to admit of any conclusions, but that they are perfectly compatible with our result: that there is a definite waste of energy derived from fat.

In another series of six experiments with a smaller amount of external work there is no apparent difference between the value of carbohydrate and fat, but the discrepancies between the single determinations are even greater than in the series reproduced.

We must mention finally the beautiful experiments made by Anderson and Lusk [1917] on a dog walking horizontally on a treadmill at rates between

<sup>1</sup> It should be remembered that the problem studied by Zuntz and his collaborators was that raised by Chauveau [1898] whether fat must be converted into carbohydrate before being utilised for muscular work. Zuntz calculated that such a conversion must involve an energy loss of at least 24 % and probably 30 % and it must be admitted that the results found by Frenzel and Reach are not compatible with the assumption of such a loss.

3900 and 4800 m. per hour. When the dog had fasted for 18 hours they found an extra energy production over the standard metabolism corresponding to 0.580 kg. meter per kg. of the dog's weight per meter. The respiratory quotient was in one case 0.74, in another 0.78 (average 0.76). After ingestion of 70–100 g. glucose the resting metabolism was increased some 20 % over the standard metabolism during the absorption, but the extra energy required for walking was nevertheless diminished to 0.550 kg. meter per kg. per meter with a quotient of 0.95 (average of three determinations). The difference is a little over 5 % and when the metabolism per unit work is assumed to be a straight line function of the quotient the waste of energy from fats works out as 8 %, without regard to the increase in resting metabolism due to the absorption of the glucose. As the part played by protein in the metabolism is unknown and may have varied it is not possible to make an exact comparison between the result of Lusk and Anderson and ours, but the essential agreement between them is unmistakable.

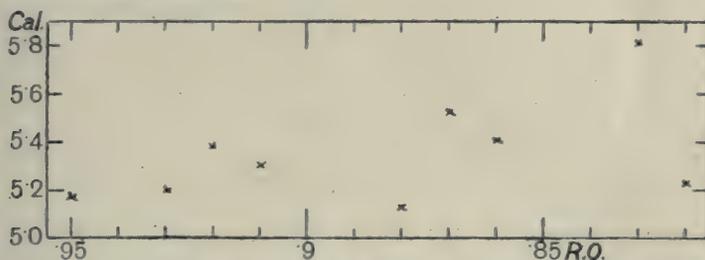


Fig. 21. Experiments from Benedict and Cathcart. Metabolism per unit work, gross.

The foregoing analysis shows that our main numerical result is not at variance with the numerical results of earlier investigations of the same problem and is actually supported by the experiments of Lusk and Anderson and Frenzel and Reach as well as by one series of Benedict and Cathcart's experiments, all of which indicate with more or less probability that the value of fat as a source of muscular energy is lower than that of carbohydrate and that the difference is in the neighbourhood of 10 %.

#### THE THEORETICAL SIGNIFICANCE OF THE WASTE OF ENERGY FROM FAT.

On the theoretical significance of the difference in value between fat and carbohydrate as a source of energy for the muscular machine, we have very little to say. According to the calculation of Chauveau (quoted by Zuntz) [1911], who assumed that fat was converted into sugar by retaining its hydrogen, splitting of some of the carbon and taking up oxygen to complete the molecule, 30 % of the energy of the fat should become lost by the conversion. It follows therefore from our result—which agrees in so far with the conclusion of Zuntz—that a conversion of this kind does not take place.

Zuntz [1911] has made another calculation, assuming that all the carbon of the fat is retained in the sugar formed. This would correspond to 191.25 g.

sugar formed from 100 g. fat. The fat could yield 950 Cal. and the sugar only 718 Cal.; that is there would be a loss of 24.4 %. This assumption too is incompatible with our determinations.

We are not convinced of the validity of any of these summary methods of calculating the waste of energy incidental to the conversion of fat into sugar or any other substance<sup>1</sup>. We believe that it will be necessary to find out about the stages through which the conversion is actually carried out and the role which may be played by interaction between intermediate products of the fats and other substances which may possibly be derived from carbohydrates.

On the other hand it is clear that our experiments cannot be used as evidence to prove that fat must be converted into sugar before being utilised for muscular work. The chemical processes actually taking place in muscles, doing work upon a fat diet, must be investigated by studies on the lines so brilliantly initiated by Fletcher, Hopkins, Hill and their collaborators, but it is our hope that the result at which we have arrived—that these processes involve a loss of energy of definite magnitude—may serve as a guide in such studies.

#### APPENDIX I

##### STATISTICAL TREATMENT OF THE DETERMINATIONS OF STANDARD METABOLISM.

We have regularly each day made two consecutive determinations of about five minutes duration of the standard metabolism of our subjects. A statistical treatment of this material can give some information about the accuracy of the method employed and about the influence of various factors upon the results.

In Table XXII we have arranged the repeated determinations of ventilation, respiratory quotient and metabolism in horizontal lines. For each person we have taken the experimental days in the order of the quotients, beginning with the lowest. The material has been arranged primarily to study the possible influence of variations in the pulmonary ventilation upon the quotient and the metabolism. From our earlier experience, especially with the recording respiration apparatus [Krogh, 1913], we know that in periods of a few minutes duration the ventilation during rest is not quite constant but shows oscillations, and this is borne out also by the present series of determinations.

*A priori* the oscillations in ventilation must be assumed to influence the quotient observed, which should increase with increasing ventilation and *vice versa* by relative washing out and storing up of carbon dioxide, while the oxygen absorption and the metabolism calculated from it should be practically unaffected.

<sup>1</sup> As a reason for suspicion against summary methods of calculating the loss of energy in question we would suggest the making up of 1 molecule of fat (tripalmitin) into 12 molecules of sugar by the addition of 21 molecules of CO<sub>2</sub> and 23 of H<sub>2</sub>O which would result in a *gain* of energy amounting to about 18 %.

Table XXII.

Subject and date	Ventilation l. per m. × 100				R.Q. × 100				Calories per m. × 100			
	I		II		I		II		I		II	
1	2	3	4	5	6	7	8	9	10	11	12	13
<b>J. L.</b>												
1. ii.	403		+ 8	411	77		+ 4	81	106		0	106
2. ii.	399	- 7		392	81	- 1		80	100	0		100
<b>G. L.</b>												
25. i.	517		+12	529	76		0	76	110		+ 1	111
26. i.	480		+ 9	489	76		+ 4	80	104		- 2	102
26. ii.	532	-59		473	80	- 1		79	104	- 8		96
<b>A. K.</b>												
20. ii.	688		+ 3	691	70		+ 3	73	115		+ 3	118
19. ii.	696	-82		614	72	+ 3		75	122	-18		104
1. iii.	618		+ 2	620	83		+ 5	88	110		0	110
12. ii.	650		+10	660	88		- 1	87	104		+ 1	105
13. ii.	651		+17	668	88		+ 6	94	106		- 3	103
<b>R. E.</b>												
25. iii.	609	-48		561	73	- 2		71	140	- 8		132
26. iii.	548			548	74		+ 2	76	124		+ 4	128
20. iii.	559	-15		544	85	- 2		83	115	- 1		114
6. iv.	640	-35		605	93	- 3		90	134	- 4		130
7. iv.	571		+20	591	93		+ 2	95	125		+ 2	127
Sums	-246		+81			- 6		+25		-39		+ 6
<b>A. M. N.</b>												
10. ii.	528			528	75		+ 3	78	112		- 4	108
9. ii.	517	-36		481	78	0		78	112	- 4		108
8. ii.	529		+ 4	533	79		- 2	77	112		+ 4	116
16. ii.	516	-22		494	79	+ 2		81	108	- 4		104
17. ii.	510		+28	538	81		- 2	79	104		0	104
30. i.	615		+23	638	82		+ 2	84	113		- 9	104
7. ii.	500		+ 7	507	83		0	83	113		- 3	110
15. ii.	530	-49		481	83	+ 1		84	106	- 6		100
29. i.	679		+51	730	84		+ 2	86	116		- 5	111
1. ii.	641	-21		620	85	+ 1		86	119	+ 1		120
19. ii.	498		+12	510	86		- 1	85	102		+ 1	103
11. ii.	505		+ 5	510	85		+ 2	87	104		- 2	102
31. i.	620	-23		597	87	0		87	109	- 4		105
6. ii.	580		+ 3	583	88		0	88	114		0	114
2. ii.	600	-40		560	90	- 2		88	122	- 6		116
3. ii.	617	- 6		611	90	- 1		89	118	+ 2		120
12. ii.	546	-33		513	94	- 4		90	108	- 2		106
18. ii.	556	-21		535	97	- 5		92	104	- 2		102
5. ii.	561		+40	601	93		+ 8	101	118		- 3	115
13. ii.	616		+ 3	619	97		+ 2	99	108		+ 2	110
4. ii.	620		+35	655	98		+ 1	99	118		+ 4	122
14. ii.	623	-23		600	106	- 3		103	110	- 2		108
Sums	-274		+211			-11		+15		-27		-15

Table XXII *continued.*

Subject and date	Ventilation l. per m. × 100				R.Q. × 100				Calories per m. × 100				
	I		II		I		II		I		II		
	1	2	3	4	5	6	7	8	9	10	11	12	13
O. H. I													
25. v.	561			+17	578	69		+ 3	72	140		+ 6	146
21.	577	- 7			570	71	- 1		70	144	+ 3		147
24.	622			+13	635	71		+ 2	73	155		- 2	153
22.	594			+31	625	71		+ 4	75	140		0	140
10.	605			+39	644	82		- 1	81	142		0	142
11.	597			+38	635	79		+ 5	84	148		- 2	146
8.	593				593	82		0	82	128		- 2	126
18.	595			+11	606	93		- 4	89	138		+ 4	142
14.	634	-13			621	92	+ 4		96	138	- 8		130
15.	624			+ 4	628	94		0	94	146		- 6	140
17.	616			+27	643	92		+ 7	99	140		- 2	138
	Sums	-20		+180			+ 3	+16			- 5	- 4	
O. H. II													
9. iv.	538	-18			520	64	+ 2		66	147	-10		137
8.	513			+54	567	70		+ 2	72	135		+ 3	138
24	646	-55			591	73	- 1		72	132	- 5		127
18.	552			+29	581	77		+ 2	79	126		+ 1	127
3.	571			+79	650	79		+ 7	86	128		0	128
6.	552	- 2			550	78	+ 2		80	128	- 3		125
4.	595	-63			532	82	- 2		80	134	-16		118
16.	578			+ 2	580	83		- 3	80	126		+ 2	128
5.	563	-24			539	84	- 2		82	132	- 2		130
10.	575	-11			564	83	+ 1		84	124	0		124
15.	636	-17			619	84	+ 2		86	128	+ 2		130
26.	603	-44			559	88	- 6		82	126	- 6		120
23.	532			+32	564	84		+ 3	87	104		+ 6	110
27.	584			+21	605	86		+ 1	87	118		+ 8	126
25.	659	-24			635	86	+ 2		88	127	- 7		120
19.	581	-14			567	90	- 2		88	123	- 1		122
20.	654	-11			643	94	+ 1		95	133	- 2		131
13.	649			+14	663	98		- 4	94	125		+ 8	133
21.	643			+ 9	652	98		0	98	125		+ 7	132
12.	647	-14			633	100	- 3		97	129	+ 2		131
11.	623			+15	638	98		+ 2	100	125		+ 3	128
14.	655			+50	705	101		- 1	100	125		+13	138
	Sums	-297		+305			- 6	+ 9			-48	+51	

In columns 3 and 4 we have arranged the differences between the ventilation in the first and second experimental period (II-I) putting the negative differences in column 3 and the positive in column 4. Though there are individual deviations (notably the first series of experiments on O. H.) we find on the whole that increases in ventilation occur to the same extent as decreases. In 30 cases out of 69 the second period shows a decrease in ventilation amounting on an average to 0.28 l. per minute while in 36 cases

there is an increase amounting on an average to 0.20 l. per minute. As a grand average we have a decrease in ventilation amounting only to 0.016 l. per minute. This means that the introductory period has been of sufficient duration to insure a practically complete quieting down of the ventilation. In the beginning of a period of rest a steady decrease of the ventilation is the general rule.

In the case of O. H. I the almost constant increase in ventilation from the first to the second period is mainly due to a slight inconvenience, caused by the mouthpiece, which became accentuated towards the end of the period and was repeatedly mentioned by the subject.

In columns 6 and 9 we have recorded the quotients found in the first and second period and in columns 7 and 8 the differences (II-I) between them. In column 7 we have put the differences corresponding to a higher ventilation in period I and in column 8 the differences corresponding to a lower ventilation in period I. As most of the differences in column 7 are negative (16 out of 30) and in column 8 positive (25 out of 37), while their sums are respectively negative (-0.20) and positive (+0.65), it follows that an increase in ventilation is correlated, as was to be expected, with an increase in quotient and *vice versa*, but, as the increases show a decided preponderance over the decreases, the final result is an increase in quotient in the second period amounting to  $0.0064 \pm 0.0036$ . This increase is nearly double its mean error and is therefore in all probability real. Since the second quotient is the higher we believe that our determinations have generally been made during that period when the probable initial washing out of carbon dioxide is being made good and take it to mean that the resting quotients as determined by us are on an average probably slightly too low.

In columns 10 to 13 we have treated similarly the determinations of metabolism. Here too we find, contrary to our expectations, a distinct correlation with the ventilation. With a decreasing ventilation there is in the greater number of cases (23 out of 30) a fall in metabolism and an increasing ventilation corresponds in 20 cases out of 37 to an increased metabolism. The average decrease in the 30 cases with decreasing ventilation is 0.040 Cal. and the average increase in the 37 cases is 0.010 Cal. On the whole the metabolism in the second period is lower than in the first by  $0.0115 \pm 0.0062$  Cal., a difference which must be assumed to be real.

The correlation between the ventilation and the metabolism might be considered as physiological, since an increase in metabolism must involve an increase in ventilation and *vice versa*. We do not believe, however, that spontaneous oscillations in the metabolism occur during rest within intervals of a few minutes, and think it safer to ascribe the correlation to the technical error in the taking of samples which we have mentioned on p. 303, and which will have just such an effect when the ventilation is not the same in the two consecutive periods.

That the metabolism is on an average lower in the second period than

in the first must probably mean that the preliminary period of rest has not always been long enough for the subject to come down to the true standard metabolism. The difference is very pronounced in the case of A. M. N., where it is on an average  $0.019 \pm 0.008$  Cal., but absent in the case of O. H.:  $0.002 \pm 0.010$  Cal. In future determinations of this kind the preliminary period of rest should be prolonged from  $\frac{1}{2}$  to  $\frac{3}{4}$  or a whole hour.

The distribution of the variations considered is wholly independent of the respiratory quotient. It is of importance from the point of view of the relation between the quotient and the standard metabolism, treated in Appendix II, that the investigation of the errors affords no ground for assuming that the determinations made at very low or very high quotients should be less reliable or infested with different systematic errors from those at intermediate quotients<sup>1</sup>.

When the two determinations made each day are treated as simple repetitions the mean error of their averages which can be called their technical error is determined by  $\tau = \frac{1}{2} \sqrt{\frac{\sum d^2}{n-1}}$ , where  $d$  is the difference between each pair of determinations.

We find

Table XXIII.

	B. Q. $\tau$	Cal. per min. $\tau$
I. L.-R. E.	0.016	0.031
A. M. N.	0.014	0.020
O. H. I	0.019	0.021
O. H. II	0.015	0.033
All experiments	0.015	0.027

The technical error of a double determination of the quotient is therefore  $\tau = 0.015$  and of the metabolism  $\tau = 0.027$  Cal. or about 2 % of the standard metabolism of our subjects.

## APPENDIX II

### THE CORRELATION BETWEEN THE RESPIRATORY QUOTIENT AND THE STANDARD METABOLISM.

In discussing the different series of experiments the standard metabolism has been treated for each subject separately and it has been shown that at very low quotients there is a slight increase in metabolism, while in some experiments a tendency towards an increase at high quotients is also apparent.

In order to obtain more definite information upon this point we have compared all our available data concerning the standard metabolism at

<sup>1</sup> The ventilation shows as might be expected a distinct increase with increasing quotient corresponding to the increasing elimination of carbon dioxide. As we have not counted the respirations and cannot therefore calculate the average depth of respiration or the alveolar ventilation a detailed study of the relation between the ventilation of the lungs and the elimination of carbon dioxide is scarcely worth attempting.

varying quotients. As the metabolism varies with the subject a direct comparison cannot be instituted, but we have calculated the average standard metabolism for each person and determined the percentage deviation from this average for each experimental day. In the case of A. M. N., whose standard metabolism showed a steady decline throughout the three weeks' experiments, we have taken the curve, Fig. 12, as our unit and determined the percentage deviation of each day's result from this curve.

All the figures thus obtained have been plotted in Fig. 22 with the respiratory quotient as abscissa and using different symbols for the different subjects as indicated. In drawing the curve representing the determinations the two lowest values at the quotients 0.82 and 0.845 have been disregarded as probably erroneous.

The curve shows unmistakably and without any apparent difference between the different subjects that the standard metabolism in subjects with a very low protein metabolism, when calculated on the basis of the caloric values for oxygen given by Zuntz, is not independent of the quotient. It has a minimum value between the quotients 0.8 and 0.94, increases by about 5% when the quotient falls from 0.8 to 0.71 and increases also by about 3% when the quotient rises from 0.94 to unity. Even between 0.8 and 0.94 it does not appear to be quite constant, but the variation found is only 1% and is within the limits of error. The dispersion of the single determinations about the curve is  $\sigma = 3.0\%$ . As the mean technical error of a double determination is according to Appendix I  $\tau = 2\%$  the individual day to day variations of each subject's standard metabolism ( $\phi$ ), which is found from the equation  $\tau^2 + \phi^2 = \sigma^2$ , works out as  $\phi = 2.2\%$ .

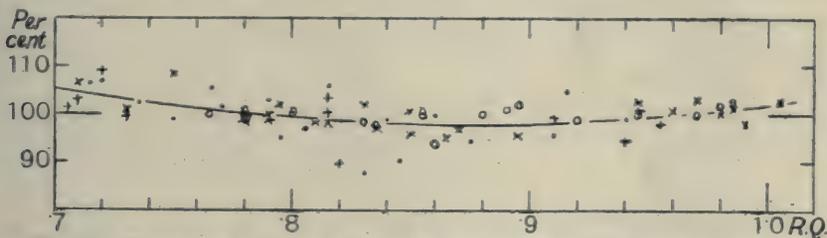


Fig. 22. Percentage variation of standard metabolism with respiratory quotient. Subjects: • J. L., G. L., A. K., R. E., o A. M. N., + O. H. 1917, x O. H. 1918.

A definite explanation of the influence of the materials catabolised upon the standard metabolism, cannot at present be given. It is conceivable, though in our opinion very improbable, that it may be unreal and due to imperfections in the indirect calorimetry employed by us. To settle this point it would be very desirable to have a sufficient number of direct calorimetric determinations of the standard metabolism of human subjects over the whole practicable range of quotients.

When the variation of the metabolism with the quotient must be accepted as real it might be thought to be due at low quotients to incomplete oxidations with excretion of keto-substances. We have repeatedly tested the urine of

Table XXIV.

Date and subject	R.Q.	Cal.	Deviation from average		Deviation from curve $\delta$
			Cal.	%	
<b>J. L.</b>					
1917					
1. ii.	·79	1·055	+0·03	+ 3	+3
2. ii.	·805	·995	-0·03	- 3	-2
Average 1·025					
<b>G. L.</b>					
25. i.	·765	1·105	+0·06	+ 5·5	+4·5
26. i.	·78	1·035	-0·01	- 1	-1
26. ii.	·795	·995	-0·05	- 5	-4·5
Average 1·045					
<b>A. K.</b>					
20. ii.	·715	1·17	+0·07	+ 6·5	+2·5
19. ii.	·735	1·13	+0·03	+ 2·5	0
5. ii.	·77	1·115	+0·015	+ 1·5	+1
1. iii.	·86	1·10	$\pm 0$	0	+2
12. ii.	·875	1·04	-0·06	- 5·5	-3
13. ii.	·91	1·05	-0·05	- 4·5	-2·5
Average 1·10					
<b>R. E.</b>					
25. iii.	·72	1·36	+0·09	+ 7	+3
26. iii.	·75	1·26	-0·01	- 1	-2·5
20. iii.	·845	1·145	-0·125	-10	-8
6. iv.	·915	1·325	+0·055	+ 4·5	+6·5
7. iv.	·94	1·26	-0·01	- 1	0
Average 1·27					
<b>A. M. N.</b>					
1918					
10. ii.	·765	1·105	$\pm 0^1$	0	-1
8. ii.	·78	1·14	+0·015	+ 1·5	+1·5
9. ii.	·78	1·105	-0·01	- 1	-1
16. ii.	·80	1·055	+0·005	+ 0·5	+1
17. ii.	·80	1·04	$\pm 0$	0	+0·5
30. i.	·83	(1·085)	(-0·125)	(-10·5) <sup>2</sup>	
7. ii.	·83	1·115	-0·02	- 2	0
15. ii.	·835	1·035	-0·02	- 2	0
29. i.	·85	(1·135)	(-0·085)	(- 7) <sup>2</sup>	
1. ii.	·855	1·195	+0·005	+ 0·5	+2·5
19. ii.	·855	1·02	$\pm 0$	0	+2
11. ii.	·86	1·03	-0·065	- 6	-4
31. i.	·87	(1·07)	(-0·13)	(-11) <sup>2</sup>	
6. ii.	·88	1·145	$\pm 0$	0	+2·5
2. ii.	·89	1·19	+0·01	+ 1	+3
3. ii.	·895	1·19	+0·02	+ 2	+4
12. ii.	·92	1·07	-0·015	- 1·5	+0·5
18. ii.	·945	1·03	$\pm 0$	0	0
5. ii.	·97	1·16	+0·005	+ 0·5	0
13. ii.	·98	1·095	+0·02	+ 2	+1
4. ii.	·985	1·20	+0·035	+ 3	+2
14. ii.	1·045	1·085	+0·02	+ 2	

<sup>1</sup> Deviations from curve, Fig. 12.<sup>2</sup> Very doubtful. See p. 325.

Table XXIV *continued.*

Date and subject	R.Q.	Cal.	Deviation from average		Deviation from curve
			Cal.	%	
O. H. I					
1917					
25. v.	-705	1.43	+0.02	+ 1.5	-3
21.	-71	1.45	+0.04	+ 3	-1
24.	-72	1.54	+0.13	+ 9	+5.5
22.	-73	1.40	-0.01	- 0.5	-3.5
10.	-815	1.42	+0.01	+ 0.5	+1.5
11.	-815	1.465	+0.055	+ 3.5	+4.5
8.	-82	1.27	-0.14	-10	-8.5
18.	-91	1.40	-0.01	- 0.5	+1.5
14.	-94	1.335	-0.075	- 5.5	-4.5
15.	-945	1.425	+0.015	+ 1	+2
17.	-955	1.385	-0.025	- 2	-1.5
Average 1.41					
O. H. II					
1918					
9. iv.	-65	(1.42)	(+0.14)	(+11) <sup>1</sup>	
8.	-71	1.365	+0.085	+ 6.5	+2.5
24.	-73	1.295	+0.015	+ 1	-2
7.	-75	1.39	+0.11	+ 8.5	+7
18.	-78	1.27	-0.01	- 1	-1
3.	-79	1.28	±0	0	0
6.	-79	1.265	-0.015	- 1	-1
17.	-795	1.31	+0.03	+ 2.5	+3
4.	-81	1.26	-0.02	- 1.5	-0.5
16.	-815	1.265	-0.015	- 1	0
5.	-83	1.31	+0.03	+ 2.5	+4
10.	-835	1.245	-0.035	- 2.5	-1
15.	-85	1.285	+0.005	+ 0.5	+2.5
26.	-85	1.23	-0.05	- 4	-2
23.	-855	(1.07)	(-0.21)	(-16.5) <sup>2</sup>	
27.	-865	1.22	-0.06	- 4.5	-2.5
25.	-87	1.24	-0.04	- 3	-0.5
19.	-895	1.225	-0.055	- 4.5	-2.5
20.	-945	1.32	+0.04	+ 3	+3.5
13.	-96	1.29	+0.01	+ 1	+1
22.	-97	1.32	+0.04	+ 3	+2.5
21.	-98	1.28	±0	0	-1
12.	-985	1.30	+0.02	+ 1.5	+0.5
11.	-99	1.26	-0.02	- 1.5	-2.5
14.	1.005	1.32	+0.04	+ 3	+0.5
Average 1.28					

<sup>1</sup> Somewhat doubtful.<sup>2</sup> Considered as erroneous. See p. 333.

our subjects for aceto-acetic acid and found no evidence of acidosis, and the rise in metabolism begins at quotients which are too high to admit of acidosis.

In our opinion the higher metabolism both at low and at high quotients is most probably due to transformation processes of carbohydrate into fat at high quotients and of fat (and protein) into carbohydrate at low quotients. Such transformations will cause at high quotients an increase in the total respiratory quotient over that of the true catabolic processes and at low quotients a decrease, provided of course that the transformation products are stored, at least provisionally.

It is significant therefore that we have occasionally observed quotients above unity, when the subjects were on a carbohydrate diet, and below 0.71, when the diet consisted chiefly of fat. We have no reason to doubt the reality of these quotients, which must mean that synthetic transformations take place, and it is a natural inference that the same transformations take place, though with diminished intensity, when the quotient is somewhat below 1.00 or above 0.71.

Additional evidence corroborating the view that a transformation of fat into carbohydrate takes place regularly, when the respiratory quotient is below 0.8, and the reverse transformation, when the quotient is above 0.9, will be given in Appendix III.

### APPENDIX III

#### THE CHANGE IN RESPIRATORY QUOTIENT TAKING PLACE ON THE TRANSITION FROM REST TO MUSCULAR WORK.

In Table XXV we have compared the average respiratory quotient found each day during rest with the quotient observed on the same day during the first experimental period of work. The experiments have been arranged as usual in the order of increasing quotients during rest.

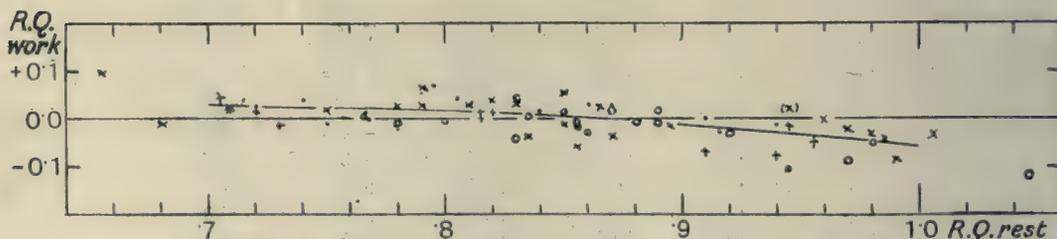


Fig. 23. Change in respiratory quotient on transition from rest to work.

Subjects: ● J. L., G. L., A. K., R. E., ○ A. M. N., + O. H. 1917, × O. H. 1918.

The results have been plotted in Fig. 23 with the quotient during rest as abscissa and the differences in quotient between rest and work as ordinates. When the quotient during work is increased over that during rest the difference has been denoted as positive. Though the individual results are somewhat

irregular it is unmistakable that at low quotients there is on the whole an increase in the quotient on the transition from rest to work, but at high (above 0.9) there is a very decided decrease, amounting, when the quotient during rest approaches unity, to about 0.05.

The dispersion of the individual results about the curve drawn is  $\sigma = \pm 0.028$  which is partly explained by the technical errors of the determinations involved. The technical error on the difference between resting and working quotient here considered is about  $\tau = \pm 0.016$ . Those variations from the curve, which are due to the subjects, are therefore  $\phi = \pm 0.023$ .

There are two known sources of systematic error in the comparison here given between the respiratory quotients during rest and work. The first is that we have compared the quotient during rest, not with the quotient at the beginning of the work, but with the quotient found during a period extending from 30 to 50 (or 60) minutes after the beginning of work. According to the results given in Appendix IV we must assume that the quotient at the beginning of the work was generally a little higher and by making a somewhat uncertain extrapolation we find it to be about 0.01 higher. A correction of this magnitude would shift the curve slightly to the right so that we should have the zero point at 0.91 instead of at 0.87.

Another systematic error is that the quotients determined during rest are (as shown in Appendix I) probably on an average slightly too low, which may possibly compensate the error on the quotients during work.

Whether the curve shall finally be shifted slightly to the right or slightly to the left the essential result remains unchanged: that on the transition from rest to constant work of moderate intensity a rise in quotient takes place only when the quotient was low beforehand, but a fall when it was high, while at intermediate quotients the average change is very slight.

Our result as here summarised agrees with the findings of Zuntz and his school, but is strikingly at variance with the results reported by Benedict and Cathcart [1913], who found that the quotients during work were generally higher than in a preceding period of rest and were increased more or less in proportion to the severity of the work performed. The quotients determined by Benedict and Cathcart during work have been adversely criticised by Lindhard [1915] on the ground that some of them are undoubtedly influenced by forced respiration and that there is good reason to think that they are generally so influenced on account of the inconvenience of breathing through a mouthpiece and into a respiration apparatus. We would point out further, that since it can be deduced from the experiments of Carpenter [1915], referred to above (p. 293), that the "unit" apparatus employed gives quotients during rest which are on an average 0.03 too high, it is quite possible that during work there is an even greater systematic error.

To explain the increase in standard metabolism both at low and at high quotients we have in Appendix II offered the hypothesis that synthetic processes take place, resulting respectively in the formation of carbohydrate or

Table XXV.

Date	R. Q. rest	R. Q. work	Diff.	Deviation from curve 100 δ	Date	R. Q. rest	R. Q. work	Diff.	Deviation from curve 100 δ
J. L.		•			O. H. I		+		
1. ii.	·79	·846	+·055	+ 4	25. v.	·705	·744	+·04	+ 1
2. ii.	·805	·846	+·04	+2·5	21.	·71	·731	+·02	- 1
G. L.		•			24.	·72	·735	+·015	- 1
25. i.	·765	·766	0	- 2	22.	·73	·717	-·015	- 4
26. i.	·78	·762	-·02	-3·5	10.	·815	·814	0	- 1
26. ii.	·795	·863	+·07	+5·5	11.	·815	·830	+·015	+0·5
A. K.		•			8.	·82	·836	+·015	+0·5
20. ii.	·715	·757	+·04	+ 1	18.	·91	·839	-·07	- 6
19. ii.	·74	·781	+·04	+1·5	14.	·94	·864	-·075	- 5
1. iii.	·86	·891	+·03	+2·5	15.	·945	·929	-·015	+ 1
12. ii.	·87	·896	+·025	+2·5	17.	·955	·904	-·05	- 2
13. ii.	·91	·909	0	+ 1	O. H. II		×		
R. E.		•			9. iv.	·655	·751	+·095	
25. iii.	·72	·747	+·025	0	8.	·71	·728	+·02	- 1
26. iii.	·75	·742	-·01	- 3	7.	·75	·768	+·02	0
20. iii.	·84	·854	+·015	+0·5	18.	·78	·808	+·03	+1·5
6. iv.	·915	·891	-·025	- 1	3.	·79	·855	+·065	+ 5
7. iv.	·94	·932	-·01	+ 1	6.	·79	·819	+·03	+1·5
A. M. N.		o			4.	·81	·840	+·03	+ 2
10. ii.	·765	·770	+·005	-1·5	16.	·82	·862	+·04	+ 3
9. ii.	·78	(·768)	-·01 <sup>1</sup>	- 3	5.	·83	·858	+·03	+ 2
8. ii.	·78	·770	-·01	-2·5	10.	·835	·797	-·04	- 5
16. ii.	·80	·793	-·005	- 2	15.	·85	·904	+·055	+ 5
30. i.	·83	·873	+·045	+3·5	26.	·85	·838	-·01	-1·5
7. ii.	·83	·787	-·045	-5·5	23.	·855	·796	-·06	-6·5
15. ii.	·835	·841	+·005	-0·5	27.	·865	·890	+·025	+2·5
29. i.	·85	·866	+·015	+ 1	25.	·87	·832	-·04	- 4
1. ii.	·855	·850	-·005	- 1	19.	·895	·880	-·015	- 1
19. ii.	·855	·844	-·01	-1·5	20.	·945	(·966)	(+·02) <sup>3</sup>	
11. ii.	·86	·829	-·03	-3·5	13.	·96	·961	0	+ 3
31. i.	·87	·890	+·02	+ 2	22.	·97	·951	-·02	+1·5
6. ii.	·88	·876	-·005	-0·5	21.	·98	·950	-·03	+ 1
2. ii.	·89	·886	-·005	0	12.	·985	·944	-·04	0
3. ii.	·89	·910	+·02	+2·5	11.	·99	·904	-·085	- 4
12. ii.	·92	·889	-·03	-1·5	14.	1·005	·974	-·03	+ 2
18. ii.	·945	·843	-·10 <sup>2</sup>	- 7					Sum - 25
5. ii.	·97	·882	-·09	-5·5					
13. ii.	·98	·930	-·05	- 1					
14. ii.	1·045	·926	-·12						

<sup>1</sup> Slightly uncertain. <sup>2</sup> Slightly uncertain.<sup>3</sup> Doubtful.

fat with consequent lowering or raising of the respiratory quotient. The quotient of the catabolic processes alone would, according to this view, be higher when the total quotient is low and lower when it is high. When work is being performed the anabolic processes may continue unaltered or may possibly become reduced, because the necessary blood supply is deflected to the working muscles, but there is no reason to think that they should be intensified in proportion to the work. If we assume with Zuntz that the

proportion between the catabolism of carbohydrate and fat remains on the whole unchanged on the transition from rest to work, while the anabolic processes going on at the time are continued without any increase proportional to the increased catabolism, their influence upon the total quotient becomes diminished, and we shall consequently obtain a rise in total quotient when it was low before the work and a fall when it was high—or just what we have found.

The theoretical conception to which our experiments appear to lead up is therefore the following, which is meant not as a proved theory but as a definite formulation of a working hypothesis.

The proportion of carbohydrate to fat catabolised is a function of the relative available quantities of the two substances, and substantially the same during rest and during muscular work.

Fat is formed from carbohydrate and stored up when the available supply of carbohydrate is in excess of the supply of fat. The formation becomes distinct at quotients above 0.9. When the total quotient during rest has reached unity the catabolic quotient is only about 0.95. The conversion of carbohydrate into fat takes place with a loss of energy and a consequent increase of a few per cent. in the total standard metabolism at high quotients.

Sugar (or substances allied to carbohydrates) is formed from fat (and protein) and stored up when the available supply of fat is in excess of the supply of carbohydrate. The formation becomes distinct at quotients below 0.8. When the total quotient during rest has reached 0.71 the catabolic quotient is about 0.74. The conversion of fat takes place with a loss of energy and a consequent increase in the standard metabolism at decreasing quotients.

It is a consequence of this hypothesis that neither fats alone nor carbohydrates alone are suitable for the supply of the energy requirements of the body, but that the catabolic disintegration of either of these substances requires the presence and the simultaneous catabolisation of the other.

While it has long been concluded from the acidosis and excretion of keto-substances in severe diabetes and during starvation that carbohydrates are necessary for the complete catabolisation of fat and our results only lend support to this view, it is, so far as we are aware, a new hypothesis that fats are likewise necessary for the catabolisation of carbohydrate.

#### APPENDIX IV

##### THE VARIATION OF THE RESPIRATORY QUOTIENT AND THE METABOLISM DURING ONE HOUR OF CONSTANT WORK.

In Table XXVI we have arranged the three work determinations of each day in horizontal lines and figured out the differences in respiratory quotient and total metabolism between the three periods. For each subject we have taken the days in the order of increasing quotients. For the sake of convenience we have multiplied the quotients by 1000 and the Calories per minute

Table XXVI.

Date	r.q. during work $\times 1000$					Metabolism during work Cal. $\times 100$				
	I	Diff.	II	Diff.	III	I	Diff.	II	Diff.	III
		II-I		III-II			II-I		III-II	
J. L.										
16. i.	767	+ 4	771	+20	791	608	- 3	605	+11	616
15. i.	772	-16	756	+ 4	760	592	+15	607	+39	646
1. ii.	846	+27	873	- 5	868	568	0	568	+ 6	574
2. ii.	846	- 3	843	- 7	836	584	+ 3	587	- 5	582
G. L.										
26. i.	762	- 5	757	+ 5	762	479	+ 2	481	+ 7	488
25. i.	766	+11	777	-26	751	463	+ 8	471	+19	490
26. ii.	863	- 6	857	-24	833	459	+19	478	-10	468
18. i.	906	-21	885	-18	867	428	+17	445	0	445
19. i.	927	-14	913	-13	900	472	- 6	466	-18	448
A. K.										
20. ii.	757	+ 5	762	- 5	757	590	+ 9	599	+ 8	607
19. ii.	781	- 7	774	-19	755	531	+19	550	- 1	549
1. iii.	891	-21	870	-24	846	530	+24	554	+ 9	563
12. ii.	896	-29	867	- 6	861	522	+ 6	528	+ 8	536
13. ii.	909	-16	893	-10	883	538	+ 3	541	+19	560
R. E.										
26. iii.	742	-12	730	+ 8	738	576	+11	587	+15	602
25. iii.	747	- 5	742	+ 9	751					
20. iii.	854	-18	836	-19	817	538	0	538	+ 6	544
6. iv.	891	+ 4	895	-16	879	523	- 1	522	+ 1	523
7. iv.	932	-14	918	-21	897	532	+ 9	541	+25	566
A. M. N.										
9. ii.	(768)	- 4	764	- 4	760	(512)	+25	537	- 1	536
8. ii.	770	- 9	761	+ 3	764	536	+ 2	538	0	538
10. ii.	770	- 7	763	- 2	761	527	+ 2	529	+ 9	538
7. ii.	787	- 5	782	+23	805	514	+11	525	- 6	519
16. ii.	793	-14	779	+23	802	516	+11	527	-14	513
11. ii.	829	+ 1	830	- 5	825	511	+ 1	512	+10	522
15. ii.	841	- 3	838	+ 4	842	499	+ 7	506	+ 5	511
18. ii.	(843)	-14	829	+ 4	833	(491)	+ 5	496	+10	506
19. ii.	844	+ 2	846	- 6	840	495	+ 6	501	+ 1	502
1. ii.	850	- 4	846	0	846	529	+ 7	536	+11	547
29. i.	866	- 6	860	-12	848	612	-14	598	+ 9	607
30. i.	873	-11	862	- 7	855	533	- 3	530	+13	543
6. ii.	876	0	876	- 8	868	511	- 2	509	+11	520
5. ii.	882	+25	907	-31	876	519	- 3	516	+10	526
2. ii.	886	-16	870	+ 2	872	520	+ 7	527	+ 6	533
12. ii.	889	- 5	884	- 8	876	496	- 1	495	+12	507
31. i.	890	- 2	888	- 5	883	520	+ 4	524	+ 8	532
3. ii.	910	-14	896	- 4	892	568	- 5	563	+ 5	568
14. ii.	926	- 2	924	+16	940	514	- 4	510	+ 5	515
13. ii.	930	- 5	925	+ 3	928	493	+ 3	496	+13	509

Table XXVI *continued.*

Date	R.Q. during work $\times 1000$				Metabolism during work Cal. $\times 100$					
	I	Diff. II-I	II	Diff. III-II	III	I	Diff. II-I	II	Diff. III-II	III
O. H. I										
22. v.	717	- 8	709	+10	719	671	- 1	670	+ 8	678
21.	731	-16	715	- 1	714	682	+ 3	685	+ 3	688
24.	735	- 1	734	+31	765	656	+ 7	663	-21	642
25.	744	-13	731	+15	746	668	0	668	0	668
10.	814	-11	803	+ 7	810	682	- 1	681	+ 1	682
11.	830	+ 5	835	-21	814	669	- 8	661	+11	672
8.	836	- 7	829	-11	818	686	- 3	683	+ 4	687
18.	839	-22	817	-18	799	656	+12	668	+ 2	670
14.	864	+ 6	870	+ 6	876	668	- 8	660	- 7	653
17.	904	- 8	896	-15	881	634	+11	645	+ 5	650
15.	929	- 7	922	- 3	919	650	+ 4	654	- 3	651
O. H. II										
8. iv.	728	+12	740	- 8	732	664	- 8	656	+11	667
9.	751	-14	737	+11	748	644	+14	658	- 7	651
7.	768	- 2	766	- 3	763	649	+ 3	652	+ 4	656
23.	796	- 8	788	-10	778	611	+10	621	+ 8	629
10.	797	+ 7	804	0	804	650	- 6	644	+13	657
18.	808	0	808	- 8	800	627	+ 1	628	+11	639
17.	816	- 5	811	-20	791	627	+ 3	630	+21	651
6.	819	+20	839	- 9	830	636	-10	626	+11	637
25.	832	-10	822	+ 7	829	614	+ 3	617	+ 3	620
26.	838	+ 8	846	+ 2	848	613	+ 4	617	- 4	613
4.	840	- 1	839	- 8	831	662	- 7	655	- 5	650
3.	855	-12	843	+ 2	845	642	+ 5	647	0	647
5.	858	-12	846	-20	826	636	- 5	631	+16	647
16.	862	-11	851	- 2	849	618	+ 6	624	0	624
19.	880	+ 4	884	-14	870	622	- 4	618	+ 6	624
27.	890	+17	907	-15	892	608	- 5	603	+ 8	611
11.	904	- 1	903	- 1	902	631	+ 1	632	+ 4	636
15.	904	- 9	895	-13	882	607	+ 6	613	+ 5	618
12.	944	+ 1	945	-28	917	622	- 6	616	+12	628
21.	950	-11	939	+31	970	605	- 3	602	- 2	600
22.	951	-16	935	+14	949	598	- 2	596	+10	606
13.	961	+ 6	967	-23	944	616	- 4	612	+14	626
20.	(966)	-31	935	+ 2	937	(594)	+18	612	+ 6	618
14.	974	-36	938	+ 2	940	610	+17	627	- 9	618

by 100 so as to have the differences in whole numbers. In the experiments on J. L., G. L., A. K. and R. E. the periods were of about 30 minutes duration; in the first series of experiments on O. H. they were of about 24 minutes and in the remainder of the experiments of 21 minutes duration.

The table shows that the quotient varies irregularly but on the whole very slightly. In most cases there is a slight decrease in quotient from the first to the second and again from the second to the third period but it does not amount to more than about 0.01, and in some cases there is an increase of similar magnitude. The largest difference observed between two periods

is 0.035. We have averaged the differences found in all the experiments (74) and those found in the most reliable series with periods of about equal length (series 2-4) with the following results:

Number	R.Q. II-R.Q. I	R.Q. III-R.Q. II	Cal. II-I	Cal. III-II
74	-0.0056	-0.0040	+0.033	+0.054
55	-0.0051 ± 0.0014	-0.0025 ± 0.0018	+0.019 ± 0.010	+0.047 ± 0.010

There is a slight fall in the quotient from the first to the second period and a still smaller from the second to the third. Though the work remains constant the metabolism increases slightly from the first to the second period and somewhat more from the second to the third. As the fall in quotient must cause an increase in the metabolism a correction can be applied to give the change in metabolism from period to period, on the assumption that the quotient remained constant. According to the average result of our determinations a decrease in the quotient of 0.01 will correspond approximately to an increase in the metabolism of 0.02 Cal. In the 55 best experiments the corrected increase in metabolism from the first to the second period will be therefore  $0.019 - 0.51 \times 0.02 = 0.009 \pm 0.010$  and from the second to the third  $0.047 - 0.25 \times 0.02 = 0.042 \pm 0.010$ .

The corrected increase in metabolism from period to period must be taken to show the influence of fatigue, and the calculations show that such an influence is practically absent in the second period but distinct, though small, in the third.

In order to see whether the diet has any influence upon the variation of the quotient during work and upon the onset of fatigue we have averaged the differences in the experiments with initial quotients between 0.7 and 0.85 and compared them with the averages derived from the remaining experiments with quotients between 0.85 and 1.00. The results are as follows:

Number	R.Q.	R.Q. II-R.Q. I	R.Q. III-R.Q. II	Cal. II-I	Cal. III-II
28	0.7 - 0.85	-0.0043 ± 0.0017	-0.0001 ± 0.0024	+0.031 ± 0.014	+0.032 ± 0.016
27	0.85 - 1.00	-0.0059 ± 0.0023	-0.0049 ± 0.0025	+0.007 ± 0.014	+0.062 ± 0.012

Caloric difference corrected for decrease in quotient

R.Q.	Cal. II-I	Cal. III-II
0.7 - 0.85	+0.022 ± 0.018	+0.032 ± 0.021
0.85 - 1.00	-0.005 ± 0.017	+0.052 ± 0.017

The fall in quotient during a one hour period of constant work is slightly larger at high quotients than at low, and the influence of fatigue upon the economy of the work appears to be very nearly the same at all quotients. All the changes observed are scarcely outside the limits of error.

In our experiments work has been performed at the same rate for 30 minutes before the beginning of the first period. An extrapolation shows that at the beginning of work the respiratory quotient has probably on an average been about 0.01 higher than in the first experimental period.

The results here given show that the organism maintains during work a remarkably constant proportion between the amounts of carbohydrate and

fat catabolised. This proportion is evidently a function of the available supplies of the two sources of energy, and the change in quotient shows that the balance between carbohydrate and fat is very slowly altered as the work progresses in favour of the latter.

#### SUMMARY.

1. Methods are described for determining in 20 minute periods the respiratory exchange of human subjects doing constant work on an ergometer placed in a Jaquet respiration chamber.

2. The technical error on determinations of total metabolism made by these methods is below 1 % and the respiratory quotient can be determined with a maximum error of 0.005.

3. Four series of experiments (about 220 determinations) on six different subjects, living during the experiments on a diet poor in protein, have been made to study the relative value of fat and carbohydrate as a source of muscular energy. All the series agree in showing that work is more economically performed on carbohydrate than on fat. When the work was sufficiently severe the subjects performed it with greater difficulty on fat than on carbohydrate and became much more tired.

4. The net expenditure of energy (standard metabolism deducted) necessary to perform one Calorie technical work on the ergometer has varied between about 5.5 and 4 Cal. At a constant quotient it varies with the subject and for the same subject it decreases with increasing training. During one hour of work it generally rises slightly from fatigue.

5. For the single subject and on a constant level of training the relation between the respiratory quotient and the net expenditure of energy per unit work can be expressed graphically as a straight line. Since the proportion of fat to carbohydrate catabolised is also a straight line function of the quotient the difference in value for muscular work between fat and carbohydrate can be expressed by a single figure: the waste of energy from fat. In the three best series of experiments the net expenditure of energy per Cal. technical work varies from about 4.6 Cal. when fat alone is catabolised (R.Q. = 0.71) to about 4.1 Cal. when carbohydrate alone is catabolised (R.Q. = 1.00). The waste of energy from fat is 0.5 Cal. or 11 % of the heat of combustion of the fat.

6. The standard metabolism (during rest, in the postabsorptive state) of a human subject is not independent of the preceding diet. When the diets are poor in protein it is lowest at intermediate quotients and increases about 5 % when the quotient falls to about 0.71 and about 3 % when the quotient rises to about unity.

7. On the transition from rest to moderate muscular work the respiratory quotient is generally altered. On an average it is increased when the quotient was low and diminished when it was high before the work. The fall at high quotients is greater (0.05) than the increase at low (0.03). At quotients between 0.8 and 0.9 the average change on the transition to work is very slight.

8. It is suggested as a working hypothesis:

that both during rest and during work the proportion of fat to carbohydrate catabolised is a function of the available supplies of these substances;

that carbohydrate is formed from fat and provisionally stored when the quotient is below 0.8 while a corresponding transformation of carbohydrate to fat takes place when the quotient is above 0.9;

that these anabolic processes make the total respiratory quotient lower than the catabolic when this is low and higher when it is high and that they give rise to an extra expenditure of energy during rest;

and finally that during work the anabolic processes (combined with storage) are not increased in proportion to the catabolic, whereby the total quotient is lowered when it was high and raised when it was low beforehand.

9. During one hour of constant work of moderate intensity the respiratory quotient decreases on an average only 0.008; slightly more at high quotients and less at low. The metabolism per Cal. technical work increases a little (0.065 Cal.). Part of the increase is due to the fall in quotient (0.01 Cal.) and the rest to fatigue. The effect of fatigue upon the economy of the work appears to show itself earlier when the quotient is low than when it is high.

The expenses of this research have been defrayed out of a grant from the Carlsberg foundation. Our thanks are due further to our collaborators, Dr G. Liljestrand who took part during his stay with us in the planning and execution of the first series of experiments, and Mr K. Gad Andresen who has made most of the gas analyses on which the accuracy of our results so largely depends, and finally to Messrs R. Ege, A. Möller Nielsen and O. Hansen who have undertaken the tedious work of acting as subjects and by their care and devotion contributed largely to the uniformity of the results.

Part of the expense of publication of this paper has been defrayed from a grant for which the Biochemical Society is indebted to the Royal Society.

## REFERENCES.

- Anderson and Lusk (1917). *Biol. Chem.*, **32**, 421.  
Benedict and Cathcart (1913). *Carnegie Publication*, No. 181.  
— and Roth (1918). *Proc. National Acad. Sc.*, **4**, 149.  
Carpenter, T. M. (1915). *Carnegie Publication*, No. 216.  
Chauveau (1898). *Compt. Rend.*, **125**, 1070; **126**, 795.  
Fletcher and Hopkins (1917). *Proc. Roy. Soc. B.*, **89**, 444.  
Frentzel and Reach (1901). *Pflüger's Arch.*, **83**, 477.  
Grafe (1910). *Zeitsch. physiol. Chem.*, **65**, 1.  
Hasselbalch (1912). *Biochem. Zeitsch.*, **46**, 403.  
Heineman (1901). *Pflüger's Arch.*, **83**, 441.  
Krogh (1913). *Skand. Arch. Physiol.*, **30**, 375.  
— (1915, 1). *Abderhalden's Handb. biochem. Arbeitsmethoden*, **8**, 537.  
— (1915, 2). The Respiratory Exchange of Animals and Man, *Monographs of Biochemistry*, Longmans, Green and Co., London  
— (1915, 3). *J. Physiol. Proc.*, **49**, xxxi.  
— (1919). *K. Danske Vidsk. Selsk. Math.-fys. Medd.*, **1**, No. 12.  
— (1920, 1). *Biochem. J.*, **14**, 267.  
— (1920, 2). *Biochem. J.*, **14**, 282.  
— A. and M. (1913). *Medd. om Grönland*, **51**, 19.  
Liljestrand (1916). *Skand. Arch. Physiol.*, **33**, 161.  
— (1917). *Skand. Arch. Physiol.*, **35**.  
Lindhard (1915). *Pflüger's Arch.*, **161**, 377.  
Loewy (1911). *Oppenheimer's Handb. Biochemie*, **41**, 281.  
Zuntz (1901). *Pflüger's Arch.*, **83**, 564.  
— (1911). Die Quellen der Muskelkraft, *Oppenheimer's Handb. Biochemie*, **4**, 826.  
— and Loeb (1894). *Arch. Anat. Physiol.*, 571.  
— and Loewy (1913). *Lehrb. der Physiologie der Menschens*, 663.  
— and Schumburg (1901). *Physiologie des Marsches*, Berlin.

# XXXI. STUDIES IN THE ACETONE CONCENTRATION IN BLOOD, URINE, AND ALVEOLAR AIR. II: THE PASSAGE OF ACETONE AND ACETO-ACETIC ACID INTO THE URINE.

BY ERIK MATTEO PROCHET WIDMARK.

*From the Physiological Institute, Lund, Sweden.*

*(Received February 10th, 1920.)*

THERE is a great difference between the conditions of diffusion in the tissues in the case of the two components of the total acetone, the acetone and the aceto-acetic acid. The aceto-acetic acid occurs within the organism as a largely dissociated salt. Of the salts of the organic acids we know that they possess only to an inconsiderable degree the power of penetrating the living cell. The tissue cells therefore constitute a certain obstacle to the free diffusion of the salt. On this account a difference in concentration may arise and remain on the two sides of a tissue membrane. Acetone on the other hand belongs to a group of substances which can with the greatest ease penetrate the living cell. It can therefore, on its appearance within the organism, diffuse without any hindrance from cells or cell membranes.

This difference appears clearly in the passage of these two substances into the urine.

The aceto-acetic acid is concentrated through the action of the kidneys. The acetone passes into the urine by means of diffusion, and no concentration of the free acetone in the urine can be brought about by the action of the kidneys.

In the urinary bladder the concentration of the aceto-acetates is considerably greater than the concentration of these substances in the blood; diffusion through the walls of the bladder is not probable. The acetone on the other hand is very readily diffused through the walls of the bladder.

In this part we give details of a number of experiments intended to throw light upon these facts.

## THE ELIMINATION OF ACETONE THROUGH THE URINE.

The idea that substances soluble in lipoids pass into the urine solely through diffusion was formulated more than ten years ago by Overton [1907]. He writes: "Wenn Amphibien in nicht tödlichen Konzentrationen von Alkohol, Aceton u.s.w. suspendiert werden, so enthält der Harn nach Ablauf einiger Zeit [die zur Einstellung des Gleichgewichts zwischen dem Gehalte

der Verbindungen im Blute und der Aussenlösung erforderlich ist] diese Stoffe in annähernd gleicher Konzentration wie die Aussenlösung [nach eigenen, noch nicht publizierten Versuchen]. Auch bei den Säugetieren fand Gréhant nach Einführung von Alkohol in den Magen fast die gleiche Alkoholkonzentration im Blute und im Harn. Wegen der schnellen Verbrennung des Alkohols bei warmblütigen Tieren ändert sich die Alkoholkonzentration im Blute recht schnell, und bei dem ungünstigen Verhältnis der absorbierenden Oberfläche der mässig oder stärker gefüllten Harnblase zu deren Volumen [namentlich bei grösseren Tieren] kann sich die Alkoholkonzentration jenes Harnes, der zu einer Zeit abgesondert wurde, wo sich viel Alkohol im Blute befand, nicht mehr ausgleichen mit der Konzentration im Blute, nachdem dieses an Alkohol arm geworden ist. Wenn umgekehrt viel Harn in der Harnblase vorhanden war, ehe die Alkoholkonzentration im Blute ihr Maximum erreicht hatte, muss zu einer bestimmten Zeit die Alkoholkonzentration in der Harnblase hinter derjenigen im Blute stehen. Ähnliches gilt natürlich für andere Vertreter dieser Gruppe von Stoffen.

“Dass viele Verbindungen, die in den Lipoiden leicht löslich sind, im Organismus durch Paarung mit anderen im Körper gebildeten Stoffen oder auf anderen Weise in Substanzen übergehen, die nicht mehr in den Lipoiden merklich löslich sind, und einer aktiven Exkretion durch die Drüsenzelltätigkeit unterliegen, ist einer Sache für sich, die in keinem Widerspruch mit dem vorher Gesagten steht. Solche organische Verbindungen die nur schwer löslich in den Lipoiden sind, wie Glycerin und Harnstoff, sind ebenfalls in gewissen Drüsen der aktiven exkretorischen Zellentätigkeit unterworfen.”

No further detailed researches have yet been published by Overton.

The passage of ethyl alcohol into the urine was investigated in detail some years ago by the author [Widmark, 1915]. Comparative estimations were then carried out between the alcohol percentage in the blood and in the urine simultaneously secreted. The results showed that the concentrations in blood and urine very nearly coincided with one another. The alcohol concentration in the urine was not affected by diuresis; an increase in diuresis caused by the taking of a quantity of water did not in any way change the alcohol concentration in the urine.

The agreement between the concentrations in the blood and in the urine has more recently been demonstrated, independently of the author, by Chabanier and Ibarra-Loring [1916], whose experiments were concerned with ethyl and methyl alcohols.

#### *Experimental.*

The experiments with acetone were carried out as follows:

The subject first completely emptied his bladder. He then drank a certain quantity of dilute acetone solution. Blood estimations were at once carried out, with a few minutes interval after each. Every 15th minute the bladder was emptied, the quantity of urine measured, and the acetone concentration

determined. In certain experiments this series of estimations began immediately after the taking of acetone; in other cases the comparative estimations were not begun until after the acetone concentration in the blood had become something like constant. In order to demonstrate the influence of diuresis, or more properly speaking to show that diuresis has no influence upon the acetone concentration in the urine, a quantity of water was taken at a certain point in the experiment, the variation in the quantity of urine secreted during the periods of 15 minutes was measured, and the acetone concentration estimated.

*Experiment 1.* Subject of experiment: the author. Acetone taken: 8 grams in about 200 cc. of water. First blood test 7 minutes after taking, first urination 15 minutes after taking. During the sixth 15-minute period 300 cc. of water at room temperature were taken. The results of the experiment are shown by Table I and Fig. 1.

Table I.

Time (Periods of 15 min.)	URINE		BLOOD Concentration of acetone ‰
	Concentration of acetone ‰	Rate of excretion cc./min.	
I	0.01	2.3	0.04
II	0.12	2.8	0.15
III	0.16	9.2	0.12
IV	0.16	10.7	0.12
V	0.14	4.5	0.12
VI	0.14	3.2	0.12
VII	0.14	5.7	0.11
VIII	0.13	10.7	0.12
IX	0.14	3.6	0.12
X	0.13	3.7	0.10
XVII	0.10	2.9	0.08

The table and figure show that the acetone concentration of the blood at first rises more rapidly than that of the urine; the highest values in the blood were obtained sooner than the highest values in the urine. The connection between the two cannot be ascertained more closely, because the periods during which the urine was collected in the bladder are too long for the estimation of the concentration in the portions obtained to express accurately the course of the curve. When the blood acetone has shown itself to be at a certain fairly constant level, the urine acetone also remains fairly constant, as far as the concentration is concerned. The concentration of acetone in the urine remains throughout somewhat higher than that in the blood. This is probably due to the fact that the acetone in the urine has about the same concentration as that in the plasma, which is somewhat higher than that of the total blood. Possibly the concentration in the urine may be even a little higher than in the plasma, on account of the fact that acetone is perhaps more easily soluble in urine than in plasma. The saltiness of the urine does not seem to play any part worth mentioning. During the experiment the excretion curve reached two maxima: the first due to the

quantity of fluid taken in connection with the taking of the acetone, the second to the quantity of water taken during the sixth period. The concentration in the urine in no way follows the variations in the excretion, but seems to proceed in complete independence of these.

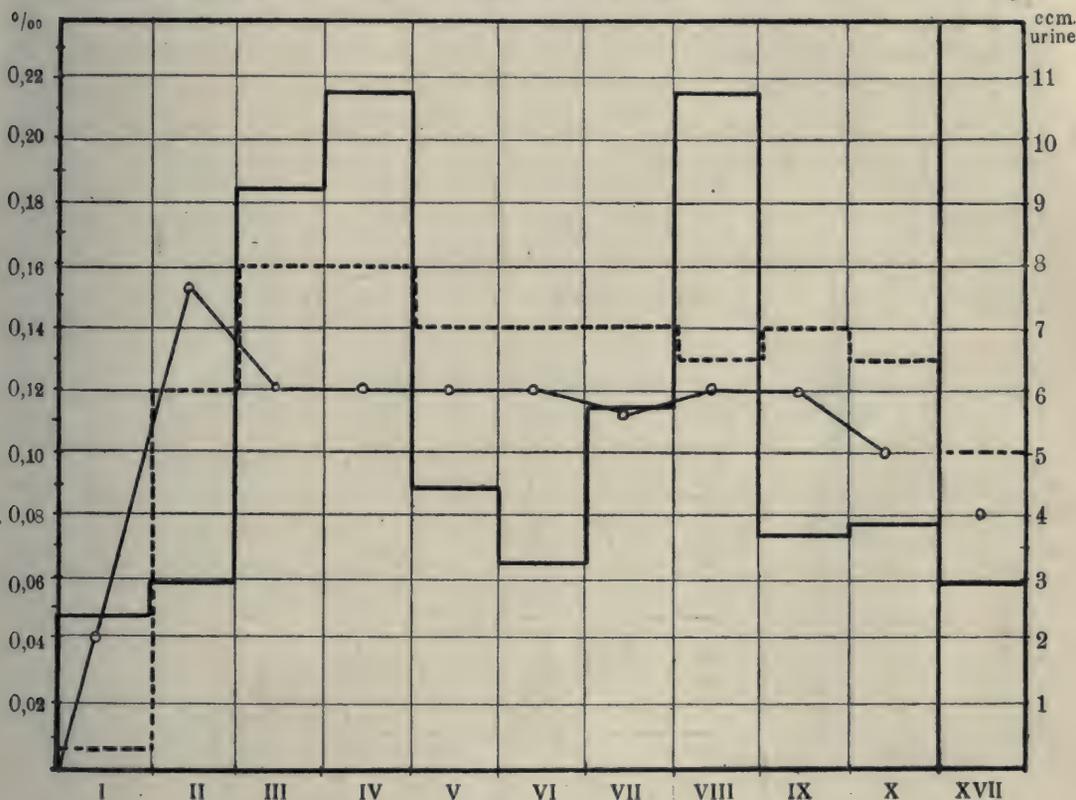


Fig. 1. — = excretion.  
 - - - = concentration of acetone in urine.  
 o—o = concentration of acetone in blood.

Abscissa: periods of analysis.  
 Ordinate: acetone concentration in %oo, excretion in cc. per min.

*Experiment 2.* Subject: the author. On March 16 at 10.45 a.m., 9.6 g. of acetone were taken in 500 cc. of water. The comparative estimations of the acetone concentration in blood and urine were begun at 1.15 p.m. At 1.45 p.m. 500 cc. of water were taken. The result is shown by Table II and Fig. 2.

Table II.

Time	URINE		BLOOD	
	Concentration of acetone %oo	Rate of excretion cc./min.	Time	Concentration of acetone %oo
1.15-1.30	0.160	1.33	1.35	0.127
1.30-1.45	0.160	1.80	2.20	0.127
1.45-2.00	0.160	2.13	2.25	0.124
2.00-2.15	0.160	8.40	2.50	0.115
2.15-2.30	0.160	7.33	—	—
2.30-2.45	0.156	2.87	—	—

This experiment shows, even better than the preceding, how completely the acetone concentration of the urine is independent of the amount excreted. Here too the concentration of acetone in the urine is somewhat higher than in the blood. The lowest value for the rate of excretion is 1.33, the highest is 8.4; although therefore the volume of the urine becomes more than six times as great, the concentration of the acetone is not in the slightest degree influenced by this fact.

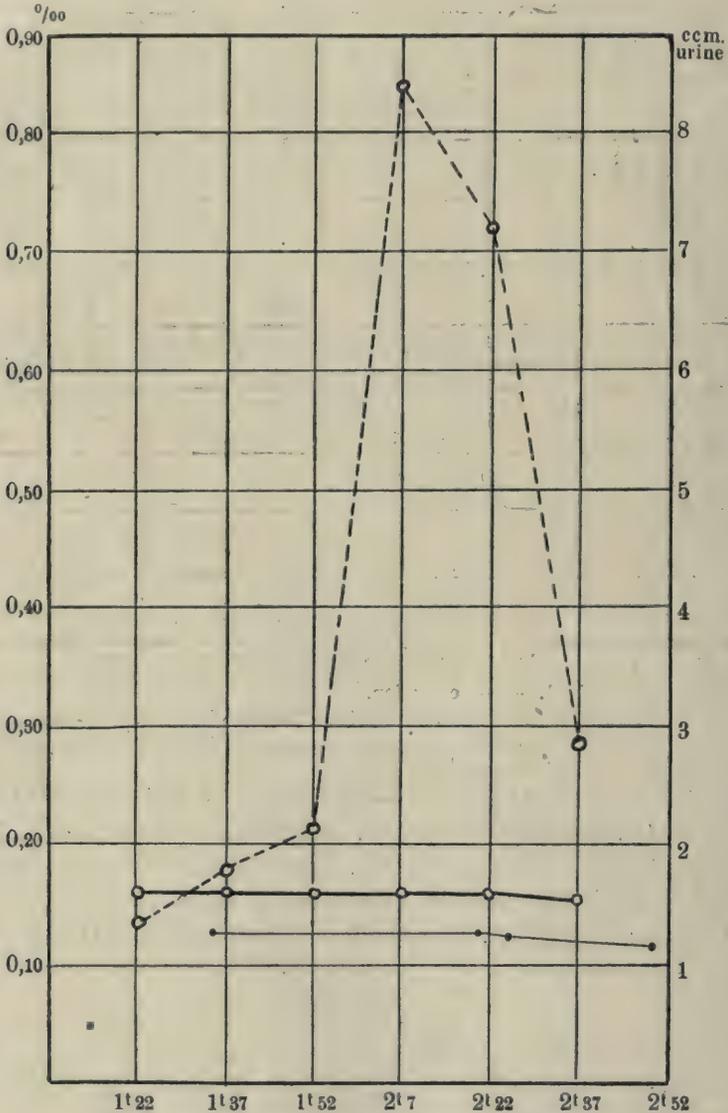


Fig. 2. o—o = concentration of the acetone in urine.  
 — = concentration of the acetone in blood.  
 o - - o = excretion.

In this graph, for the sake of clearness, both the excretion and the estimations of the acetone concentration of the urine are represented by points lying in the middle of the period in question.

*Experiment 3.* Subject: the author. On March 7, 1917 at 9.45 a.m., 16 g. of acetone were taken in 600 cc. of water. The comparison between the acetone concentration in blood and urine was begun at 1.15 p.m. At 2.35 p.m. 500 cc. of water were taken.

Table III.

Time	URINE		Time	BLOOD Concentration of acetone ‰
	Concentration of acetone ‰	Rate of excretion cc./min.		
1.15-1.30	0.28	1.87	1.15	0.22
1.30-1.45	—	1.67	—	—
1.45-2.00	0.29	1.80	—	—
2.00-2.15	—	1.87	—	—
2.15-2.30	0.27	1.67	—	—
2.30-2.45	0.27	1.87	—	—
2.45-3.00	0.25	3.73	—	—
3.00-3.15	0.26	9.67	3.14	0.21
3.15-3.30	0.25	10.53	—	—
3.30-3.45	0.23	5.73	4.0	0.18

*Experiment 4.* Subject: B. J. Body weight, 63 kg. At 11.40 a.m. 8 g. of acetone were taken in 500 cc. of water. The comparison between the acetone concentration in blood and in urine was begun at 2.30 p.m. At 2.35 p.m. 500 cc. of water were taken.

Table IV.

Time	URINE		Time	BLOOD Concentration of acetone ‰
	Concentration of acetone ‰	Rate of excretion cc./min.		
2.30-2.45	0.15	1.60	2.12	0.15
2.45-3.00	0.16	4.93	3.15	0.13
3.00-3.15	0.17	8.80	4.12	0.12
3.15-3.30	0.16	8.40	—	—
3.30-3.45	0.17	8.67	—	—

This experiment, carried out on a different subject from the preceding, shows the same facts as have been already described.

#### THE ELIMINATION OF ACETO-ACETIC ACID THROUGH THE KIDNEYS.

If an experiment in the same direction as those recorded above is made with a diabetic patient with acidosis—*i.e.* if he is allowed to drink a certain quantity of water, and comparative measurements are then taken of the total acetone concentration in the blood and in the quantity of urine passed during each 15-minute period—quite a different result is obtained. We know that by far the greatest part of the total acetone of the urine occurs in the form of aceto-acetates. Only a small part is free acetone. It has already been pointed out that the conditions of diffusion for these salts are quite other than for the acetone. The salts are not diffused through the kidneys but may evidently be subjected to an active excretion, through which the concentration of these substances in the urine may become many times greater than their

concentration in the blood. With an increased quantity of urine resulting from the resorption of a certain amount of water by the subject, the amount of the aceto-acetates secreted is not increased in the same proportion as the amount of urine. The consequence of this is that with a water-diuresis the total acetone concentration sinks rapidly, to rise again later as soon as the rate of excretion of urine returns to its normal value. This is shown by the two following experiments.

*Experiment 5.* August 16, 1916. Subject: Mrs L., diabetic with pronounced acidosis. 1 litre of water was taken on an empty stomach. The total acetone concentration was then estimated in portions of urine obtained every quarter of an hour. The estimations were carried out according to the method of Embden and Schliep. The reaction of the urine was alkaline or amphoteric.

Table V.

Time (Periods of 15 min.)	Total acetone concentration of urine ‰	Rate of excretion of urine cc./min.
I	0.696	0.53
II	0.658	0.60
III	0.482	0.93
IV	0.189	2.73
V	0.193	3.60
VI	0.244	1.80
VII	0.310	1.67

As soon as the rate of excretion is increased, the total acetone concentration in the urine simultaneously falls to a considerable extent. After the rate has reached its maximum and has begun to sink, the total acetone concentration rises.

*Experiment 6.* Subject: J. N., diabetic with acidosis. At 1.45 p.m. on March 14, 1917, one hour after a meal, 500 cc. of water at room temperature were taken. The reaction of the urine was alkaline or neutral. The results are given in Table VI and Fig. 3.

Table VI.

Time	URINE		BLOOD Total acetone ‰
	Total acetone ‰	Rate of excretion cc./min.	
1.45-2.00	—	0.60	—
2.00-2.15	> 0.95	0.86	2.20 { 0.109 0.103
2.15-2.30	0.31	4.26	—
2.30-2.45	0.19	7.73	—
2.45-3.00	0.25	6.40	3.20 { 0.126 0.121
3.00-3.15	0.72	1.60	—

This experiment shows that the total acetone concentration in the urine is throughout considerably higher than in the blood. When the diuresis sets

in the concentration in the urine at once sinks to a considerable extent, and rises again as soon as the quantity of urine is diminished. There is therefore no correlation between the acetone curves in the blood and in the urine, and the passage of the aceto-acetic acid into the urine is determined by the incidental secretive activity of the kidneys<sup>1</sup>.

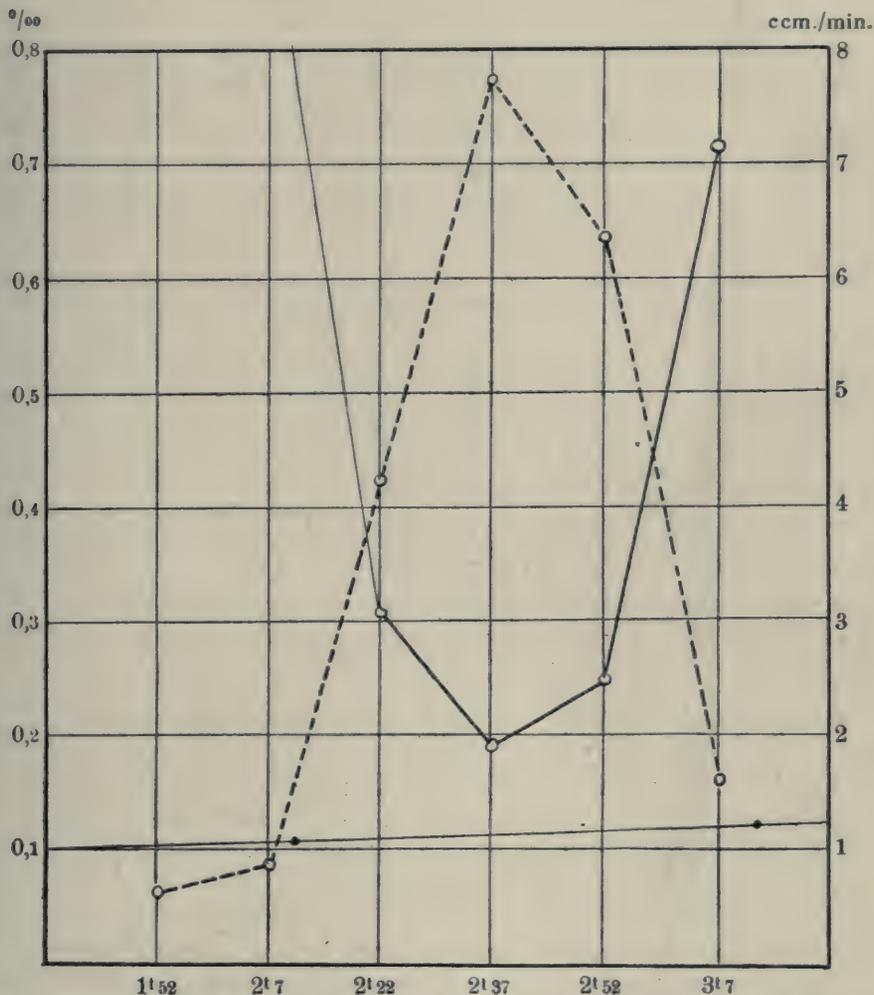


Fig. 3. Relation between the excretion of urine and the total acetone concentration in the urine in diabetes, according to Exp. 6. o - o = excretion of urine; o - o = total acetone concentration in urine; — = total acetone concentration in blood.

<sup>1</sup> It is possible that the *reaction* of the urine may also play a certain part in the elimination of aceto-acetic acid. The free acid may possibly be more easily diffused than the salt through the kidney epithelium; a difference in concentration will in that case less easily arise. In this circumstance may perhaps lie the explanation of the fact which many writers claim to have demonstrated, that the acetone bodies pass over into the urine in greater quantities if the patient is given bicarbonate.

ON THE RELATION OF THE ACETONE TO THE WALLS OF THE  
URINARY BLADDER.

It was formerly believed that the walls of the healthy urinary bladder are, practically speaking, impermeable to the substances which pass into the bladder by way of the kidneys, and that these substances can only leave the bladder by one way, viz. through the urethra [compare Cohnheim, 1909].

Novicka [1913] has however quite incontestably shown that alcohol is diffused with great ease through the walls of the bladder, the direction of diffusion depending upon the higher concentration. For this substance therefore the rule does not always apply that the amount of alcohol found in the daily quantity of urine is also the daily quantity of alcohol coming from the kidneys. The following cases, for instance, may be imagined to occur:

(a) The alcohol concentration in the bladder and the blood is the same during the period between two urinations. The quantity of alcohol which has come from the kidneys is not altered in the bladder by diffusion.

(b) The alcohol concentration is lower in the blood than in the bladder during the time that elapses between two urinations. The quantity of alcohol which has come from the kidneys is diminished in the bladder by diffusion from bladder to blood.

(c) The alcohol concentration is higher in the blood than in the bladder during the period between two urinations. The quantity of alcohol which has come from the kidneys is increased by direct diffusion from blood to bladder.

If the time between the urinations is short, the difference in concentration between blood and urine will be inconsiderable, because the alcohol, as we have previously emphasised, passes into the urine in the same concentration as into the blood, and no considerable fluctuations in concentration can appear. If the time between two urinations is long, it may happen that the alterations in the concentration of the blood are of so high a degree that cases (b) and (c) may occur.

It is evident that this property possessed by ethyl alcohol of diffusion through the walls of the bladder is merely an evidence of its power to penetrate the living cell and organic tissues in general. It is therefore to be expected that all substances having the same properties as alcohol will form an exception to the rule as to the impermeability of the walls of the bladder<sup>1</sup>.

In the following we shall show that the relation of acetone to the walls of the bladder is exactly the same as that of alcohol.

<sup>1</sup> Thus, for example, methylene blue diffuses with the greatest ease through the bladder walls. If the ureters of a rabbit are bound up and a dilute solution of methylene blue is injected into vena jugularis, it will be found very soon—18 minutes after the injection—that the urine is coloured deep blue.

*Demonstration of the diffusion of acetone from  
bladder to blood.*

*Experiment 7.* August 24, 1916. Rabbit ♂. A half-solid catheter was introduced into the bladder and allowed to remain throughout the experiment. 45 cc. of urine were drawn off. 50.5 cc. of 2.42 % acetone in physiological salt-solution—*i.e.* 122 mg. of acetone—were then introduced. After one hour the bladder was tapped and rinsed out three times with 5 cc. of physiological salt-solution. In the mixed quantity the acetone was determined according to Messinger-Huppert and the amount found to be 71.4 mg.

<i>Result:</i>	Introduced	122 mg.
	Found	71 mg.
	Quantity lacking after one hour	51 mg. = 41.8 %

The blood contained 1.54 mg. of acetone per 100 cc. Post mortem examination showed an intact bladder mucous membrane. Otherwise nothing pathological.

*Experiment 8.* March 5, 1917. Rabbit 1330 g. ♂. At 11.10 a.m. a few cc. of a 5 % acetone solution were injected by means of a catheter into the animal's bladder. A series of blood estimations was then undertaken. The result is shown by Table VII.

Table VII. The passage of acetone from the contents of a rabbit's bladder into the blood.

Time	Total acetone of blood %
11.0 a.m. before the injection	0.010, 0.012 = 0.010
12.15 p.m.	0.10
1.25	0.11
2.25	0.13
3.20	0.09

The experiment shows that acetone introduced into the bladder can be demonstrated in the blood as soon as one hour afterwards.

Even though it is highly probable that there is no important dissimilarity between the conditions of diffusion in the urinary bladder of rabbits and of human beings, the demonstration of the diffusion in human beings is nevertheless of importance. For the introduction of a definite amount of acetone into the bladder of human beings a modification of Janet's method is used. The arrangement of the apparatus is shown by Fig. 4. *A* is a vessel that can be raised and lowered, attached by a tube to the pressure-flask *B*, communicating with the burette *C*, which has a glass tap at either end. The discharging-tube of the burette is provided with a perforated rubber cone, which can be introduced into fossa navicularis penis so as to close the orifice of the urethra. At the beginning of the experiment all the vessels contain sublimate solution (1 : 2000). By lowering *A* beneath the level of *B* and giving *C* a suitable position with the fluid above the tap *b*, the sublimate

solution can be conveniently sucked out of the burette and sterile acetone solution sucked in up to a certain height. When the pressure has been equalised by raising *A*, the taps are closed, and the level in the burette is read off. The burette is now brought into contact with the urethra, the taps are opened once more, and *A* is gently raised to a suitable height. When a sufficient quantity of fluid has passed into the bladder, *A* is lowered and the amount of fluid present in the distended urethra flows back into the burette, with the exception of a very small quantity (0.5–1.0 cc.), which can be caught and measured when the burette is removed from the penis. Before this is

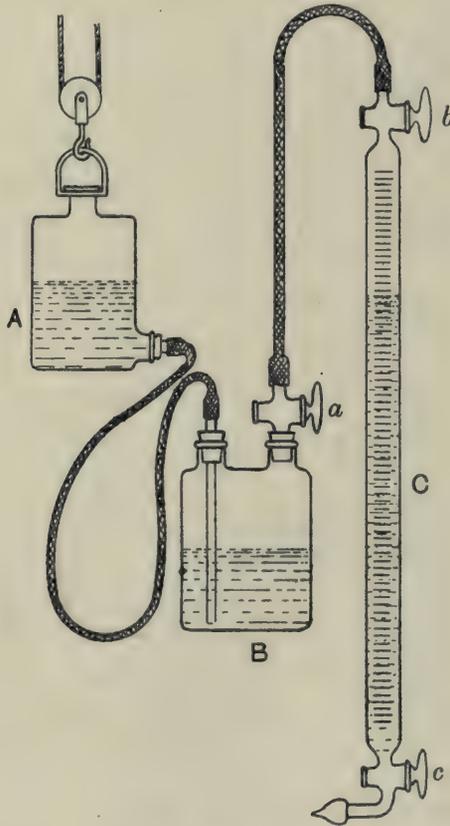


Fig. 4.

done the taps are closed and the burette is read off. By this arrangement an accurately measured quantity of fluid can be introduced into the bladder without pain or any appreciable discomfort. The fluid in the burette can be kept at body temperature. After the injection the concentration of the acetone in the burette is determined. The burette has all the time been completely closed, except during the injection. This removes the possibility of an error which might arise owing to the evaporation of the acetone solution from an open vessel.

*Experiment 9.* Subject: E. W. August 25, 1916. 37 cc. of a 1 % solution of boracic acid containing 0.74 ‰ acetone were introduced into the bladder. An hour after this the bladder was emptied. In order to wash out as much as possible of the acetone remaining, the bladder was emptied a couple of times more, with 5–10 minutes' interval between. The total quantity of urine amounted to 294 cc. It contained 22.8 mg. of acetone.

<i>Result:</i>	Introduced	27.4 mg. acetone
	Found	22.8 „
	Amount lacking after one hour	4.6 „ = 16.8 %

This experiment suffers from two deficiencies: (1) the amount of acetone introduced is too small, and (2) the experiment was carried out immediately after a meal, so that the excretion of urine was comparatively great. For both these reasons the pre-formed iodine-binding substances present in the urine give rise to a relatively great source of fallacy. The result of the experiment may therefore easily lead to the conclusion that a less complete re-absorption takes place than is actually the case. The following experiment, in which a larger quantity of acetone was introduced during a period of low rate of excretion, gives a clearer idea of the diffusion.

*Experiment 10.* Subject: E. W. August 26, 1916. 57 cc. of a 2.77 ‰ acetone solution were introduced. After one hour the bladder was emptied as in the previous experiment. The total quantity of urine, 178 cc., contained 114 mg. of acetone.

<i>Result:</i>	Introduced	158 mg.
	Found	114 mg.
	Amount lacking after one hour	44 mg. = 27.8 %

The experiments show that acetone is able to pass quite easily through the walls of the human bladder also. It should however be pointed out that a certain quantity of acetone may have diffused into the blood in the passage through the urethra during the introduction of the liquid. The walls of the ureters are of course permeable to a far higher degree, for many substances, than the walls of the bladder. Nevertheless, that there is a diffusion through the walls of the bladder can hardly be denied.

*Demonstration of the diffusion of acetone from blood to urine  
with tied ureters.*

*Experiment 11.* August 23, 1916. Rabbit ♂. Weight 2640 g. Narcosis with ethylene urethane. After ventral incision in the middle line both ureters were brought forward and tied immediately above the bladder. The wound was provisionally closed. A half-solid catheter was then introduced through the urethra and 46 cc. of urine were drawn off. 50 cc. of physiological salt-solution were introduced.

Now, for a period of ten minutes, 15 cc. of about 50 % acetone in physiological salt-solution were injected drop by drop through the vena jugularis

externa. The rabbit showed strong dyspnoea during the injection. Five minutes after the injection was over Legal's acetone test on 2 cc. of the contents of the bladder showed uncertain reaction. After five minutes more the test was clearly positive, and 15 minutes after the injection strongly positive: 30 minutes after the injection a blood sample was taken and the urine left in the bladder drawn off. Acetone estimations on both samples according to Messinger-Huppert showed:

In the blood:  $3.2 \text{ ‰}$  acetone.

In the contents of the bladder:  $1.1 \text{ ‰}$  „

Section showed an intact bladder mucous membrane. In other respects nothing noteworthy.

These experiments show therefore that free acetone can pass with considerable ease through the walls of the urinary bladder. The direction is determined by the side of the bladder walls on which the greater concentration prevails. The quantity diffused will depend on the concentration and the extent of the absorbing surface.

In diabetes the changes in the percentage of free acetone in the blood probably take place somewhat slowly. In all probability no considerable difference in concentration occurs between the free acetone of the blood and that of the bladder, nor, consequently, is there any considerable change in the amount of acetone in the urine owing to diffusion through the walls of the bladder. On the other hand it is conceivable, especially if the urine is strongly acid, that part of the aceto-acetic acid is already decomposed in the bladder. On account of the higher acetone concentration which thus appears in the bladder, a diffusion of acetone into the blood may take place. The extent of the decomposition of the aceto-acetic acid under such circumstances is not known. No kinetic measurements of the decomposition of the acid under different circumstances are yet forthcoming. We cannot decide, therefore, how great a diminution is undergone by the amount of aceto-acetic acid secreted by the kidneys as a result of the fact that part of it is already decomposed in the bladder and the acetone arising therefrom diffused through the bladder walls.

I have performed no experiments with regard to the relation of aceto-acetic acid to the walls of the urinary bladder. From the fact that the total acetone concentration of the urine is usually higher than that of the blood we may conclude that diffusion through the walls of the bladder cannot take place to any considerable extent.

#### FORMULA FOR THE ELIMINATION OF ACETONE THROUGH THE KIDNEYS.

Free acetone is eliminated—as soon as more violent variations in the acetone concentration of the blood have ceased to take place—in a concentration which lies very near to that of the acetone concentration in the plasma. This concentration remains unchanged even though the amount of urine excreted during the unit of time varies.

The amount of acetone,  $M_u$ , coming from the kidneys in the unit of time depends therefore upon the following three factors:

The concentration of free acetone in the plasma,  $a$ ,

The partition coefficient urine/plasma,  $\kappa$ ,

The rate of excretion, quantity of urine per unit of time,  $v$ ,

or  $M_u = a \cdot v \cdot \kappa$ .

If free acetone exclusively is present in the blood, the quantity eliminated may be approximately estimated from the above factors. With constant blood acetone concentration the amount of acetone eliminated will be increased proportionally to the increase in the quantity of urine. If this quantity of urine is increased to twice as much, *e.g.* because the subject of the experiment drinks a certain quantity of water, the amount of acetone eliminated will also be doubled. With a constant quantity of urine the amount of acetone eliminated increases proportionally to the increase in the concentration in the blood, provided that this increase does not proceed too quickly.

$\kappa$  for urine/plasma is in all probability very nearly 1. For urine/total blood—according to calculation from the values given by the published experiments—it is 1.235, with  $E = \pm 0.0670$  and  $e_1 = \pm 0.0186$ .

If, as well as the free acetone, aceto-acetic acid simultaneously occurs, the total quantity of acetone eliminated through the urine cannot be calculated in the same simple fashion. The amount of aceto-acetic acid secreted depends not only on the aceto-acetic acid concentration in the blood but also on the capacity of the kidneys, a factor which cannot at present be determined. The formula for the elimination of the total acetone,  $M_t$ , in the unit of time may (for a purpose to be mentioned later) be written:

$$M_t = a \cdot v \cdot \kappa + c,$$

where  $c$  is unknown.

By throwing light on this proposition we shall be able to arrive at some explanation of the relations between the acetone and the aceto-acetic acid in the urine.

As is well known, Folin [1907] and Embden [1907] have found that the pre-formed acetone in the urine generally constitutes a quite inconsiderable proportion of the total acetone of the urine. From Folin's measurements we take the following table in which all the values are reckoned in ‰.

Table VIII. Some of Folin's estimations concerning the relation between acetone and aceto-acetic acid in the urine of diabetics. All the values are expressed in ‰.

No.	Total acetone	Free acetone	Acetone from aceto-acetic acid
I	0.67	0.16	0.51
II	0.68	0.25	0.43
III	2.83	0.36	2.47
IV	0.79	0.24	0.55
V	2.15	0.26	1.89
VI	1.67	0.22	1.45
VII	1.02	0.10	0.92

Embden finds that often only aceto-acetic acid occurs in the urine. In general the amount of acetone in his estimations constituted less than 25 % of the total acetone, and most frequently considerably under 10 %.

Now we know that the free acetone—provided that no very considerable decomposition of the aceto-acetic acid takes place in the bladder—can never reach values lying appreciably higher than the concentration of the free acetone in the blood. It is only in very rare cases that the total acetone in the blood of diabetics is as much as 0.40 ‰; the free acetone therefore has still lower values. Hence it follows that the acetone in the urine can never reach any very high concentration. But the aceto-acetic acid, which is secreted by the kidneys, may on the other hand be present in the urine in a concentration many times greater than that of the blood.

#### SUMMARY.

Acetone passes into the urine by way of diffusion. The concentration in the blood and in the urine is therefore usually the same.

Aceto-acetic acid passes into the urine by way of the concentrating activities of the kidneys. The concentration in the urine is therefore higher than in the blood.

#### REFERENCES.

- Chabanier and Ibarra-Loring (1916). *Compt. Rend. Soc. Biol.*, **79**, 8.  
Cohnheim (1909). Zuntz and Loewy's *Physiologie des Menschen*, p. 588.  
Embden (1907). *Verhandl. Kongr. f. inn. Med.*, **24**, 252.  
Folin (1907). *J. Biol. Chem.*, **3**, 177.  
Novicka (1913). *Contribution à l'étude de la perméabilité et du pouvoir absorbant de la vessie*, Thèse, Paris.  
Overton (1907). "Ueber den Mechanismus der Resorption und der Sekretion," Nagel's *Handbuch d. Physiol.*, **2**, 744.  
Widmark (1915). *Skand. Arch. Physiol.*, **33**, 55.

## XXXII. STUDIES IN THE ACETONE CONCENTRATION IN BLOOD, URINE, AND ALVEOLAR AIR. III: THE ELIMINATION OF ACETONE THROUGH THE LUNGS.

BY ERIK MATTEO PROCHET WIDMARK.

*From the Physiological Institute, Lund, Sweden.*

*(Received February 10th, 1920.)*

In the following an attempt is made at an exact analysis of the passage of acetone into the expiratory air, and in so doing I have tried in the main to follow the method of analysis adopted in modern experiments in regard to the exchange of respiratory gases<sup>1</sup>.

If the passage of acetone into the alveolar air is a pure diffusion process, the partial pressure of the acetone vapour in the latter should never be higher than the partial pressure in the blood. But if, on the contrary, an energetic, secretory activity is contributed by the lungs, the partial pressure of the acetone in the alveolar air may be higher than in the blood. The decision of these points becomes much more simple in the case of acetone than in the case of the respiratory gases. The latter are partly dissolved and partly in chemical combination in the blood, and their concentrations are subjected to great variations in the venous and the arterial blood. The acetone, on the other hand, is exclusively dissolved in the blood, and the concentration remains pretty nearly constant for hours [Widmark, 1919]. Certainly after resorption no demonstrable difference in concentration occurs in the arterial and venous blood.

In the physiology of respiration the absorption coefficient, as defined by Bunsen [1877], has been used in the estimation of the pressure of the gases in blood and air. The custom of thus expressing by the absorption coefficient the partition of a gas between a fluid and a gas above the fluid is associated with the fact that the gas analyses have chiefly been carried out by the eudiometric method.

The absorption coefficient may evidently be regarded as a partition coefficient for the gas in question with reference to a fluid and a gaseous phase, respect being taken to the particular pressure and temperature of the gases. If we estimate in the direct chemical way *the concentration* of a substance which is divided between a gaseous and a fluid medium it is to a certain

<sup>1</sup> No historical review is given in this paper. The reader is referred to my book [1917].

extent a roundabout method to express the division by the absorption coefficient. It is simpler to express it in the same way as when we are dealing with the distribution of a substance between two fluid media. Here the concentration of the substance in the gaseous phase must naturally be calculated upon the volume which the gas has when diffusion equilibrium is attained.

As far as acetone is concerned, therefore, we need not express the concentration in partial pressure; it is simplest to give the quantity by weight of acetone per cc. of air and per cc. of fluid. The coefficient of partition ( $\lambda$ ) therefore takes the following expression:

$$\lambda = \frac{[\text{acetone in air}]}{[\text{acetone in fluid}]}$$

This method of expression is made use of in the following pages. Naturally the values can easily be calculated again in mm. of mercury-pressure. In that case we should have to do with fractions of a mm., although the exposition would not gain in clearness thereby.

The above method of expression can naturally only be used when the partial pressure of the binary fluid complex is directly proportional to the dilution. It is not deducible *a priori*—nor indeed is it probable—that such a simple state of affairs exists, in regard to acetone, throughout the whole dilution from 100–0 %.

For our purpose, however, it is only of interest to know the coefficient of partition between air and fluid within the domain represented by the acetone concentration in the fluid from 0–1 ‰. This is consequently only a thousandth part of the whole curve of partition, and in regard to this part we may assume that it will be approximately straight, *i.e.* that a constant relationship will exist between the acetone concentration in fluid and in air. This is also shown by the experiments.

The partial pressure in the case of the system water-acetone has previously been studied by Carveth [1899]. He has determined the composition of the vapour from this binary fluid and has calculated the partial pressure of the acetone. His measurements however cannot be used for the particular relationships in question. It is therefore necessary to elucidate these experimentally.

Our first object then will be to determine the partition of acetone between air and fluid for solutions under 1 ‰. Further, in order that it may be possible to apply the measurements to physiological conditions, the coefficient of partition should be determined for a temperature of 38°.

The determination of the coefficient of partition has been carried out in two different ways, first by determining the quantity of acetone which an acetone solution loses when shaken with a certain volume of air at 38° (method *A*), and secondly by an analysis of air shaken at the same temperature with acetone solution of known strength (method *B*).

After determining  $\lambda$  for water, blood, and serum I have proceeded to examine whether the acetone concentration in alveolar air shows a value that is in agreement with this coefficient.

A. DETERMINATION OF  $\lambda$  BY ESTIMATIONS OF THE CONCENTRATION OF THE ACETONE SOLUTION BEFORE AND AFTER SHAKING WITH A DEFINITE QUANTITY OF AIR.

For these determinations a litre flask was used, the neck of which was drawn out into a glass tube about 13 cm. long and 5 mm. in diameter. Over the tube was passed a glass cup which was fixed to the lower end of the tube by a piece of rubber tubing. The cup was filled with mercury up to a certain mark. An inverted test-tube was fitted over the glass tube, its opening reaching down into the mercury. The test-tube was supported by the bottom of the cup and was further fixed by means of a perforated rubber stopper passing over the test-tube and fitting into the orifice of the cup, so that this became perfectly air-tight. The space thus enclosed was accurately measured with water and found to contain 1230 cc.

The flask was shaken in a machine driven by a motor. It was enclosed in a case which could be kept at a constant temperature of 38°, at which the shaking was performed. The temperature was regulated by a spirally twisted bimetallic spring which changed its curvature with variations in temperature, and according to the movements of this an electric current was shut off or opened, this current in its turn affecting an electro-magnetic key which regulated the flow of gas to a burner placed inside the case. The temperature was regulated to about 0.1°. The apparatus was constructed at the Lund Physiological Institute.

The experiments were carried out as follows. In the flask was placed an accurately measured quantity of acetone solution, usually 12 cc., in some cases 3 cc. The flask was then closed, placed in the shaking-machine, and shaken for one hour at 38°. A test experiment showed that this period was long enough to admit of the establishment of a balance between the acetone concentration in the air and in the fluid. After the shaking the flask was taken out of the machine, the mercury in the cup poured off, and the flask turned upside down so that the acetone solution might run down quickly into the test-tube. Samples for analysis were then taken from the latter. In experiments with pure aqueous solutions the analysis was performed according to Messinger; in experiments with blood and serum the estimation was carried out after distillation in the usual way, either by the macro- or the micro-method.

Full details are here given of one experiment. The other estimations were carried out in exactly the same way.

*Experiment 1.* February 27, 1917. In the flask was placed 12 cc. of 0.809 % acetone solution. After one hour's shaking at 38° the solution was

found to be 0.648 ‰. By heating from 20–37° the volume of the solution was diminished through evaporation of water from 12 cc. to 11.965 cc. (calculated according to *Chemiker-Kalender*, II, 1917, Table 88).

Amount of acetone introduced	12 × 809 = 9708 γ
Found in the solution after shaking	11.965 × 648 = 7753 γ
Amount of acetone evaporated	1955 γ

The volume of the flask was 1230 cc. After the introduction of 12 cc. of fluid, therefore, the volume of air over the fluid became 1230 – 12 = 1218 cc.

The quantity of acetone per cc. of air is therefore  $\frac{1955}{1218} = 1.605 \gamma$ .

From this we calculate

$$\lambda = \frac{1.605}{648} = 0.00248.$$

In addition to this estimation two further estimations were carried out on pure acetone solutions. These were performed exactly as has just been described. In the table given below all the estimations will be found set out together.

Table I. Summary of estimations of the partition of acetone between air and water. The second column contains the results of the analysis of the acetone solution after the shaking, the third column gives the calculated quantity of acetone per cc. of air, and the fourth the values of  $\lambda$ .

	Acetone found in solution after shaking γ/cc.	Acetone in air (by difference) γ/cc.	λ
1	648	1.61	0.00248
2	646	1.62	0.00251
3	342	0.82	0.00240
			Average: 0.00246

The coefficient of partition for acetone between water and air at 38° is therefore, according to these experiments, 0.00246.

The coefficient of partition *between serum and air* ought presumably to be somewhat higher than for pure aqueous solutions, on account of the saltiness of the serum. An estimation however showed no certain difference.

*Experiment 2.* For this estimation a quantity of defibrinated calves' blood was centrifuged, the serum pipetted off, and a small quantity of pure acetone added. Into the flask was introduced 3 cc. of serum, which was shaken for one hour at 38°. The acetone concentration was determined in 0.2 cc. by the micro-method, and was

Before shaking ‰	After shaking ‰
0.392	0.199
0.387	0.184
0.378	0.187
Average: 0.386	0.190

This gives  $\lambda = 0.00255$ .

In the determination of the coefficient of partition between *air and total blood* higher values were obtained for  $\lambda$ .

*Experiment 3.* 15 cc. of defibrinated cow's blood was shaken for one hour at 38°. The determination of the acetone concentrations was carried out by the macro-method. The following values were obtained:

Acetone concentration before shaking, 0.826 and 0.823, average 0.825 ‰.

Acetone concentration after shaking, 0.661 ‰.

Hence we get for the acetone concentration in the air, 2.04  $\gamma$ /cc., and for  $\lambda$  0.00309. Another measurement gave 0.00329.

The measurement of the coefficient of partition between total blood and air therefore gives a value considerably higher than those previously found. This is easily understood if we assume that the acetone concentration in the blood corpuscles is lower than in serum—an assumption the probability of which is strengthened by other experiments. The total blood should, theoretically speaking, yield the same amount of acetone per cc. of air as the plasma obtained after centrifugalisation. But on the other hand the partition coefficient comes to be somewhat higher for the total blood, since the determination of the acetone concentration in the total blood gives a lower value than in serum.

#### B. DETERMINATION OF $\lambda$ BY AN ANALYSIS OF AIR SHAKEN WITH ACETONE SOLUTION.

From the experiments just described it follows that the proportion between the amount of acetone per cc. of air and per cc. of solution is about 3:1000. In 100 cc. of air we ought therefore to find about three times as much acetone as in 0.1 cc. of solution. Consequently there should be quite sufficient quantities of acetone in the air for an estimation by the micro-method to be possible, and the error can therefore be actually three times as great as in the blood estimations without making the error per cent. any greater than in these.

The analysis of air shaken with acetone solution was carried out as follows. In a tube of about 120 cc. capacity, provided with glass taps at both ends, were placed about 50 cc. of acetone solution of known concentration. The tube was then shaken in the shaking-machine for 1½ hours at 38°. A test experiment showed that after one hour's shaking equilibrium had already been set up between the acetone concentration in the air and in the fluid. A couple of times during the shaking process one of the taps of the tube was opened in order to equalise the pressure, which had been increased by the heating from room temperature to 38°. After the shaking the tube was taken out and placed in a small chamber heated by a gas flame to 38°, the temperature of which was kept constant by means of a regulating arrangement constructed like that of the shaking-machine. The air in the tube was here passed over into a Hempel gas burette, provided with correction-tube and manometer. Acetone solution of the same strength as that used in the

shaking served as fluid in the pipette. When the amount of air had been measured at the temperature and pressure which prevailed during the shaking, the air was passed over into three test-tubes, arranged like a series of wash-bottles. The glass tubes, which reached down to the bottom of the test-tubes, were drawn out into fine capillary points. Each of the test-tubes contained 3 cc. of  $N/2$  sodium hydroxide + 2 cc. of  $N/200$  iodine solution (or 2 cc. of  $N/100$  iodine solution in the estimation of greater quantities of acetone). The air was passed very slowly through the solutions in the three test-tubes bubble by bubble.

The titre proved to be diminished when 100 cc. of air from the laboratory had been passed through. In the first tube this diminution amounted to about 0.04 cc. of  $N/200$  iodine solution; in the two others it was somewhat less, but still quite perceptible. The change of titre was determined before every series of experiments, and the results of titration were corrected by the values thus obtained from the blind tests.

The acetone was absorbed almost completely by the iodine solution of the first test-tube. Into the second 5–10 % usually passed over, in the third the formation of iodoform could not as a rule be demonstrated with certainty.

The acetone solution used—50 cc.—had in proportion to the volume of air so great a volume that no change in the concentration of the solution after shaking could be observed by titrating. In the calculation I have therefore used the original concentration of the acetone solution.

As an example of the method the following report is adduced.

*Experiment 4.* January 26, 1917. 50 cc. of 0.164 ‰ acetone were introduced into the shaking tube and shaken for  $1\frac{1}{2}$  hours at 38°. The volume of the air analysed was 57.2 cc. and it contained 24.5  $\gamma$  acetone, *i.e.* per cc.

$$\frac{24.5}{57.2} = 0.43 \gamma \text{ acetone.}$$

Hence

$$\lambda = \frac{0.43}{164} = 0.00262.$$

In this way 27 estimations were carried out, in which the strength of the acetone solution varied from 0.164 to 0.822 ‰. Details of these estimations are given in the following table.

Table II. Determinations of the partition coefficient of acetone between air and water at 38°.

Acetone per cc. solution $\gamma$	Acetone per cc. air						Average acetone per cc. air $\gamma$	$\lambda$
	0.43	0.46	0.44	0.40	0.43	0.39		
164	0.43	0.46	0.44	0.40	0.43	0.39	0.425	0.0026
328	0.94	1.01	0.93	0.99	0.80	0.68	0.891	0.0027
369	0.98	1.11	0.92	0.87	1.03	0.87	0.963	0.0026
576	1.31	1.49	1.30	1.61	1.34	1.21	1.377	0.0024
822	2.15	1.87	2.14	—	—	—	2.053	0.0025

The weighted mean of these values for  $\lambda$  is  $0.00257 \pm 0.000055$ . The values show no rise or fall in the case of these solutions, therefore a constant relationship may be considered to exist between the concentration in the air

and in the solution. Fig. 1 (p. 387) gives a graphic representation of the position of the different values in regard to the mean. The abscissa represents the concentration in the aqueous solution expressed in  $\gamma/cc.$ , the ordinate the concentration in the air in  $\gamma/cc.$  The values obtained are represented by crosses. The circles represent the values obtained according to method A. The straight line is drawn in accordance with the weighted mean value for  $\lambda$  in method B. The error is somewhat considerable:  $\pm 0.00028$ . This is probably due to the fact that it is very difficult to determine satisfactorily the titre of the blind tests. It has been found also that both rubber tubes and greased glass taps have an extremely injurious effect upon the determinations and cause the values to become irregular; possibly rubber tubes and grease give off small quantities of iodine-binding substances or absorb the acetone. The determination of the mean value is however of sufficient accuracy to admit of the use of this value in the investigation which follows. The values obtained by this process are somewhat higher than those given by the method previously described; the difference however is unimportant.

### C. ESTIMATION OF ACETONE IN THE ALVEOLAR AIR.

If the elimination of acetone through the lungs is a pure diffusion-process we should find—provided that a complete balance of diffusion can be set up—in determinations of the acetone concentration in alveolar air and blood a coefficient of partition which is in agreement with that obtained by the shaking experiments with blood. This will naturally be the case only if free acetone exclusively is found in the blood. If aceto-acetic acid is also present we ought to obtain a coefficient lower than that just mentioned, on account of the fact that in the estimation in the blood a total acetone value is obtained. If, on the other hand, an active secretion of the acetone occurs, we should obtain a coefficient higher than that obtained in the shaking experiments. In the following a simple method for determining the acetone concentration in the alveolar air is described. The results of the determinations obtained by this method show that the coefficient in the case of blood/alveolar air has a value which agrees so closely with those obtained in the shaking experiments that *the elimination of the acetone may be entirely explained as a diffusion-process*. There is not the slightest reason to suppose that any secretion takes place.

The method seems to promise sufficient accuracy to permit of the use of the alveolar air analyses in determining the percentage of free acetone in the blood. Thus in a diabetic, by a combined blood estimation and analysis of alveolar air, we may arrive at an understanding of the relationship between the free acetone and the total acetone in the blood. The method has this great advantage, that the relationship between the acetone and the aceto-acetic acid can in no way be disturbed by the analysis: the separation of the free acetone from the aceto-acetic acid is effected so to speak with the organism itself as distillation apparatus.

*Method.*

A necessary presupposition, if these measurements are to give exact and reliable results, is that the acetone must not come into contact with rubber tubes and greased glass taps. This condition is fulfilled by the following method, which, with all its simplicity, seems to give quite satisfactory results.

The apparatus is composed of the following parts:

(a) A Haldane tube, made entirely of glass, length about 1.3 metres, diameter 23 mm. The mouth of the tube is produced by squeezing together one end of it, so that the tube may conveniently be stopped up by the tip of the tongue. 1 dm. inside the mouth of the tube another tube 5 cm. long is melted on at right angles to it. The diameter of this tube is 7 mm.

(b) A well calibrated collecting pipette for the air, about 100 cc. in capacity. This can be made out of an ordinary 100 cc. pipette by cutting off the lower end 4 cm. under the bulb and drawing it out into a  $5\frac{1}{2}$  cm. long capillary tube. On the tube is placed a small cup intended to serve as a mercury seal in the manner shown by Fig. 2. The other end of the pipette is attached to a rubber tube and mercury receiver. Into the rubber tube a glass tap is fitted. The rubber tube can further be compressed with a clamp.

(c) A test-tube prepared by fusing together the upper part of a wider and the lower part of a narrower test-tube. The upper part has a length of  $4\frac{1}{2}$  cm. and a diameter of 18 mm., the lower part a length of 10 cm. and a diameter of about 6 mm. (cp. Fig. 3).

(d) A doubly bent glass tube, one end of which is somewhat widened and the other drawn out into a 10 cm. long capillary tube of even thickness. This tube can be fixed in the test-tube by means of a perforated cork so that the point of the capillary reaches to within a couple of mm. of the bottom of the test-tube. The cork has also another perforation into which a short rectangularly bent tube is fitted (Fig. 3).

(e) A small V-shaped capillary tube.

When the sample of alveolar air is to be taken, the pipette is placed in such a position in regard to the Haldane tube (horizontally supported by two stands) that its capillary reaches up through the T-tube to about 1 cm. above the inner orifice of the latter (see Fig. 2). The lower orifice of the T-tube will then be closed by the mercury seal of the pipette. In this position the pipette is now filled with mercury by raising the receiver up to a certain position ascertained by experiment beforehand.

The subject of the experiment is now allowed to take the mouth of the tube between his lips. After one inspiration the nose is closed and he must now hold his breath for 20 seconds, after which he should breathe out as much air as possible into the tube. Immediately after this expiration he closes the opening of the tube with the tip of his tongue. He must now in this position breathe through the nose while the mercury is made to run out quickly from

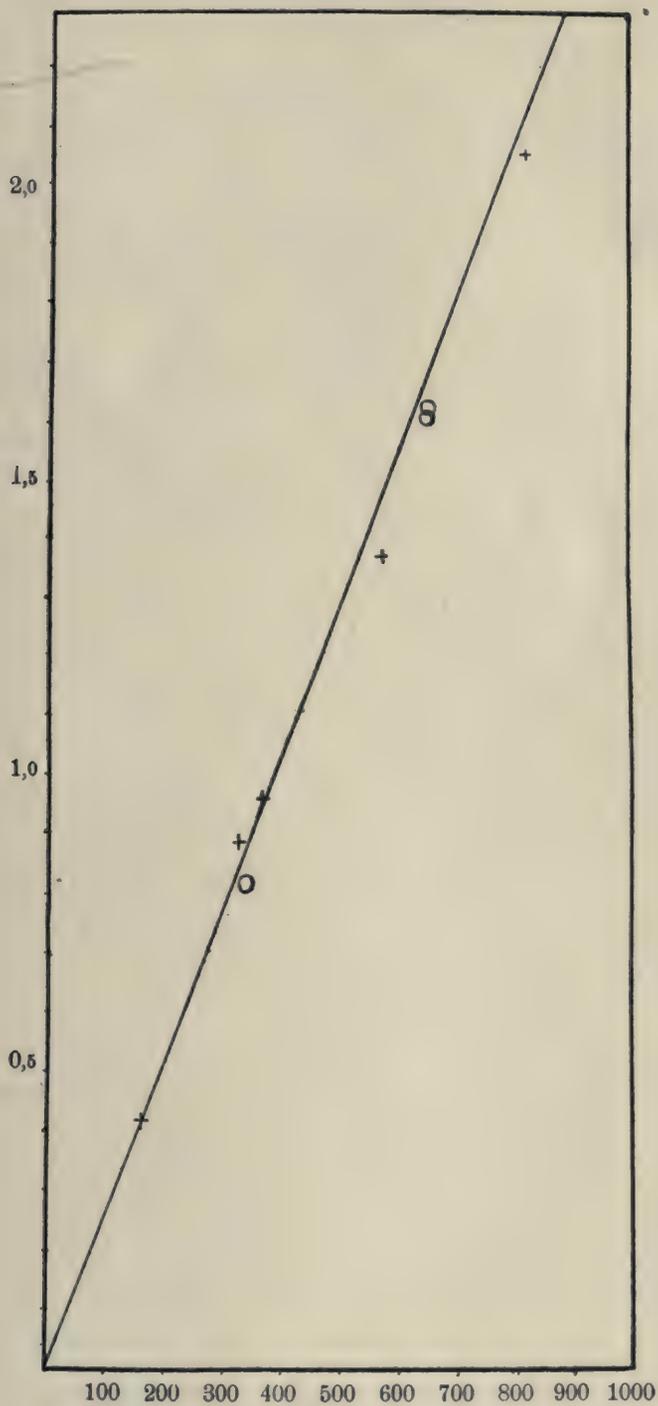


Fig. 1.

the pipette by lowering the vessel. The mercury must sink down to a certain mark on the lower tube of the pipette.

When this is done the glass tap is closed and the pipette promptly lowered so that the bent tube (*d*) can be pushed over the capillary tube of the pipette and closed by the mercury. The test-tube, containing 2 cc. of *N*/200 iodine solution and 3 cc. of *N*/1 NaOH, can be placed in position beforehand. During the whole operation the pipette should not be touched with the hands but should be held by a stand or clamps in the lowering and raising processes.

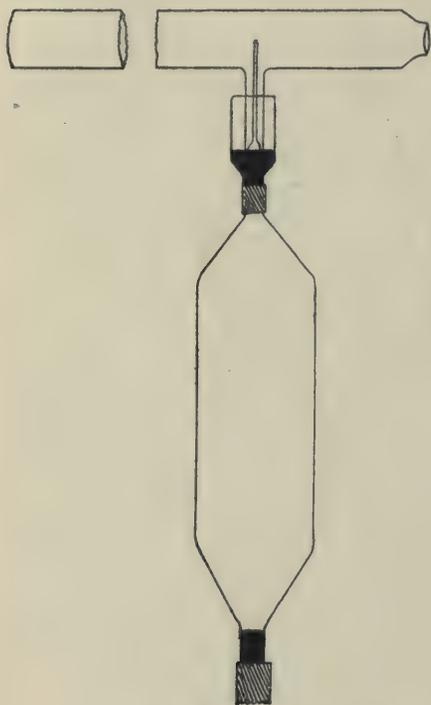


Fig. 2.

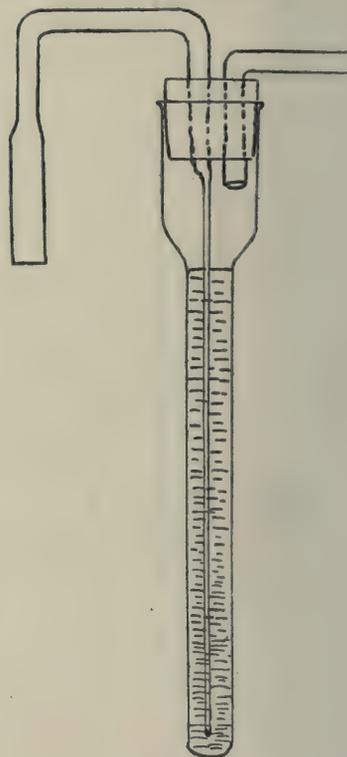


Fig. 3.

The mercury vessel is now raised, the clamp screwed on, and the glass tap opened, after which the speed of flow of the mercury is regulated by a screw-clamp so that the air is driven bubble by bubble through the alkaline iodine solution. The passage of 100 cc. ought to take about an hour. After all the air has been driven out of the pipette it is necessary to pass through the iodine solution the volume of air—amounting to about 1.5 cc.—present in the connecting tube. For this purpose the small bent glass tube in the cork of the test-tube is attached to a short piece of rubber tubing provided with a mouth-piece consisting of a capillary tube 4 cm. long. By gently sucking through this the mercury is first made to rise half a centimetre from the seal up into the connecting tube. Then the small V-shaped capillary tube is dipped into the mercury and manipulated so that one end of it emerges above the

surface into the connecting tube. A few more cc. of air are now sucked through the iodine solution, and in this way the acetone gas present in the connecting tube is washed out.

The absorption of the acetone in the alkaline iodine solution becomes in this fashion practically complete; I have not been able to find, by coupling several test-tubes together, that any acetone passes the first test-tube. The conditions for the absorption are more favourable with this apparatus than with that previously described, partly because the narrower test-tube makes the layer of fluid through which the air bubbles have to pass deeper, and partly because the stronger hydroxide increases the speed of reaction in the iodoform formation.

It was found, however, that the passage of 100 cc. of room air is sufficient to change the titre of the iodine. This change is very constant and amounts to a diminution of 0.05 cc. of  $N/200$  iodine. It should always be determined by means of a blind test. It is further of great importance to provide carefully that both test-tube and connecting tube shall be extremely well cleaned and rinsed out with freshly distilled water immediately before the beginning of the experiment.

Before the titration, which in other respects proceeds in the manner previously described, the fluid is acidified with 3.5 cc. of  $N$ /sulphuric acid. The mixture is at once stirred and titration carried out immediately.

In the case of healthy persons, in whom an estimation by the micro-method reveals no trace of acetone in the blood, we do not obtain by the alveolar air test any greater binding of iodine than we find in the blind tests with room air. The percentage of carbonic acid in the alveolar air therefore plays no part in the estimations, and the normally occurring amount of acetone is too inconsiderable to be demonstrated.

The measurement of the acetone contained in the alveolar air is performed at room temperature. In the calculation of the acetone concentration in the air we must therefore reckon the volume of the air from room temperature to 38°, at which temperature the air is assumed to be saturated with water. This calculation is most easily performed by the aid of the tables in "Landolt and Börnstein." The air is assumed to have stood the whole time under atmospheric pressure; the slight increase of pressure arising in the lungs before the expiration into the Haldane tube may be neglected in the calculation. We add complete details for one case.

*The acetone in the alveolar air after the administration of acetone.*

*Experiment 5.* March 16, 1917. Bar. 768 mm. Room temperature 16°. Volume of the analysing pipette 95.86 cc. Subject: the author. At 10.45 a.m. about 9.5 g. of acetone were taken in 500 cc. of water. Alveolar tests at 2.22 p.m. with 20 seconds' breathing pause. The volume of the air measured, saturated with water at a pressure of 768 mm. and a temperature of 38°, was 108.3 cc.

Total quantity of acetone found: 46.9  $\gamma$ .

Acetone concentration in the alveolar air:  $\frac{46.9}{108.3} = 0.433 \gamma/\text{cc.}$

Acetone concentration in the blood: at 2.20 p.m. 0.127 }  
 " " " " at 2.25 p.m. 0.124 } 0.126 ‰.

$$\lambda = \frac{0.433}{126} = 0.00344.$$

In the same way a number of other determinations of the partition of the acetone between blood and alveolar air in experimental acetonaemia were carried out. These measurements are collected in the following table.

Table III.

Blood acetone $\gamma/\text{cc.}$	Air acetone $\gamma/\text{cc.}$	$\lambda$
222	0.59	0.00266
220	0.72	0.00327
210	0.63	0.00300
208	0.62	0.00298
199	0.59	0.00296
143	0.45	0.00315
126	0.43	0.00341
94	0.28	0.00298
63	0.16	0.00254
60	0.18	0.00300
Average:		0.002995
$E = \pm$		0.000257
$e = \pm$		0.000081

All the determinations were carried out with the author as subject. Most of the values for the blood acetone concentration were obtained by twofold estimations. The error in these blood estimations may be estimated at about  $\pm 15 \gamma$  per cc. of blood. The mean error for  $\gamma$  in the separate estimation is 0.000257. The average for  $\lambda$  is 0.002995 with  $e = \pm 0.000081$ . The acetone percentage of the alveolar air therefore agrees very well with the concentration obtained by shaking air with blood containing acetone. In the experiments referred to (p. 383) the two values 0.00309 and 0.00329 were obtained.

The error in the estimation of  $\lambda$  arises partly from the error in the determination of the concentration in the blood and partly from the error in the determination of the concentration in the air. If the mean error in the blood estimation is assumed to be  $\pm 15 \gamma$ , the error in the blood analysis alone will amount to about  $\pm 0.00015$ . The air analysis cannot therefore give any appreciable error.

Hence it follows that the estimation of the free acetone in the blood can be performed with about the same accuracy by an analysis of the alveolar air as the total acetone estimation in a blood sample by means of the micro-method.

It is probable that the establishment of diffusion equilibrium between blood and alveolar air takes place almost instantaneously. In an experiment upon myself with 20 seconds' breathing pause I obtained  $\lambda = 0.00296$ , and upon expiration into the Haldane tube in immediate succession to a normal expiration I found the values 0.00284 and 0.00273. With another person, B. J., as subject, the value 0.0034 was obtained after 20 seconds' breathing pause, 0.0032 after 10 seconds' and 0.0032 after a normal expiration. Since the mean error of the separate estimation is  $\pm 0.00026$  we cannot therefore demonstrate that the diffusion equilibrium has not been able to establish itself in normal respiration. Probably the difference between the acetone partial pressure of the blood and the acetone partial pressure of the alveolar air is quite inconsiderable. The experiments show at all events that a breathing pause of 20 seconds is quite sufficient to allow diffusion equilibrium to be established. This time is further taken with so wide a margin that it must be presumed that even in persons whose lungs may offer less favourable conditions for diffusion than the normal, equilibrium must be established. For further information upon these points we refer to Marie Krogh [1914].

If the acetone within the organism is found in diffusion equilibrium in the different fluids of the tissues, *the acetone in these fluids should have a partial pressure agreeing with that of the blood.* These fluids should therefore give off to the air an amount of acetone per cc. equal to that found in the alveolar air. This may be shown indirectly by the following experiment:

*Experiment 6.* March 16, 1917. Subject: the author. After taking a certain quantity of acetone the alveolar air contained at 2.22 p.m. 0.433  $\gamma$ /cc.

Of the amount of urine collected in the bladder from 2.15–2.30 p.m. 3 cc. were shaken in a flask according to method *A*.

Concentration in the urine before shaking was 0.168  $\frac{\circ}{\infty}$  (three estimations).

Concentration in the urine after shaking was 0.085  $\frac{\circ}{\infty}$  (three estimations).

From these values  $\lambda$  for the urine is found to be 0.00241. The 0.168  $\frac{\circ}{\infty}$  urine can therefore give off:  $168 \times 0.00241 = 0.405 \gamma$  per cc. of air, which is only 7% lower than the value found in the alveolar air. The small difference may possibly be due to the circumstance that method *A* regularly gives somewhat lower values than method *B*. The urine sample was obtained during strong diuresis (7.33), and if the value 0.00262—which was the mean value of the determinations with aqueous solutions according to method *B*—be used for  $\lambda$ , we obtain the value 0.437 for the acetone concentration in the air.

#### THE ACETONE IN THE ALVEOLAR AIR OF DIABETICS.

These air analyses, as has already been pointed out, render possible *the determination of the concentration of the free acetone in the blood of diabetics.*

This determination is carried out precisely in the manner previously described for the alveolar air analyses. The percentage of free acetone in the

blood is calculated by dividing the air acetone concentration by the value for  $\lambda$ , *i.e.* approximately 0.003<sup>1</sup>.

The estimation of the percentage of aceto-acetic acid in the blood can be carried out in the usual way by a simultaneous blood estimation; the value of the free acetone is subtracted from the total acetone value.

In the following I give a couple of examples of these estimations of the percentage of free acetone and aceto-acetic acid in the blood of diabetics<sup>2</sup>. The estimations are only intended to demonstrate the possibility of the process here described. A systematic investigation of the relationship between the acetone and the aceto-acetic acid in the blood is reserved for a later work.

*Experiment 7.* March 14, 1917. Subject: J. N., diabetic with moderate acidosis. Breathing pause 20 seconds. Three estimations were made:

- (1) At 1.30 p.m. the alveolar air contained 0.108  $\gamma$ /cc.
- (2) „ 2.15 „ „ „ „ 0.117 „
- (3) „ 3.17 „ „ „ „ 0.135 „

From these values the free acetone in the blood was calculated, using the factor 0.003:

- (1) 0.032 ‰.
- (2) 0.035 ‰.
- (3) 0.041 ‰.

In the same case estimations of the total acetone concentration in the blood were carried out.

2.25 p.m.	0.102 0.109	}	0.106 ‰.
3.20 p.m.	0.121 0.126	}	0.124 ‰.

The complete analysis of the relationship between the acetone and the aceto-acetic acid in the blood is given in the following table:

Table IV. Relationship between acetone and aceto-acetic acid in J. N. according to Exp. 6.

Time	Total acetone in blood ‰	Acetone in air $\gamma$ /cc.	Free acetone in blood ‰	Combined acetone in blood ‰	Free acetone in per cent. of total acetone
1.30 p.m.	—	0.108	0.032	—	—
2.15 p.m.	0.106	0.117	0.035	0.071	33
3.17 p.m.	0.124	0.135	0.041	0.083	33

<sup>1</sup> A certain irregularity in the values may of course arise if the proportion between the volume of the blood corpuscles and that of the plasma departs excessively from the normal. This is however not usually the case with diabetics.

<sup>2</sup> In both examples the total acetone concentration in the blood is rather low, so that the estimations lie very near the limits of error of the method. Unfortunately while these experiments were being carried out I had not access to any case with a higher degree of acidosis.

*Experiment 8.* March 21, 1917. Subject: J. N., diabetic with moderate acidosis. Breathing pause 20 seconds. Two estimations were carried out:

Time: 2.40 p.m. 0.119  $\gamma$ /cc.

3.35 p.m. 0.119  $\gamma$ /cc.

The blood estimations gave the following results:

Time: 3.00  $\left. \begin{array}{l} 0.073 \\ 0.068 \end{array} \right\} 0.071 \text{ } \text{‰}$ .

3.40  $\left. \begin{array}{l} 0.077 \\ 0.080 \end{array} \right\} 0.079 \text{ } \text{‰}$ .

All the estimations are shown in Table V.

Table V. Relationship between acetone and aceto-acetic acid in J. N. according to Exp. 8.

Time	Total acetone in blood ‰	Acetone in air $\gamma$ /cc.	Free acetone in blood ‰	Combined acetone in blood ‰	Free acetone in per cent. of total acetone
3.00 p.m.	0.071	0.119	0.036	0.035	51
3.40 p.m.	0.079	0.119	0.036	0.043	46

These estimations give values which may be quite well compared with those obtained by Marriott [1913]. In the four estimations published by him the pre-formed acetone constituted 63, 30, 61 and 28 % of the total acetone.

#### FORMULA FOR THE ELIMINATION OF ACETONE THROUGH THE LUNGS.

A simple expression similar to that derived in Part II for the elimination of the acetone through the kidneys may also be obtained for its elimination through the lungs.

Assuming that the acetone in the alveolar air is in diffusion equilibrium with the acetone in the blood, the quantity of acetone,  $M$ , eliminated through the lungs per unit of time is evidently directly proportional to the concentration of the free acetone in the plasma and to the alveolar ventilation, *i.e.* the alveolar air exchanged during the unit of time:

$$M_t = a \cdot V \cdot \lambda,$$

where  $a$  = the concentration of the free acetone in the plasma;  
 $V$  = the alveolar ventilation;  
 $\lambda$  = the coefficient of partition between air and plasma.

An increase in the ventilation, *e.g.* in coma, consequently increases the amount of acetone eliminated through the lungs. Naturally this law only applies so long as the ventilation does not exceed a degree which would make it impossible for diffusion equilibrium to be set up. In the latter case the amount eliminated through the lungs is estimated not only from the factors here mentioned but also from the lung diffusion constant for acetone, and from other factors which we shall not go into here (see Marie Krogh [1914]).

The formula given above naturally applies to the free acetone only. The aceto-acetic acid does not take part in the elimination through the lungs, since its salts are not volatile. Nor is there any reason to suppose that a greater decomposition of the aceto-acetic acid takes place in the lungs than in other organs of the body<sup>1</sup>.

As far as the mutual relationship between the amount of acetone given off through the lungs and through the kidneys is concerned, it is obvious that this can be estimated from the factors here mentioned.

If in the blood free acetone alone is found, the relationship will be as follows:

$$\frac{M_l}{M_n} = \frac{aV\lambda}{av\kappa} = \frac{V\lambda}{v\kappa}$$

where  $\lambda$  and  $\kappa$  may be regarded as constants. Besides these we therefore determine the relationship between two factors that are independent of one another: the ventilation and the quantity of urine. With increased ventilation the ratio is altered to the advantage of the elimination through the lungs; with an increased quantity of urine it is changed in the opposite direction. The quotient is always greater than 1.

If aceto-acetic acid occurs in the blood we obtain the following expression:

$$\frac{M_l}{M_t} = \frac{a \cdot V \cdot \lambda}{a \cdot v \cdot \kappa + c}$$

where  $a$  still represents the concentration of the free acetone in the blood.

Since the aceto-acetic acid ( $c$ ) is given off exclusively through the kidneys, the quotient will of course become smaller the more aceto-acetic acid there is in the blood. This explains why the quotient in diabetes is less than in the experimental acetonæmia induced by the taking of acetone. In severe cases of diabetes it is small for the same reason.

#### SUMMARY.

By determining the acetone concentration in the alveolar air it is established:

- (1) that the elimination through the lungs is a pure diffusion process;
- (2) that from a simple determination of the acetone concentration in the alveolar air it is possible to calculate the free acetone concentration in the blood.

#### REFERENCES.

- Bunsen (1877). *Gasometrische Methoden*, 2nd ed., Braunschweig.  
 Carveth (1899). *J. Physical Chem.*, **3**, 193.  
 Krogh, M. (1914). *Luftdiffusionen gennem Menneskets Lunger*, Diss., Copenhagen.  
 Marriott (1913). *J. Biol. Chem.*, **18**, 507.  
 Widmark (1917). *Acetonkoncentrationen i blod, urin och alveolärluft samt några därmed sammanhängande problem*, Gleerupska bokhandeln, Sweden.  
 — (1919). *Acta Medica Scandinavica*, **52**, 87.

<sup>1</sup> For further details see Widmark [1919].

# XXXIII. THE METABOLISM OF CARBOHYDRATES. PART I.

## STEREOCHEMICAL CHANGES UNDERGONE BY EQUILIBRATED SOLUTIONS OF REDUCING SUGARS IN THE ALIMENTARY CANAL AND IN THE PERITONEAL CAVITY.

BY JAMES ARTHUR HEWITT AND JOHN PRYDE.

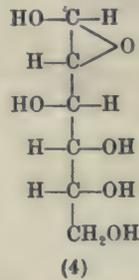
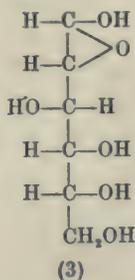
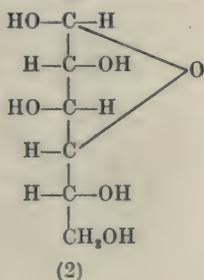
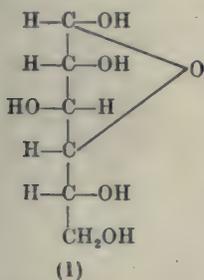
*From the Physiology Laboratory, The University, St Andrews.*

(Received March 20th, 1920.)

THE following communication describes a number of abnormal and unexpected results encountered in an experimental study of the rates of absorption of simple carbohydrates by living tissues. As originally formulated, the scheme of research included the determination of the relative rates of absorption of simple reducing sugars such as glucose, and also of closely related derivatives such as the synthetic alkyl glucosides. The opportunity was open to use in these experiments a number of carbohydrates of exceptional purity, and it was also hoped that the influence of configuration on the rate of absorption would be determined by the employment of definite  $\alpha$ - and  $\beta$ -derivatives of glucose and by the substitution of galactose and mannose for glucose in such derivatives.

At the same time it was recognised that all problems connected with the reactions of sugars are much affected by the recent recognition of the specially reactive type of which the so-called " $\gamma$ -glucose" is a representative, and as the study of " $\gamma$ " sugars is comparatively recent, it may be advisable to review briefly the essential facts regarding their structure.

The expressions  $\alpha$  and  $\beta$  are normally applied to the crystalline varieties of glucose which display downward and upward mutarotation respectively, and the following structural formulae (1) and (2) which represent the compounds as butylene oxides are applied to them:



Formulae corresponding with (1) and (2) represent the isomeric crystalline methyl glucosides, but in 1914 Fischer [1914] described a third methyl glucoside which Irvine, Fyffe and Hogg [1915], who also encountered the compound simultaneously, were able to characterise as a mixture of two methyl glucosides wherein the ring is present as in an ethylene oxide.

Following Fischer's nomenclature the parent sugar was thus provisionally described as " $\gamma$ -glucose" and evidence is available to show that this variety of the sugar also exists in two isomeric forms (3) and (4) which are presumably mutually related as the  $\alpha$ - and  $\beta$ -glucoses.

This capacity of reducing sugars to form numerous isomerides according to the position assumed by the internal oxygen linkage is far from uncommon and what is said above seems to be of general application.

The most outstanding feature of this newly-described type of sugar is the speed and range of the reactions it undergoes. For example condensation with an alcohol to form the corresponding glucoside takes place in a few minutes and the similar reaction with acetone is practically instantaneous. By comparison, the normal forms of reducing sugars are relatively stable, inert substances and the possibility thus suggests itself that in the rapid transformations of carbohydrate in living tissues  $\gamma$ -sugars may play an important, if not an essential, part in both animal and vegetable metabolic processes.

In commencing the present work this possibility was not overlooked and consequently in the experiments afterwards described the amount of sugar introduced into and returned from the intestine, peritoneal cavity, etc. was estimated not only volumetrically but also polarimetrically. In this way it was possible both to determine the amount of sugar present in any solution and also to ascertain the stereochemical condition of the sugar.

It may at once be said that the original scheme of the research was realised in so far that accurate comparative measurements have been made of the rates of absorption of glucose and fructose, but this section of the work has been suspended in view of the unexpected results which indicate, with some precision, that, in contact with certain living tissues ordinary glucose (consisting of the  $\alpha$  and  $\beta$  forms in equilibrium) is converted into the " $\gamma$ " form in amount sufficient to be detected polarimetrically.

Incidentally it may be pointed out that quantitative estimation of glucose by reduction of cupric or other metal salts affords no indication of the presence in solution of  $\gamma$ -glucose. To detect this sugar use must be made either of the polarimeter or of qualitative tests involving the decolorisation of dilute solutions of  $\text{KMnO}_4$  or of methylene blue [Armstrong and Hilditch, 1919]. These two colour tests have certain disadvantages when employed in experiments of the nature carried out in this work, as it is found that a dilute salt solution isotonic with mammalian tissues acquires after contact with these tissues the capacity to decolorise dilute  $\text{KMnO}_4$  solutions, owing presumably to traces of organic matter. The reduction of methylene blue by living tissues is well known.

As experimental animals rabbits mostly have been used; the general results have been confirmed on rats.

The routine methods of investigation employed were:

(a) *Intestine*. The animal being fully anaesthetised<sup>1</sup>, a convenient loop of intestine was isolated, ligatured where desired and two glass cannulae inserted. The loop was then thoroughly washed with Ringer-Tyrode's solution [Bayliss, 1915] made up without glucose, until the washings were clear. To facilitate this process it was found advisable to starve the animal for 24 hours before the experiment. The sugar solution was then run in and retained in the intestine for the length of time desired, usually five minutes. As a rule the abdomen was then temporarily closed by clips or forceps. The blood and nerve supply to the loop were kept intact and all solutions used were at body temperature.

In view of the extreme fragility of the intestine of the rabbit it is essential that it be handled as little as possible, and further that it be not abnormally distended.

When the specified time had elapsed the loop was emptied and washed out with salt solution two or three times; the united solutions were then filtered, made up to known volume with distilled water and examined polarimetrically as soon as possible.

The following parts of the alimentary canal have been investigated: mouth (human), duodenum, small intestine (all parts) and colon.

(b) *Peritoneal cavity*. In this case the sugar solutions were introduced by means of a trochar cannula and were allowed to remain in for varying periods up to 30 minutes. As it is impossible to recover all the solution run in, an amount sufficient only for polarimetric observations was withdrawn and filtered. The sugar solutions were examined as recovered and were not diluted.

Experiments have been carried out with *d*-glucose, *d*-fructose and *d*-galactose. In view however of difficulties experienced with the two latter sugars the large majority of the observations have been made with glucose. In all cases the solutions of the sugars employed were allowed to remain at the temperature of the room until the observed rotation attained a constant value. In each case this corresponded with the permanent specific rotations accepted as standard in the literature. An indication of the state of purity of the materials used may be obtained by referring to the figures given below in the experimental part for the initial rotations of the glucose and fructose in aqueous solution.

Examination of the glucose solutions after contact for five minutes with the mucous membrane of the living intestine showed that disturbance of equilibrium had taken place; initial readings corresponding with a specific

<sup>1</sup> All the animal experiments were carried out by one of us (J. A. H.). The remainder of the work was in greater part done by the other. At the conclusion of each animal experiment the rabbit or rat was destroyed by an overdose of anaesthetic.

rotation much lower than  $+52.5^\circ$  were obtained and the solutions then underwent upward mutarotation to a value corresponding with that of *d*-glucose in equilibrium.

As the original solution contained the  $\alpha$ - and  $\beta$ -glucoses in equilibrium and as the former is highly dextrorotatory and the latter feebly so, the most obvious explanation is that the  $\alpha$  form had been preferentially absorbed thus leaving excess of the  $\beta$ -isomeride in solution.

The range of mutarotation of pure  $\beta$ -glucose is from  $+19^\circ$  to  $+52.5^\circ$ , and the true initial value cannot be observed by direct experiment but is obtained by extrapolation. It follows then that even were all the  $\alpha$  form completely absorbed and the  $\beta$  form unabsorbed, an initial reading corresponding with a specific rotation of  $+19^\circ$  could not be obtained owing to mutarotation commencing immediately the solution was withdrawn from contact with the absorbing tissue. As a certain length of time must necessarily elapse before observations can be made and as solutions on removal from intestine or peritoneal cavity cannot be used for polarimeter work without being filtered, attention was directed to minimising the time taken for this process in order to observe the mutarotation from as early a stage as possible. Even in the first experiments where no particular attention was paid to rapidity of work, extrapolation of the curves in which the specific rotations were plotted against time, indicated that mutarotation commenced from an initial specific rotation less than  $+19^\circ$ . Considerable difficulty was experienced in filtering the solutions rapidly owing presumably to traces of colloidal substances secreted during the experiments.

When the unavoidable delay in observing the optical changes had been reduced as far as possible, readings were obtained on the average seven minutes after removal from the intestine. On one occasion the first reading was taken in four minutes. Under these conditions extrapolation of the curves obtained indicated that mutarotation commenced at a value which was distinctly laevo. (In all cases zero time was taken as the mean of the times of commencing and finishing removal of the sugar solution from contact with the living tissue.) In the great majority of cases also, the first reading obtained corresponded with a specific rotation much too close to  $+19^\circ$  to be due to the presence of  $\beta$ -glucose alone. Not only so, but on one occasion in which glucose was used a laevo initial value was recorded, and on several occasions the initial specific rotations actually obtained lay between  $0^\circ$  and  $+19^\circ$ .

It is clear therefore that the upward mutarotation observed on removal from the intestine cannot solely be due to a preferential absorption of  $\alpha$ -glucose with the subsequent establishment of equilibrium *in vitro*.

One deduction from the experimental results described is that, in contact with certain living tissues, glucose solutions must undergo a downward mutarotation, and if the above explanation be accepted, this change is due in part at least to the transient formation of  $\gamma$ -glucose. This is supported by the additional observation that  $N/100 \text{ KMnO}_4$  in neutral solution is de-

coloured appreciably more rapidly by glucose after contact with the living intestine, than by either glucose of the same concentration in aqueous solution, or by washings from the intestine, and further that the absorption of glucose takes place in the peritoneal cavity without any stereochemical changes being manifest.

A point of considerable importance is that the sugar solutions on withdrawal from the intestine are of necessity alkaline, whilst on the other hand it would appear that faintly acid conditions are most favourable to the formation of  $\gamma$ -sugars. The possibility cannot be excluded however, that this particular type may be formed in feebly alkaline media so long as the alkalinity is lower than that found necessary by Lobry de Bruyn to promote the aldose  $\rightleftharpoons$  ketose transformation.

During the course of each experiment the sugar solutions employed were inevitably contaminated both with intestinal secretions and also with Ringer-Tyrode's solution. It became a matter of importance therefore to determine the effect, if any, of these substances on a glucose solution in equilibrium and on the rate of mutarotation of a freshly prepared solution of glucose. Further as the experiments were carried out at body-temperature ( $37^\circ$ ), and as the rotations were taken at  $11^\circ$ – $15^\circ$ , the effect of this alteration in temperature must be considered.

It has not been found possible to effect an alteration in specific rotation of a glucose solution in equilibrium either by the addition of Ringer-Tyrode's solution, of extracts of intestinal mucous membrane or by Ringer-Tyrode washings from the intestine. While the final equilibrium of a glucose solution is not disturbed by these substances the speed of mutarotation of a freshly prepared solution of *d*-glucose is very appreciably increased. The final permanent value obtained is the same as that of  $\alpha$ -glucose dissolved in distilled water.

Temperature variations of from  $22^\circ$ – $26^\circ$  may, in the case of glucose, be ignored [v. Lippmann, 1904]. Even in the case of fructose, which is notoriously sensitive in this respect, only at  $87.3^\circ$  does the specific rotation reach the value of  $-52.5^\circ$  [Armstrong, 1919], and it is considered that the changes in optical activity obtained with this sugar cannot wholly be due to temperature variations. Pending further work with a constant temperature apparatus, it is not proposed to discuss either fructose or galactose in greater detail.

The fact that the final permanent specific rotation attained after removal from the intestine is the same as that given by the sugar before being introduced precludes also any possibility of these alterations in rotatory power being due to formation of disaccharides or other sugar complexes. As an additional check the concentration of glucose as estimated from the optical value was verified volumetrically by Benedict's method. Close agreements were found in each case.

It should be mentioned that in some of the experiments in which glucose solutions were employed the mutarotation effects were ill-defined and in

some cases were completely negative; it is thus advisable to describe as closely as possible the conditions adopted when positive results were obtained.

In experiments on the absorption of glucose by the small intestine, solutions have been employed hyper-, iso- and hypo-tonic to mammalian tissues. In hypo-tonic solutions only have the changes been as described. In solutions of higher concentration the range of mutarotation *in vitro* has not been so great. In the large intestine also, with dilute solutions of glucose the results obtained were in no way so well-marked as in the case of glucose of similar concentration in the small intestine.

Examination of glucose solutions retained intra-peritoneally for 15–30 minutes showed that the equilibrium of the various isomerides was not disturbed.

After being retained in the mouth (human) for five minutes polarimetric evidence was obtained in the case of glucose that stereoisomeric changes had taken place. Mutarotation was then observed similar to, but much less well-marked than, that noted with dilute solutions of glucose in the small intestine.

#### EXPERIMENTAL.

The general methods adopted in these experiments have been described above.

In connection with the difficulty experienced at times in obtaining solutions sufficiently clear for polarimetric observations it may be said that after many trials of various types of filter papers, shaking with silica and centrifuging, etc. the most satisfactory procedure was found in filtering directly into a graduated flask, under very slight suction, through a "filter-hat" manufactured by Messrs W. and R. Balston, Ltd. These filter-hats are thick and spongy in texture and, provided they are well washed with water before use, yield, in the great majority of cases, solutions clear enough for accurate readings in a 2 dm. tube with sodium light.

In certain very few cases, even after careful filtration, the solution was not sufficiently clear for sodium light to be used and recourse had to be made either to incandescent gas flame or to the electric arc. As a routine proceeding all three sources of light were available by rotating the polarimeter on the table to the light desired. Monochromatic sodium light was always tried first and then in order the incandescent flame and the arc. In the latter case light filters for yellow light were employed [Landolt, 1898].

Polarimetric examination of the glucose and fructose employed in these experiments gave the following initial specific rotations three minutes after commencement of solution of the sugar:

$$d\text{-glucose, } 2.868 \% [\alpha]_D^{12^\circ} = + 103.4^\circ$$

$$d\text{-fructose, } 3.215 \% [\alpha]_D^{12^\circ} = - 107.8^\circ.$$

At the end of  $1\frac{1}{2}$  hours the fructose had not attained equilibrium, and after six hours the glucose gave readings corresponding with a specific rotation

of  $+54.9^\circ$ . This is an important point, as the speed of the optical change varies enormously according to the nature of the glass vessels employed and to other unavoidable factors.

On the graph given below (Fig. 1), are plotted:

- I. The velocity of mutarotation of *d*-glucose in aqueous solution.
- II. " " " " filtered intestinal washings.
- III. " " " " Ringer-Tyrode's solution.

It will be seen that the equilibrium rotation is attained more rapidly in the presence of either of these two solutions than when the glucose is dissolved in water only.

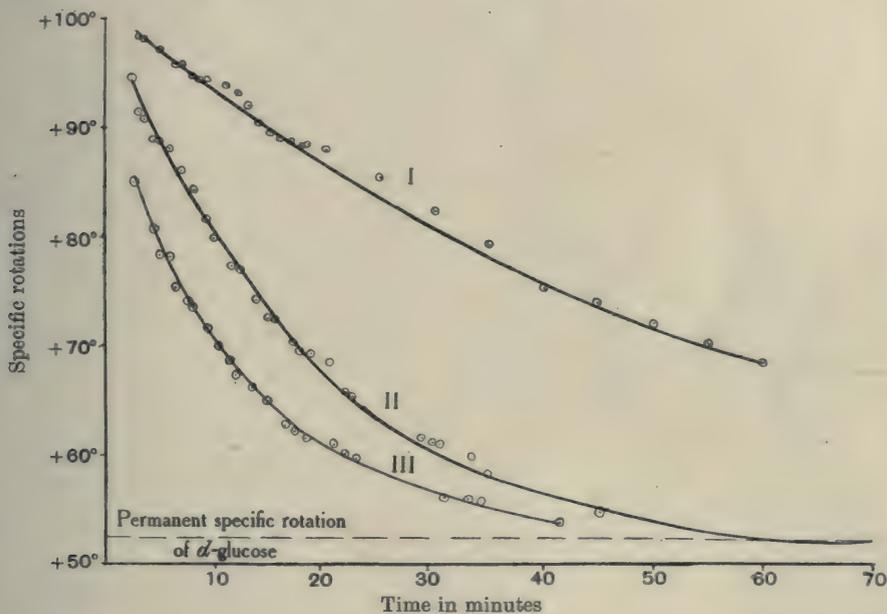


Fig. 1.

- I. Glucose 2.907% in aqueous solution.
- II. " 2.924% in filtered intestinal washings.
- III. " 2.887% in Ringer-Tyrode's solution.

### 1. Small intestine.

(a) *d*-Glucose, hypo-tonic aqueous solutions; 1.571% and 2.120%.

Graphic records of three experiments which show the optical changes most markedly are given below (Figs. 2, 3 and 4). On these are plotted the specific rotations at varying intervals after withdrawal of the solution from the intestine. After the readings had become steady a trace of caustic soda was added and the optical value then observed was taken as the permanent rotation.

As stated above, the final readings before and after the addition of the trace of alkali gave a concentration of sugar which agreed remarkably well with the concentration as determined from volumetric estimations. The

Benedict's solution employed was previously standardised against the sugars used.

(b) *d*-Glucose, iso- and hyper-tonic aqueous solution, 5.258 % and 8.040 %.

Regarding these it can be said that evidences of disturbance of equilibrium are shown, but are slight. In no case has a reading indicating a specific rotation below +40° been obtained under similar conditions to those already described above.

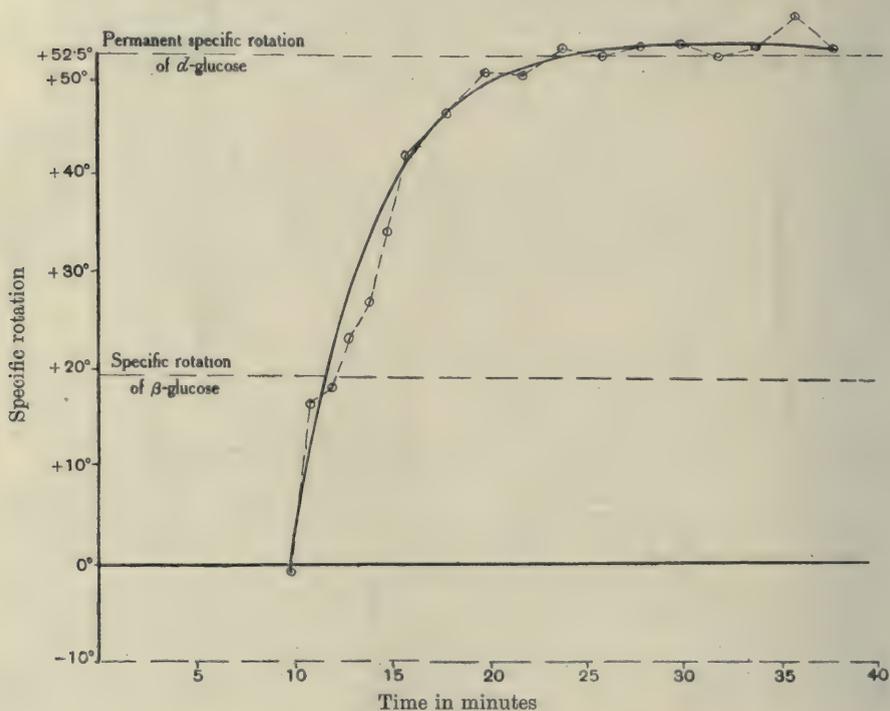


Fig. 2. *d*-Glucose.

In this and in all subsequent graphs (except no. 5) the values plotted represent the average of at least two readings taken in as short a time as possible. Readings were continuous on the part of one author, verified as a rule at intervals by the other. Below each graph are given the rotations from which the specific rotations were calculated. All rotations are dextro unless otherwise stated.

*Observed rotations*

-0.017°, 0.156°, 0.166°, 0.213°, 0.247°, 0.315°, 0.383°, 0.425°, 0.468°, 0.468°, 0.500°, 0.485°, 0.500°, 0.500°, 0.485°, 0.490°, 0.520°, 0.500°.

*After addition of a trace of alkali.* Mean of nine readings, 0.483°.

(c) *d*-Fructose, hypo-tonic aqueous solution, 2.01 %.

One graph is reproduced (Fig. 5). Other experiments have shown, in the initial observations, greater deviation from the equilibrium rotation, but the tracings are complicated by what appears to be the existence of two simultaneous reactions.

(d) *d*-Galactose, hypo-tonic aqueous solution, 2.317 %.

Evidence has been obtained that the specific rotation of this sugar in equilibrium is disturbed through contact with living intestine. The alteration in rotation observed is, however, small.

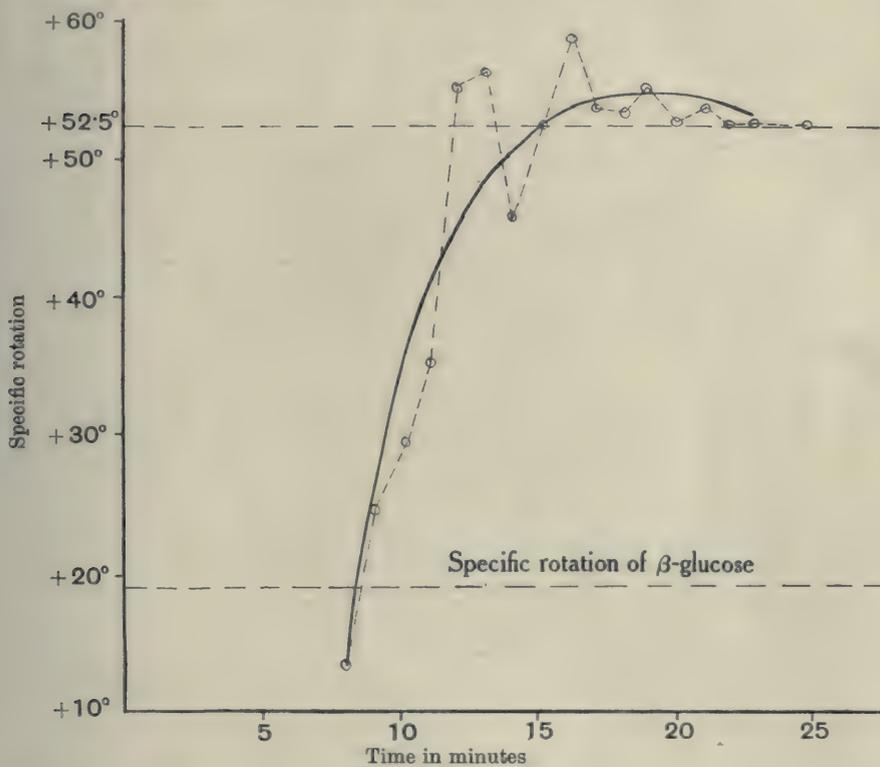


Fig. 3. *d*-Glucose.

Observed rotations

0.083°, 0.150°, 0.250°, 0.217°, 0.342°, 0.350°, 0.283°, 0.325°, 0.367°, 0.333°, 0.333°, 0.342°, 0.325°, 0.333°, 0.325°, 0.325°.

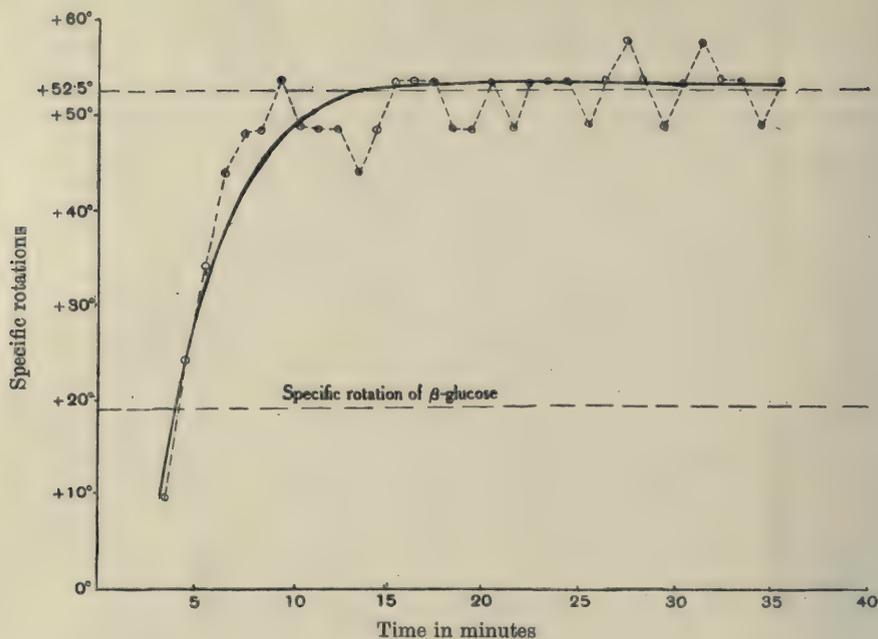
After addition of a trace of alkali. Mean of seven readings, 0.325°.

## 2. Large intestine.

In the rabbit with a 3 % solution of glucose, an initial specific rotation of + 43.03° has been obtained nine minutes after removal from the intestine. After 14 minutes the value + 52.31° was found. In experiments where the normal activity of the intestine was impaired, either by killing the animal previously or by interrupting the blood supply to the observed loop, no alteration of rotation was detected.

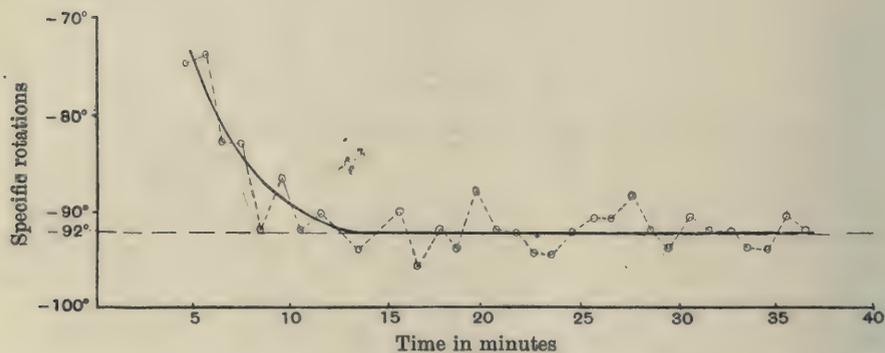
## 3. Mouth (human).

Dilute glucose solutions on being examined after being retained in the mouth for five minutes show upward mutarotation. The range of the mutarotation is small.

Fig. 4. *d*-Glucose.*Observed rotations*

0.033°, 0.083°, 0.117°, 0.150°, 0.167°, 0.167°, 0.183°, 0.167°, 0.167°, 0.167°, 0.150°, 0.167°, 0.183°, 0.183°, 0.183°, 0.167°, 0.167°, 0.183°, 0.167°, 0.183°, 0.183°, 0.183°, 0.167°, 0.183°, 0.200°, 0.183°, 0.167°, 0.183°, 0.200°, 0.183°, 0.183°, 0.167°, 0.183°.

*After addition of a trace of alkali.* Mean of 10 readings,  
0.180°.

Fig. 5. *d*-Fructose.*Observed rotations*

-0.070°, -0.066°, -0.750°, -0.750°, -0.833°, -0.783°, -0.833°, -0.816°, -0.833°, -0.850°, -0.833°, -0.816°, -0.866°, -0.833°, -0.850°, -0.800°, -0.833°, -0.833°, -0.850°, -0.850°, -0.833°, -0.816°, -0.816°, -0.800°, -0.833°, -0.850°, -0.816°, -0.833°, -0.833°, -0.850°, -0.850°, -0.816°, -0.833°.

*After addition of a trace of alkali.* Mean of 14 readings,  
-0.831°.

Considerable difficulty was experienced in filtering the solutions owing to admixture of saliva.

#### 4. *Peritoneal cavity.*

No evidence has been obtained from polarimetric observation that alteration of rotation of glucose in equilibrium takes place in the peritoneal cavity of either the rabbit or the rat.

### SUMMARY AND CONCLUSIONS.

1. Solutions of *d*-glucose in equilibrium when introduced into the intestine of the living animal undergo stereochemical changes.

2. The change in the intestine is a rapid downward mutarotation of the sugar solution to optical values corresponding with specific rotations much below  $+52.5^\circ$ . Several specific rotations below  $+19^\circ$ , and one laevo rotation have been recorded.

3. After withdrawal from the intestine these solutions undergo slower upward mutarotation to a permanent value corresponding with the specific rotation of  $\alpha$ - and  $\beta$ -glucoses in equilibrium.

4. The stereochemical changes in the intestine cannot be attributed to the production in excess of  $\beta$ -glucose through preferential absorption of the  $\alpha$  form, or to the formation of sugar complexes such as disaccharides.

5. Contact with the mucous membrane of the living intestine probably effects the production of  $\gamma$ -glucose in excess of any amount normally present in glucose solutions which have attained a permanent specific rotation.

6. The above stereochemical changes do not occur in sugar solutions which have been introduced into the peritoneal cavity.

The authors thank Professor J. C. Irvine, F.R.S. for his constant advice and criticism on the purely chemical aspects of the work, and Professor P. T. Herring for similar kindness on the physiological side.

The expenses of the work have been defrayed by a grant to one of the authors from the Research Fund of the Carnegie Trust for the Universities of Scotland.

### REFERENCES.

Armstrong (1919). *The simple Carbohydrates and the Glucosides*, Longmans and Co., Ltd.

— and Hilditch (1919). *J. Chem. Soc.*, **115**, 1410.

Bayliss (1915). *Principles of General Physiology*, Longmans and Co., Ltd.

Fischer (1914). *Ber.*, **47**, 1980.

Irvine, Fyfe and Hogg (1915). *J. Chem. Soc.*, **107**, 524.

Landolt (1898). *Das optische Drehungsvermögen.*

v. Lippmann (1904). *Die Chemie der Zuckerarten.*

# XXXIV. THE EXISTENCE IN THE BILE OF AN INHIBITOR FOR HEPATIC ESTERASE, AND ITS NATURE.

BY GEORGE MACFEAT WISHART.

*From the Department of Physiology, University of Glasgow.*

*(Received March 23rd, 1920.)*

It is generally accepted that the addition of bile to pancreatic juice accelerates its lipolytic activity. As is well known, there can be extracted from the liver an enzyme capable of rapidly hydrolysing the lower esters, and the object of the present research was to discover whether a similar effect would be produced on this esterase by the addition of bile.

The research was commenced in 1914 at the suggestion of Prof. Cathcart, and the greater part of the work was done at that time. I was then unaware of Loevenhart's [1906, 1, 2] investigations on the effect of bile-salts on the liver esterase. My work had to be left uncompleted, and it was not until its resumption at the end of the past year that Loevenhart's paper on the above subject came to my notice.

Various methods of preparing the liver extract were tried, the first attempts being directed towards obtaining a dry preparation of liver by extraction with acetone and ether, or alcohol and ether. Such dry preparations were found, however, to possess very slight esterolytic properties (Exp. 1). This is in accordance with the experiences of Loevenhart [1906, 2, p. 433].

## *Exp. No. 1. Cat's Liver.*

Quantities used:		Minutes	Cc. N/10 NaOH
		5	0.7
Ethyl butyrate	0.25 cc.	10	1.1
Extract	0.5 g.	15	1.3
Water	5.0 cc.	20	1.5
Phenolphthalein	4 drops	25	1.6
		30	1.6

The method of obtaining a dry active preparation described by Loevenhart [1906, 2, p. 432], which is a modification of a method cited by Magnus [1904], was not tried. Ramond's [1904] ether extraction method was found to be unsatisfactory.

10 % aqueous extracts were found to be quite satisfactory for the work in view, and were generally used. 5 g. of liver were ground in a mortar with a suitable amount of sand and water, and the extract made up to 50 cc. with water. The whole was then strained through muslin into a stoppered bottle and the bottle well shaken before pipetting off any quantity for use.

Such extracts were found to be highly active, and, despite the fact that, even by thorough shaking it would seem impossible to obtain a homogeneous mixture, successive quantities of equal amount withdrawn from the bottle were found to have practically the same esterolytic activity (Exps. 2 and 3). If a small amount of toluene were added to the extract, the diminution in its activity with age was remarkably slow, as shown in Exp. 4. Throughout the work, however, fresh extracts were used for each experiment.

*Exp. No. 2. Cat's Liver. 10 % Extract.*

Quantities in each tube:	Minutes	Cc. N/10 NaOH	
		First 5 cc.	Second 5 cc.
Ethyl butyrate 0.5 cc.	5	1.4	1.4
Extract 5.0 cc.	10	2.5	2.6
Water 5.0 cc.	15	3.6	3.5
Phenolphthalein 4 drops	20	4.6	4.5
	25	5.3	5.4
	30	6.1	6.0

*Exp. No. 3. Cat's Liver. 10 % Extract.*

Quantities:	Minutes	Cc. N/10 NaOH	
		First 5 cc.	Second 5 cc.
	5	2.3	2.2
	10	4.3	4.2
As in No. 2	15	6.0	5.9
	20	7.6	7.5
	25	8.9	8.7
	30	9.9	9.8

*Exp. No. 4. Cat's Liver. 10 % Extract.*

Quantities:	Minutes	Cc. N/10 NaOH	
		14th Dec.	22nd Dec.
	5	1.3	1.4
	10	2.9	2.6
As in No. 2	15	4.5	3.8
	20	5.6	4.6
	25	6.7	5.2
	30	7.7	5.7

Ethyl butyrate was the ester used in all cases, and, as the butyric acid set free rapidly destroyed the hepatic enzyme, the following method which, to a certain extent, obviated this, and also, it was thought, gave a closer approximation to the *in vivo* conditions, was adopted.

Tubes containing the ester, indicator (phenolphthalein), and water were placed in a water-bath at 38° for five minutes. The extract was then added and the contents of the tubes exactly neutralised with N/10 NaOH. Having been replaced in the water-bath, a burette was fixed above each containing N/10 NaOH, and titrations of the acidity developed were carried out every five minutes during a period of half-an-hour. Under such conditions esterolysis was found to be much more active than when titration of the mixture was performed only at the end of the period of incubation.

## A. INHIBITION OF THE HEPATIC ESTERASE BY BILE.

The following experiments show the effect of adding bile to the enzyme-containing liver extract (Nos. 5 to 13).

A summary of all results with the conclusions drawn therefrom will be found at the end of the paper.

*Exp. No. 5. Dog's Liver. 10 % Extract.*

Quantities in each tube:		Cc. N/10 NaOH				
		Without bile		With bile		
		Lobe A	Lobe B	Lobe A	Lobe B	
Ethyl butyrate	0.25 cc.	Minutes				
Extract	1.0 cc.	5	0.9	0.9	0.7	0.6
Water	4.0 cc.	10	1.4	1.4	0.8	0.65
Phenolphthalein	4 drops	15	1.65	1.6	0.8	0.65
Amount of bile used	0.15 cc.	20	1.7	1.7	0.8	0.65
		25	1.8	1.75	0.85	0.65
		30	1.85	1.8	0.85	0.7

*Exp. No. 6. Cat's Liver. 10 % Extract.*

Quantities:		Cc. N/10 NaOH		
		Minutes	Without bile	With bile
		5	0.3	0.3
		10	0.5	0.35
		15	0.7	0.35
		20	0.85	0.4
		25	0.95	0.45
		30	1.05	0.5

*Exp. No. 7. Same extract as No. 6. Tubes kept at room temperature.*

Quantities:		Cc. N/10 NaOH		
		Minutes	Without bile	With bile
		5	0.2	0.2
		10	0.35	0.3
		15	0.45	0.4
		20	0.55	0.45
		25	0.65	0.55
		30	0.75	0.6

*Exp. No. 8. Rabbit's Liver. 10 % Extract.*

Quantities:		Cc. N/10 NaOH				
		Without bile		With bile		
		Lobe A	Lobe B	Lobe A	Lobe B	
		Minutes				
		5	1.0	0.95	0.6	0.55
		10	1.7	1.7	0.85	0.85
		15	2.3	2.3	1.05	1.05
		20	2.85	2.8	1.2	1.25
		25	3.3	3.25	1.4	1.4
		30	3.65	3.6	1.55	1.5

*Exp. No. 9. Rabbit's Liver. 10 % Extract.*

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.5	0.3
As in No. 5	10	1.0	0.45
	15	1.35	0.65
	20	1.75	0.85
	25	2.1	1.0
	30	2.45	1.15

*Exp. No. 10. Rabbit's Liver. 10 % Extract.*

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.7	0.65
As in No. 5	10	1.3	1.1
	15	1.8	1.5
	20	2.3	1.9
	25	2.7	2.2
	30	3.1	2.5

*Exp. No. 11. Rabbit's Liver. 10 % Extract.*

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.9	0.2
As in No. 5 except that	10	1.75	0.2
0.5 cc. bile used	15	2.3	0.2
	20	2.9	0.2
	25	3.2	0.3
	30	3.6	0.3

*Exp. No. 12. Rabbit's Liver. 10 % Extract.*

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.9	0.2
As in No. 11	10	1.5	0.3
	15	1.95	0.3
	20	2.5	0.4
	25	2.9	0.4
	30	3.3	0.4

*Exp. No. 13. Guinea-pig's Liver. 10 % Extract.*

	Minutes	Cc. N/50 NaOH	
		Without bile	With bile
Quantities:	5	1.8	1.2
As in No. 5	10	3.0	1.9
N/50 NaOH used for titration	15	3.7	2.6
	20	4.2	3.2
	25	4.5	3.5
	30	4.8	3.7

That the dilution due to the addition of the bile has a negligible effect on the reaction is shown in Exp. No. 14.

*Exp. No. 14. Cat's Liver. 10 % Extract.*

Quantities:	Minutes	Cc. N/10 NaOH	
		Without water	With 0.15 cc. water
As in No. 5	5	1.45	1.4
	10	2.55	2.55
	15	3.45	3.4
	20	4.3	4.3
	25	5.05	5.0
	30	5.75	5.65

Control tubes containing ester without extract, and extract without ester, treated in an exactly similar manner, developed in half-an-hour an acidity of comparatively negligible amount.

## B. IS THE INHIBITING SUBSTANCE SPECIFIC FOR EACH SPECIES?

Exps. Nos. 15 to 21 were carried out in an endeavour to find whether the liver extract of one animal would be inhibited by the bile of another animal of a different species, and if the inhibition by the foreign bile would be as marked as that produced by the animal's own bile.

*Exp. No. 15. Livers from Cat, Dog, and Rabbit. 10 % Extracts.*

## A. Cat's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH			
	Without bile	With cat's bile	With dog's bile	With rabbit's bile
5	0.35	0.25	0.25	0.35
10	0.55	0.35	0.35	0.5
15	0.65	0.35	0.35	0.6
20	0.8	0.35	0.4	0.7
25	0.85	0.4	0.5	0.8
30	0.9	0.45	0.5	0.8

## B. Dog's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH			
	Without bile	With dog's bile	With cat's bile	With rabbit's bile
5	0.65	0.4	0.5	0.5
10	1.0	0.45	0.65	0.7
15	1.15	0.45	0.7	0.85
20	1.25	0.5	0.75	0.9
25	1.35	0.5	0.75	0.9
30	1.4	0.5	0.85	1.0

## C. Rabbit's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH			
	Without bile	With rabbit's bile	With dog's bile	With cat's bile
5	1.1	0.65	0.75	0.85
10	2.2	1.05	1.3	1.6
15	3.15	1.4	1.7	2.3
20	3.9	1.65	2.1	2.8
25	4.5	1.85	2.2	3.4
30	5.1	2.0	2.3	3.9

*Exp. No. 16. Cat's Liver Extract.* Quantities:—As in No. 2. Bile 0.15 cc.

Minutes	Cc. N/10 NaOH			
	Without bile	With cat's bile	With rabbit's bile	With dog's bile
5	3.9	1.6	2.0	2.6
10	8.1	3.0	4.4	5.7
15	12.0	4.5	6.9	8.8
20	15.4	5.8	9.7	12.0
25	18.4	7.1	12.1	15.1
30	20.9	8.2	14.4	17.6

*Exp. No. 17. Cat's Liver. 10% Extract.* Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	1.7	1.2	1.2
10	3.2	2.2	2.3
15	4.7	3.1	3.3
20	6.0	3.5	4.0
25	7.0	3.8	4.3
30	7.9	4.1	4.8

*Exp. No. 18. Rabbit's Liver. 20% Extract.* Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With rabbit's bile	With ox bile
5	2.4	1.6	1.6
10	5.5	2.8	3.4
15	8.5	4.0	5.0
20	10.9	5.05	6.4
25	13.5	6.1	7.8
30	15.7	6.9	9.0

*Exp. No. 19. Cat's Liver. 10% Extract.* Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	1.0	1.1	0.75
10	1.9	2.2	1.7
15	3.0	3.2	2.7
20	4.2	4.3	3.7
25	5.3	5.0	4.6
30	5.9	5.3	5.3

*Exp. No. 20. Cat's Liver. 10% Extract.* Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	2.3	1.7	1.9
10	4.3	3.7	3.8
15	6.0	5.4	5.3
20	7.6	6.9	6.3
25	8.9	8.0	7.4
30	9.9	9.0	8.4

*Exp. No. 21. Livers from Dog and Cat. 10 % Extracts.*

## A. Dog's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH		
	Without bile	With dog's bile	With cat's bile
5	0.7	0.4	0.7
10	1.2	0.5	1.1
15	1.5	0.55	1.3
20	1.7	0.6	1.4
25	1.8	0.6	1.5
30	1.95	0.65	1.55

## B. Cat's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With dog's bile
5	0.3	0.25	0.2
10	0.45	0.4	0.2
15	0.6	0.5	0.3
20	0.7	0.6	0.35
25	0.8	0.65	0.35
30	0.85	0.65	0.4

## C. THE NATURE OF THE INHIBITOR.

It has been shown by various workers [von Fürth and Schütz, 1906; Hewlett, 1905; Loevenhart and Souder, 1906; and others] that a solution of bile-salts will produce an acceleration of the hydrolysis of both fats and the lower esters by pancreatic juice, similar to that obtained on adding the whole bile. Further it has been demonstrated by Magnus [1906] that bile-salts synthetically prepared have the same action. The same author showed in a previous paper [1904] how the esterolytic ferment of the liver could be rendered inactive by dialysis, and that the dialysable portion was thermostable, soluble in alcohol but insoluble in ether, and destroyed by incineration. Magnus used as zymolyte amyl salicylate and his results were confirmed by Loevenhart [1906, 1, 2], who further discovered that the activity of the non-dialysable portion could be restored by the addition of bile-salts.

Loevenhart found, however, that while this was true of the hydrolysis of amyl salicylate, ethyl butyrate behaved in a different manner. Dialysis did not cause the liver extract to lose its property of hydrolysing the latter ester; and when bile-salts were added to a non-dialysed liver extract its activity towards ethyl butyrate was diminished.

The following experiments were performed without knowledge of this part of Loevenhart's work and are fully confirmatory of his results.

Exps. 22 to 24 were carried out to find if the inhibitory substance were dialysable. A quantity of bile was dialysed and the effect on the esterase of both dialysate and residue determined (Nos. 22 and 23). Part of the dialysate was again dialysed. The effect of this double dialysate is shown in No. 24.

*Exp. No. 22. Dog's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH		
	Normal	Dialysate added	Residue added
5	1.5	1.4	1.2
10	2.7	2.0	1.8
15	3.2	2.6	2.1
20	3.9	3.0	2.5
25	4.2	3.3	2.8
30	4.6	3.7	3.1

*Exp. No. 23. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH		
	Normal	Dialysate added	Residue added
5	2.1	1.9	2.2
10	4.5	4.1	4.1
15	7.0	6.1	6.0
20	9.0	7.8	7.6
25	10.6	9.2	9.2
30	12.0	10.6	10.7

*Exp. No. 24. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH	
	Normal	Double dialysate added
5	2.2	2.2
10	4.5	4.4
15	6.8	6.5
20	8.7	8.2
25	10.5	9.5
30	12.0	10.8

It is evident that the inhibitory substance is dialysable, though the residue from dialysis still shows considerable inhibitory power. In the light of subsequent work it would seem that the bile was not allowed to dialyse for a sufficient length of time.

An alcoholic extract of the bile was made, the extract evaporated to dryness, and the material so obtained tested for inhibitory power. *Exp. No. 25* shows the marked inhibition obtained on adding a small quantity of this dried alcoholic extract.

*Exp. No. 25. Rabbit's Liver. 20 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH	
	Normal	Alcoholic extract added
5	2.4	1.4
10	5.3	2.0
15	8.5	2.8
20	11.1	3.3
25	13.5	3.8
30	15.7	4.4

Exps. Nos. 26 to 30 show the inhibition obtained on adding progressively smaller quantities of the evaporated alcoholic extract (A.E.). In No. 30 only 1 mg. of the material was used. In these only the difference between the acidity of the normal tube and the tube with alcoholic extract added (*i.e.* the amount of inhibition expressed in cc. *N*/10 NaOH) is given.

*Cat's Liver. 10 % Extract.*

Minutes	Inhibition in cc. <i>N</i> /10 NaOH				
	Exp. 26 ca. 10 mg. A.E.	Exp. 27 ca. 8 mg. A.E.	Exp. 28 ca. 6 mg. A.E.	Exp. 29 ca. 4 mg. A.E.	Exp. 30 1 mg. A.E.
5	0.3	0.4	0.3	0.0	0.0
10	0.6	0.7	0.5	0.0	0.2
15	1.0	1.0	0.6	0.1	0.2
20	1.1	1.2	0.6	0.1	0.2
25	1.4	1.2	0.8	0.4	0.2
30	1.4	1.3	0.9	0.5	0.1

That the alcohol-soluble portion of the bile contains all the inhibitory substance, none remaining in the residue, is shown in Nos. 31 and 32.

*Exps. Nos. 31 and 32. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. <i>N</i> /10 NaOH		Cc. <i>N</i> /10 NaOH	
	Normal	Residue from alcoholic extraction	Normal	Residue from alcoholic extraction
5	1.8	1.9	1.9	2.0
10	4.0	4.0	4.1	3.9
15	6.2	6.1	6.3	6.2
20	8.0	8.0	8.1	8.0
25	9.8	9.7	10.0	9.9
30	11.3	11.2	11.5	11.5

A solution in water of the dried alcoholic extract was then treated with ether. The precipitate was dried and the filtrate evaporated to dryness. Solutions in water of the material from each were investigated. The inhibitory substance was evidently insoluble in ether (No. 33).

*Exp. No. 33. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. <i>N</i> /10 NaOH		
	Normal	Filtrate	Residue
5	2.5	2.6	1.0
10	5.3	5.8	1.9
15	7.4	7.8	2.5
20	9.3	9.6	3.2
25	11.5	11.8	3.7
30	13.7	14.1	4.2

Exps. Nos. 34 and 35 show the inhibition obtained, using a 3 % aqueous solution of Plattner's crystals, while No. 36 shows that the inhibitory power is not diminished but rather increased when the bile-salts are purified by recrystallisation.

*Exps. Nos. 34 and 35. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH		Cc. N/10 NaOH	
	Normal	0.15 cc. bile-salt solution added	Normal	0.15 cc. bile-salt solution added
5	1.4	1.1	1.4	1.1
10	2.5	2.0	2.6	2.0
15	3.6	2.8	3.5	2.8
20	4.6	3.4	4.5	3.6
25	5.3	3.8	5.4	4.5
30	6.1	4.2	6.0	5.0

*Exp. No. 36. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH		
	Normal	Before recrystallisation	After recrystallisation
5	1.6	1.2	1.3
10	2.8	2.4	2.3
15	4.3	3.5	3.4
20	5.5	4.5	4.4
25	6.8	5.6	5.4
30	7.8	6.4	6.2

The sensitiveness of the hydrolysis of ethyl butyrate to the inhibiting action of bile-salts was found to be very considerable (Nos. 37 to 43, amounts of inhibition in cc. N/10 NaOH only given). In this connection Loevenhart [1906, 2] found that concentrations of 0.02 % and under, accelerated rather than inhibited the reaction. I have been unable to confirm this, finding inhibition to be produced in concentrations as low as 0.005 %. Concentrations lower than this were not tried, as it was thought that the method of estimating the activity of the enzyme was not sufficiently accurate to allow of this being done.

*Exps. Nos. 37 to 43. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Inhibition caused by bile-salts in concentration of:

Minutes	Inhibition caused by bile-salts in concentration of:						
	2 %	1 %	0.5 %	0.1 %	0.05 %	0.01 %	0.005 %
5	0.8	0.5	0.4	0.2	0.1	0.0	0.0
10	1.5	1.6	1.1	0.3	0.2	0.0	0.1
15	2.5	2.6	1.7	0.8	0.5	0.1	0.4
20	3.5	3.5	2.7	1.1	0.7	0.3	0.4
25	4.4	4.2	3.4	1.6	1.0	0.6	0.6
30	5.2	5.4	4.1	2.4	1.2	0.8	0.7

#### D. OTHER INHIBITORS.

Rosenheim and Shaw-McKenzie [1910] found that sodium cholate, saponin, and serum, among other substances, acted as accelerators to the pancreatic lipase in its action on olive oil. The presence of cholesterol inhibited this acceleration. Von Fürth and Schütz [1906] also showed that sodium cholate was an active accelerator of the hydrolysis of fat by pancreatic juice. The activation of pancreatic lipase by saponin was investigated by Flohr [1919],

who showed that the acceleration produced was proportional to the lowering of surface-tension caused by the saponin. In the case of ricin lipase, however, saponin caused inhibition, and this, he concluded, was due to a poisoning action on the enzyme.

Exp. No. 44 shows the effect of cholalic acid and its derivatives, choleic and deoxycholeic acids. These substances were preparations made and kindly supplied by Dr S. R. Schryver. The first two produced inhibition, the latter, acceleration.

*Exp. No. 44. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH			
	Normal	Cholalic acid	Choleic acid	Deoxycholeic acid
5	2.6	1.8	2.4	2.6
10	5.6	3.8	4.8	5.8
15	8.3	5.6	6.4	8.8
20	10.7	7.5	8.1	12.0
25	13.0	9.2	9.6	15.9
30	15.1	10.8	11.1	19.4

That the sensitiveness of the reaction to cholalic acid was as great as to the bile-salts is shown by Exps. 46 to 52 (amount of inhibition in cc. N/10 NaOH only given).

*Exps. Nos. 45 to 51. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Inhibition caused by cholalic acid in concentration of:						
	2 %	1 %	0.5 %	0.1 %	0.05 %	0.01 %	0.005 %
5	0.2	0.0	0.0	0.0	0.1	0.0	0.0
10	0.3	0.4	0.2	0.1	0.2	0.0	0.0
15	1.1	1.0	0.7	0.6	0.3	0.2	0.2
20	1.7	1.4	1.4	1.0	0.6	0.4	0.3
25	2.6	2.0	1.6	1.4	0.7	0.8	0.4
30	3.3	2.6	2.1	1.7	0.9	1.1	0.6

The effect of saponin was investigated and found to produce slight acceleration in large concentration (3 %, Exp. 52); in weaker concentrations (0.5 %, Exp. 53) little change was noted.

*Exps. Nos. 52 and 53. Rabbit's Liver. 10 % Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH		Cc. N/10 NaOH	
	Normal	Saponin (3 %)	Normal	Saponin (0.5 %)
5	2.6	2.8	2.6	2.7
10	5.8	6.2	5.3	5.2
15	8.8	9.7	8.4	8.4
20	12.1	13.0	11.5	11.4
25	15.1	16.3	14.8	14.6
30	17.9	19.4	17.7	17.4

Cholesterol showed no marked activity in either direction (Nos. 54 and 55).

*Exps. Nos. 54 and 55. Cat's Liver. 10% Extract. Quantities:—As in No. 2.*

Minutes	Cc. N/10 NaOH		Cc. N/10 NaOH	
	Normal	Cholesterol	Normal	Cholesterol
5	2.1	2.2	1.5	1.6
10	4.5	4.7	2.9	3.0
15	7.0	7.1	4.4	4.6
20	9.0	8.9	5.5	5.8
25	10.6	10.5	6.5	7.0
30	12.0	11.9	7.4	8.1

#### SUMMARY AND CONCLUSIONS.

1. There has been found to be present as a constant constituent in the bile of all the animals examined, a substance possessing the power of inhibiting the hepatic esterase.

2. Investigation of the action, on the esterase of one animal, of the bile of other species, showed that the inhibitor is probably of the same nature in all biles. At the same time, the results suggest that the concentration of the inhibitor in the bile of a particular animal is that best adapted for producing inhibition of the esterase in that animal.

3. Chemically the inhibiting substance was found to be soluble in alcohol, but insoluble in ether. A solution of Plattner's crystals was found to be as active as the bile in producing inhibition, and, since purification of the crystals by recrystallisation led to no loss of inhibitory power, the inhibitor may be concluded to be the bile-salts.

4. Esterolysis by the hepatic enzyme is extremely sensitive to the presence of bile-salts, as low a concentration as 0.005 % producing inhibition. Since the reaction is also inhibited by cholalic acid and is equally sensitive to this substance, it may be concluded that the bile-salts act as inhibitors by reason of the cholalic acid portion of their molecule.

5. The inhibition does not appear to be in any way due to the physical properties of bile-salts in lowering surface-tension as saponin does not produce a similar result.

6. Cholesterol does not inhibit the action of the esterase.

In conclusion I should like to record my great indebtedness to Prof. E. P. Cathcart for much help and guidance in the prosecution of the research.

Part of this work was done during the tenure of a part-time Carnegie Scholarship.

#### REFERENCES.

- Flohr (1919). *Arch. Néerland. Physiol.*, **3**, 182.  
 von Fürth and Schütz (1906). *Centralb. Physiol.*, **20**, 47; *Beiträge*, **9**, 28,  
 Hewlett (1905). *Johns Hopkins Hosp. Bull.*, **16**, 20.  
 Loevenhart (1906, 1). *J. Biol. Chem.*, **2**, 391.  
 ——— (1906, 2). *J. Biol. Chem.*, **2**, 427.  
 ——— and Souder (1906). *J. Biol. Chem.*, **2**, 415.  
 Magnus (1904). *Zeitsch. physiol. Chem.*, **42**, 149.  
 ——— (1906). *Zeitsch. physiol. Chem.*, **48**, 376.  
 Ramond (1904). *Compt. Rend. Soc. Biol.*, **2**, 142.  
 Rosenheim and Shaw-McKenzie (1910). *J. Physiol.*, **40**. *Proc.* viii, xii.

## XXXV. A SERIES OF ABNORMAL LIESEGANG STRATIFICATIONS.

By EMIL HATSCHEK.

(Received March 23rd, 1920.)

(With Plate VI.)

THE experiments described in the present paper represent preliminary work for an investigation in which it was necessary to produce Liesegang stratifications in gelatin specimens having volumes up to 300 cc. and completely submerged in the aqueous solution of the one component. It was therefore desirable to employ a material less expensive than silver nitrate and, for reasons apart from the cost, to produce in the gelatin a precipitate more closely corresponding to some of the mineral constituents of organic structures. I accordingly determined to study the deposition of calcium phosphate in gelatin gel.

The reaction between alkaline phosphates and calcium nitrate has been used by Stephane Leduc [1911]. He used Liesegang's procedure, which consists in placing a drop of the solution of one reaction component on a film of gelatin gel containing the other, and obtained stratifications of such fineness that they acted as a diffraction grating. It has been shown conclusively by Liesegang that this effect is due to wrinkling of the surface of the gel [1913]. This particular effect would have been useless for my purpose, which was to produce stratifications extending through a considerable depth of gel, and I accordingly studied the reaction in test tubes. This procedure is now well known: a test tube is partly filled with the sol containing one of the components in suitable concentration, the sol is allowed to set completely, and the aqueous solution of the other component—which must, in all cases, have a molar concentration greatly in excess of that in the gel—poured on.

After some preliminary experiments, which it is not necessary to describe in detail, it was found that the best results were obtained with gels containing 10 g. of gelatin in 100 cc. of trisodium phosphate solution, the concentration of the latter being 3, 2.5 or 2 % of the crystallised salt ( $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ , *i.e.* respectively 7.87, 6.57 and 5.26 millimoles of  $\text{Na}_3\text{PO}_4$ ), on which were placed solutions of calcium chloride, calcium nitrate or mixtures of calcium chloride and sodium chloride. The use of such mixtures, one constituent of which takes no part in the reaction, is a well known device for reducing the concentration of the reacting constituent, while keeping the total molar concentration—which settles the rate of diffusion into the gel—sufficiently high.

In preparing the gelatin sol a somewhat unusual procedure was adopted, except in a few instances, which will be mentioned specially. The usual course—dissolving the salt in the sol—proved objectionable, as all five brands of gelatin used in the investigation gave a, more or less copious, precipitate with trisodium phosphate. If the salt, as is usually done, was added to the sol, the protective effect of the latter rendered the precipitate so fine that the greater portion passed through the Chardin paper usually employed for filtering gelatin. The gelatin leaf was therefore allowed to swell in the phosphate solution itself: in this way whatever precipitate is produced forms in the gelatin gel, which renders it coarser even than it would be in aqueous solution. After 24 hours' swelling the gelatin was dispersed by heating to about  $90^{\circ}$  and then filtered on a hot water funnel, at about the same temperature, through Chardin paper. Treated in this way all five gelatins gave filtrates as clear as those obtained from the same gelatins without the addition of phosphate. The filtered sol, while still warm, was poured into the test-tubes and allowed to set for about 24 hours before the aqueous solutions were poured on. In some instances, which will be specially referred to, the sol was allowed to set, and the resulting gel re-melted for filling into test-tubes.

The times allowed for swelling, dispersion and gelation in the test-tubes were strictly adhered to throughout, unless a departure from the standard procedure is expressly mentioned; the time required for filtering different brands, which varied considerably, was unfortunately beyond control. As the phosphate solution is markedly alkaline, the difference in the times during which the sols were exposed to the filtering temperature of about  $90^{\circ}$  (this time varying from 30 to 90 minutes for 200 cc. of different brands) and the consequent difference in hydrolysis may, in part, account for the astonishing divergence in the results obtained with different brands in conditions otherwise alike. It may be well to add that stratifications are obtained only with trisodium phosphate, and not with either  $\text{Na}_2\text{HPO}_4$  or  $\text{NaH}_2\text{PO}_4$ .

Five brands of gelatin were investigated: (1) a pre-war German table gelatin of unknown origin and moderate "hardness," (2) an English calf gelatin, (3) and (4) two different brands of English gelatin of the same maker, and (5) a French photographic gelatin, the last four brands being all markedly hard. A selection of the most striking results is illustrated in Pl. VI, figs. 1 to 12.

Figs. 1, 2 and 3 show stratifications of the normal type, but of unusual perfection as regards both the regularity of the strata and the entire absence of precipitate in the clear spaces between them. The number of strata is unusually large in No. 3. The preparations were obtained as follows: No. 1, gelatin (2) with 3 % of trisodium phosphate, the aqueous solution consisting of equal parts of 10 % solutions of calcium chloride and sodium chloride; No. 2, gelatin (1) with 2 % of trisodium phosphate, the aqueous solution consisting of four parts of 10 % calcium chloride and one part of 10 % sodium chloride solution; No. 3, gelatin (1) with 2 % of trisodium phosphate, the aqueous solution containing 10 % of crystallised calcium nitrate.

Anomalous features first show themselves in No. 4. The strata are extremely numerous and regular, but are curved, the *upper* side being convex. This is quite unusual: if strata are curved at all, they are concentric with the meniscus of the gelatin gel, *i.e.* the concave side is turned upwards. In addition, many of the strata show a small opening about the centre. This specimen was obtained with gelatin (1), containing 3 % of trisodium phosphate, and an aqueous solution of 20 % of crystallised calcium nitrate.

A different anomaly is exhibited by No. 5, gelatin (4), with 3 % trisodium phosphate and a 20 % solution of calcium nitrate. Below the strata there appear rings of precipitate which are confined to the glass surface. As this brand of gelatin exhibited the same tendency with other concentrations of phosphate and other aqueous solutions, its further use was abandoned.

No. 6 is a very remarkable specimen. The gelatin was (2), with 2 % of trisodium phosphate, while the aqueous solution was a mixture of two parts of 10 %  $\text{CaCl}_2$  and three parts of 10 % NaCl solution. The last four strata alternate with layers of macroscopic aggregates, and the distances, especially between the last two strata, are remarkably great. This alternation between zones containing a large number of small, and a small number of large, particles, has been observed before on a microscopic scale [Hatschek, 1911], but never in the striking fashion exhibited by the present specimen.

Even more striking anomalies are, however, exhibited by Nos. 7 to 12. Some of these appear to have been caused purely by slight (intentional) variations in the procedure adopted in soaking the gelatin etc. Thus in No. 7, the gelatin, phosphate concentration and the aqueous solution were exactly the same as used for No. 3. The gelatin was, however, soaked in water for 30 minutes only, dispersed by warming and the trisodium phosphate added to the sol, which was then filtered. The strata, although spaced normally, show a very marked convexity upwards, and a tendency to the formation of central stems. Similar convex strata are shown by No. 8, but these are arranged quite regularly in pairs. In this case the gelatin and the phosphate concentration were the same as in No. 1, but an aqueous solution of 20 %  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  was used. Nos. 9, 10 and 11 show an even more striking feature not observed hitherto: the formation of wide and narrow bands of precipitate, which alternate regularly in No. 9 and—apparently—quite erratically in Nos. 10 and 11. In these three cases the gelatin was soaked for only 45 minutes: Nos. 9 and 11 were gelatin (2) with 2 % of trisodium phosphate, the aqueous solution being respectively 10 %  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  and 7.5 %  $\text{CaCl}_2$ . No. 10 contained 2.5 % of trisodium phosphate, the brand of gelatin and the aqueous solution being the same as used in No. 9. The gel used for No. 9 had been re-melted about 24 hours after filtration.

No. 12 finally shows the result obtained in a gelatin gel *hardened by formaldehyde before* the solution of the calcium salt was allowed to diffuse into it. The gel was gelatin (1) with 2 % trisodium phosphate, which was hardened by allowing a solution containing 1 % formaldehyde and 2 %

1

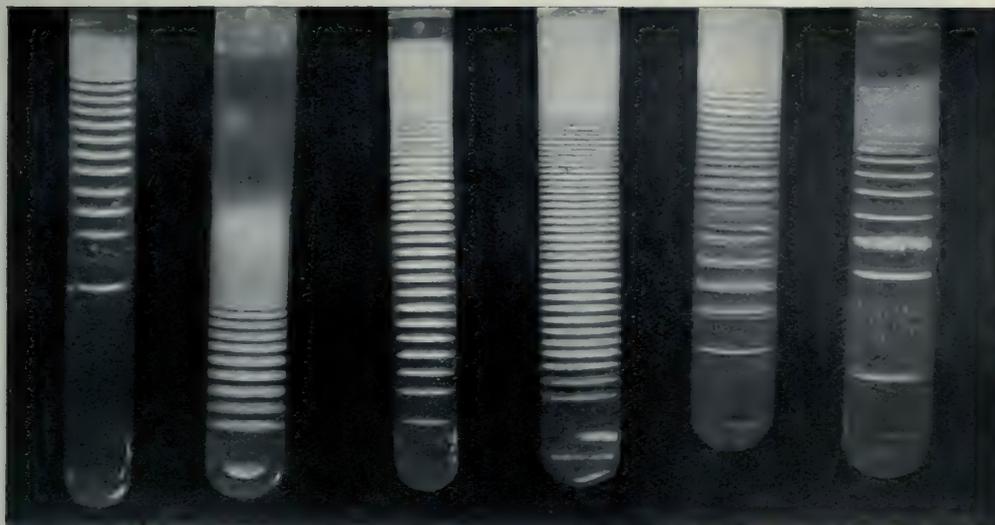
2

3

4

5

6



7

8

9

10

11

12





trisodium phosphate to diffuse into it for about five days. The addition of phosphate to the formaldehyde solution is of course necessary to prevent the salt from diffusing out of the gel. After five days the formaldehyde solution was poured off and was replaced by a 10 % solution of  $\text{CaCl}_2$ . The curious pseudo-helical arrangement has never, to my knowledge, been observed before in test-tube experiments: it recalls, however, the spirals obtained by Liesegang [1914], with the silver chromate reaction carried out in his usual manner on plates, in gelatin gels of suitable acid and gelatose content.

In the present unsatisfactory state of our knowledge regarding the normal phenomenon it is impossible even to attempt an explanation of the extremely complicated structures just described. Other reactions, such as Liesegang's original example, are also sufficiently sensitive to small variations in the brand of gelatin, its acid and gelatose content, in the concentration of the reacting solutions and in the procedure adopted. The resulting stratifications differ, however, generally speaking, in number, in the width of the chromate bands and of the spaces separating them and in the amount of diffuse precipitate found in the latter: such anomalies as bands distinctly grouped in pairs, or alternately wide and narrow, have never been described. While the results therefore enormously increase the difficulty—sufficiently great even in view of the material published hitherto—of propounding a general theory of the phenomenon, they may also enhance its importance to the biologist and geologist by showing that structures of a much more complicated nature than any hitherto obtained in such experiments may be the result of simple diffusion into a gel.

## REFERENCES.

- Hatschek (1911). *J. Soc. Chem. Ind.*, No. 5, 30.  
Stephane Leduc (1911). *Theorie physico-chimique de la vie*, Fig. 16.  
Liesegang (1913). *Kolloid. Zeitsch.*, 12, 181.  
— (1914). *Zeitsch. physikal. Chem.*, 88, 1.

## XXXVI. THE ACIDITY OF ROPY MILK.

By KATHLEEN FREEAR

AND

ELFRIDA CONSTANCE VICTORIA VENN.

*From the Research Institute in Dairying, University College, Reading.*

(Received March 25th, 1920.)

### ROPY MILK.

IN 1916 two samples of ropy milk from two Lancashire farms were sent to the laboratory by Miss Stubbs, Lancashire County Council Dairy School, Hutton.

The samples were plated out upon neutral lemco agar and from both of them a gram-positive *Coccus* showing also diplococcal forms, and in fluid cultures short chains, was obtained. Sometimes the cocci appeared lanceolate and possibly capsulated, the latter appearance being most marked in glucose broth cultures. The cultures which were obtained were called *Bury* and *Accrington* since the farms from which the samples came were in the neighbourhoods of these towns.

After satisfactory isolation had been carried out the bacteriological characteristics of two strains B<sub>12</sub> and B<sub>15</sub> from the Accrington farm and two others RM<sub>4</sub> and RM<sub>5</sub> from the Bury farm were studied. The results obtained appeared to show that these organisms were either identical with or closely related to the *Streptococcus Hollandicus*, and they were used as starters with satisfactory results in the preparation of Edam cheese.

For purposes of comparison strains of *Streptococcus Hollandicus* were obtained from Prof. Orla Jensen and also from Hoorn and Delft. The reactions of these strains were studied upon solutions of sugars, alcohols, etc. which were prepared in the following way. Tubes of media containing 1 % peptone and 0.5 % NaCl were neutralised to litmus; they were then coloured with litmus and 0.5 % of the "sugar" was added except in the cases of inositol, sorbitol and dulcitol, of which only 0.25 % was used. The tubes were then sterilised by steaming on three consecutive days. The reactions produced by the various strains upon these media are shown in Table I. It does not appear that the strains which had been isolated from the milk obtained from Accrington and Bury differed in any material respect from those found with the strains of *Streptococcus Hollandicus*, especially when the method of preparation of the media, and the difficulty of obtaining constant reactions with streptococci upon such media are considered.

Table I.

	ROPY MILK.				
	February 21, 1917.				
	Accrington	Bury	Hoorn	Delft	Jensen
Saccharose	—	Sl. acid	—	—	?
Mannitol	Acid	Acid	—	—	V. sl. acid
Galactose	Acid	Acid	Acid	—	—
Adonitol	—?	—	—	—	?
Dextrose	Acid	Acid	Acid	Sl. acid	?
Glycerol	Sl. acid?	—	—	—	?
Dextrin	Acid	Acid	Acid	—	—
Raffinose	—	—	—	—	—
Lactose	Acid	Acid	Acid	—	—
Inulin	Sl. acid?	V. sl. acid?	—	—	?
Sorbitol	—?	—	?	—	—
Inositol	—?	—	V. sl. acid	?	Sl. acid
Dulcitol	—?	Acid	—	?	?
Salicin	Acid	V. sl. acid?	Acid	—	Acid?

While the bacteriological examinations of the strains were being carried out an occasional determination of the acidity of samples of the ropy milk was made, and was always found to fall within narrow limits. It was therefore decided to study the range of acidity within which the isolated strains produced ropiness and with this object the following experiments were carried out.

The milk under investigation was said to be "Ropy," when definite threads could be drawn out from its surface by means of a sterile platinum needle. Under the conditions of experiment very long ropy threads were not obtained and the ropiness was considered to be "good" when the length of the threads approximated to an inch. When the threads were considerably shorter than this but nevertheless distinguishable from mere stickiness, the ropiness was described as "slight." During the last series of determinations the degree of ropiness was further estimated by means of simple viscosity determinations. The method was not sufficiently accurate to justify the inclusion of the results in the text, but it may be mentioned that simultaneously with the appearance of "good rope" there was a sudden and very marked increase in viscosity. With the diminution of "ropiness" there was a corresponding decrease in viscosity, until with its disappearance the viscosity value obtained closely approximated to that of the uninoculated milk.

In the first series of experiments, the results of which are shown in Fig. 1, flasks of sterile milk, which had been inoculated with the strains isolated from the samples of ropy milk, were incubated at 22°. Samples were withdrawn, tests for ropiness made, and determinations of acidity with *N*/9 NaOH using phenolphthalein as indicator were carried out every 24 hours for a period of 7 days. Fig. 1 shows that ropiness was found on the first day after inoculation except in the case of B<sub>15</sub> when it did not appear until the second day, and that it occurred between acidities which varied between 0.44 and 0.75 (% lactic acid calculated from titrations with *N*/9 NaOH). Ropiness was well marked

ROPY MILK - ACIDITY.  
DETERMINED BY  $\frac{1}{10}$  N NaOH. 28.8.1917

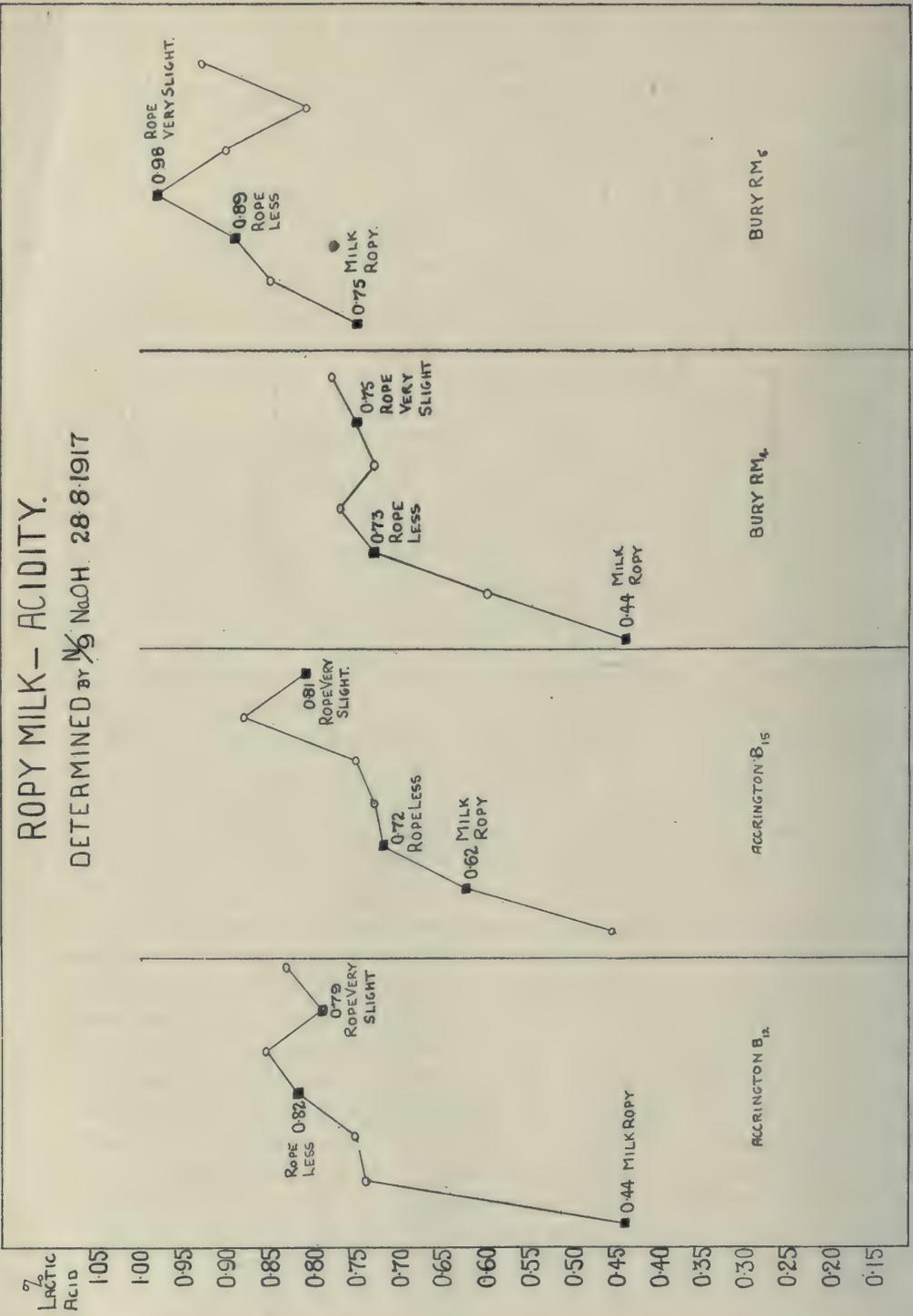


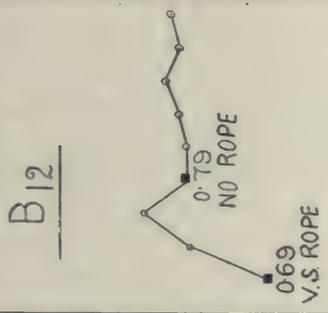
FIG. 1

ROPY MILK ACIDITY  
DETERMINED BY N/9 NaOH

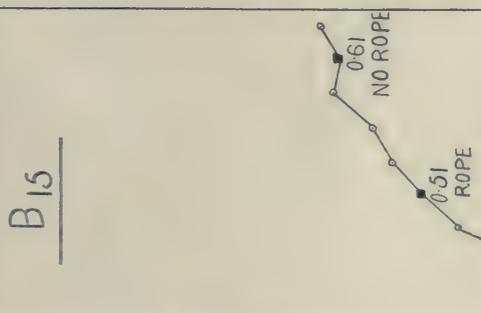
% LACTIC ACID

1.00  
0.95  
0.90  
0.85  
0.80  
0.75  
0.70  
0.65  
0.60  
0.55  
0.50  
0.45  
0.40  
0.35  
0.30  
0.25  
0.20  
0.15

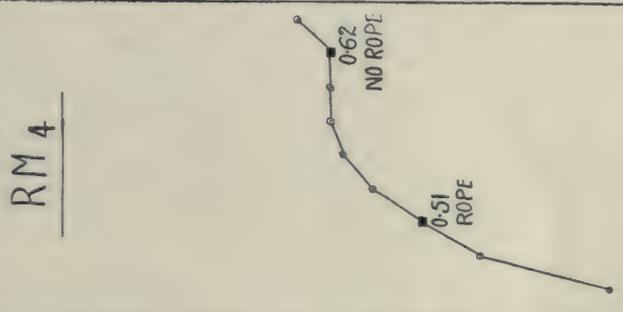
ACCRINGTON  
B 12



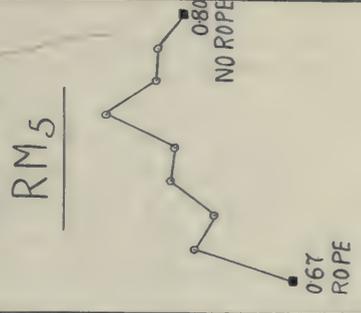
ACCRINGTON  
B 15



BURY  
RM 4



BURY  
RM 5



DAYS  
1 2 3 4 5 6 7 8 9

7-16. XI. 1917

Fig. 2.

for the first three or four days, at the end of which time the acidities varied between 0.72 and 0.89; subsequently the ropiness diminished without any marked increase of acidity, but was still present to a very slight extent at the end of the experiments. A second series of experiments was then undertaken, partly to confirm the results already obtained, and partly to prolong the duration and, if possible, to find the period at which the ropiness really disappeared. Fig. 2 shows that sometimes ropiness did not appear until the fourth day after inoculation. It continued to be present in gradually diminishing amount for periods which varied from three to eight days. The range of acidity over which ropiness was present was 0.51 to 0.87. At the end of the experiments the ropiness had disappeared, though no appreciable alteration in the acidity had taken place.

In continuation of this series two strains from each of the cultures which were under investigation were studied and with them two controls of sterile inoculated milk. Fig. 3 shows that the acidities of the controls lay within the values 0.17 and 0.20. In all the inoculated flasks ropiness was found at the end of 24 hours, the acidity varying at this time from 0.59 to 0.82. The ropiness remained well marked for periods which varied from one to three days with acidities which lay between 0.59 and 0.96 except upon one occasion when the acidity was found to be 1.435. It is doubtful whether this reading does not represent an experimental error. The ropiness began to diminish from two to four days after inoculation and became gradually less until it ceased to be present on the sixth to the ninth day. In all cases the ropiness had disappeared at the end of the experiment which was carried out over a period of nine days. The diminution of ropiness was accompanied by an acidity which varied between 0.83 and 1.0. The disappearance of ropiness was not accompanied by any material variation in the acidity.

In these experiments the examinations of the samples were first made 24 hours after inoculation of the milk, by which time the ropiness was in many cases well established. In all later experiments, therefore, the inoculations were made at midnight and the milk was tested at six hour intervals for the first 18 hours, and afterwards at intervals of 12 hours. The third series of experiments was carried out under these conditions and Fig. 4 shows that at the 30th hour after inoculation the acidity was 0.30 and no ropiness was found, but at the 42nd hour the acidity was 0.73 with fair ropiness. At the end of another 12 hours, when the acidity was 0.9 the ropiness was already decreasing, and continued to decrease until it had completely disappeared 102 hours after the inoculation when the acidity was 0.97. The highest acidity recorded during the period of the experiment was 1.00.

It was thought that the initial acidity at which ropiness occurred might be fixed with greater accuracy if more continuous determinations were made, but it was not possible to carry out satisfactory titration experiments at night. In the later experiments, therefore, the acidity determinations were made not only by titrating with  $N/9$  NaOH but also electrometrically.

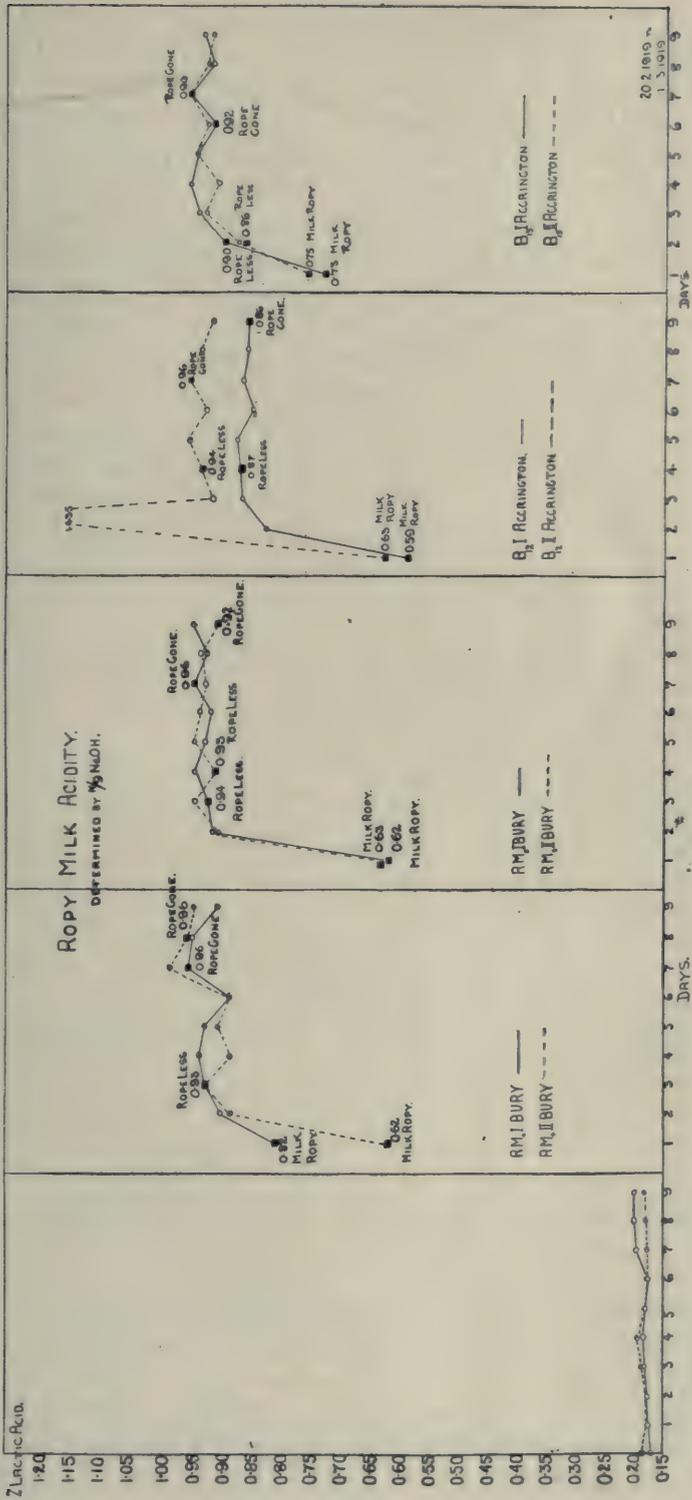
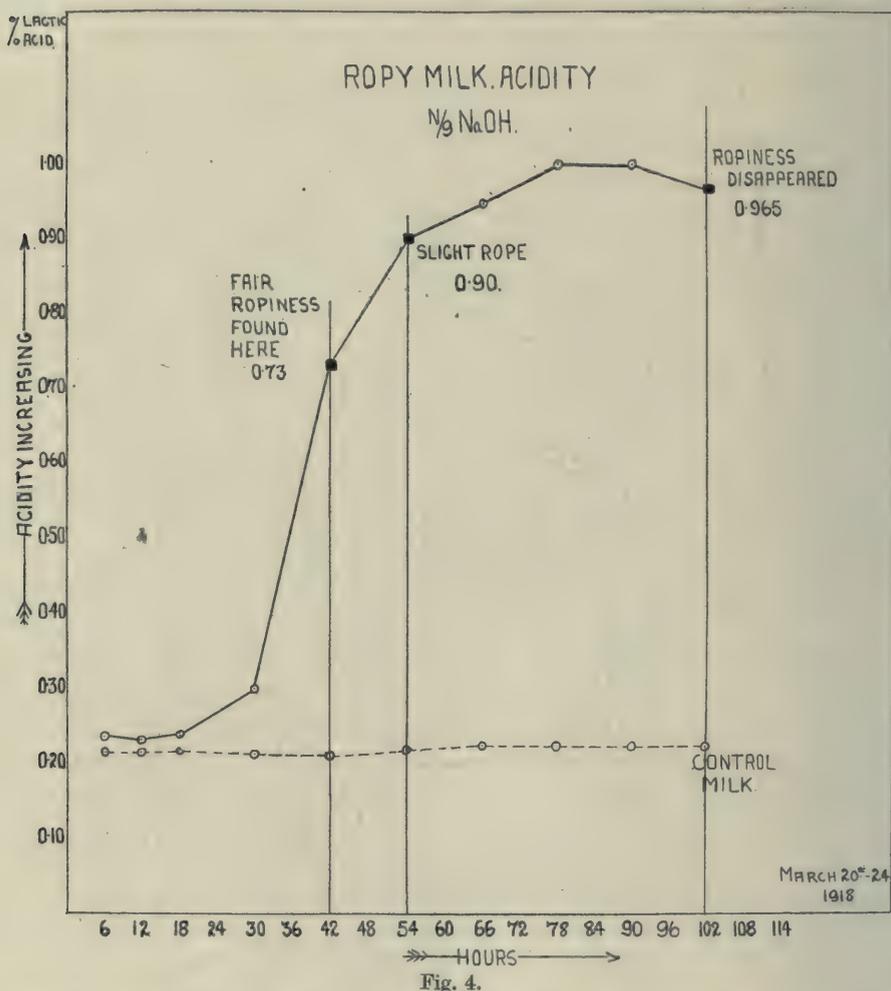


Fig. 3.

Figs. 5 and 6 show the results obtained by both methods in the course of the next experiments. It is seen that ropiness was present at the 18th hour after inoculation, when the percentage of lactic acid was 0.43 and the  $P_H$  was 5.0. It continued to be well marked for 102 hours, when it was found to be diminishing. During this period the acidity as determined by  $N/9$  NaOH rose from 0.43 to 0.81, and the  $P_H$  changed from 5.0 to 4.0. After the 102nd hour the ropiness diminished, while the acidity remained practically unchanged. The quantity of milk available was insufficient to carry on the



experiment beyond 138 hours, and it was therefore repeated with a much larger bulk of milk. In order to ensure sterility of such a volume the heating was necessarily prolonged with the result that a certain amount of charring took place, rendering the end point of the titrations with  $N/9$  NaOH indeterminate, for which reason these readings are not included. The results of the electrometric determinations are shown in Fig. 7 from which it appears

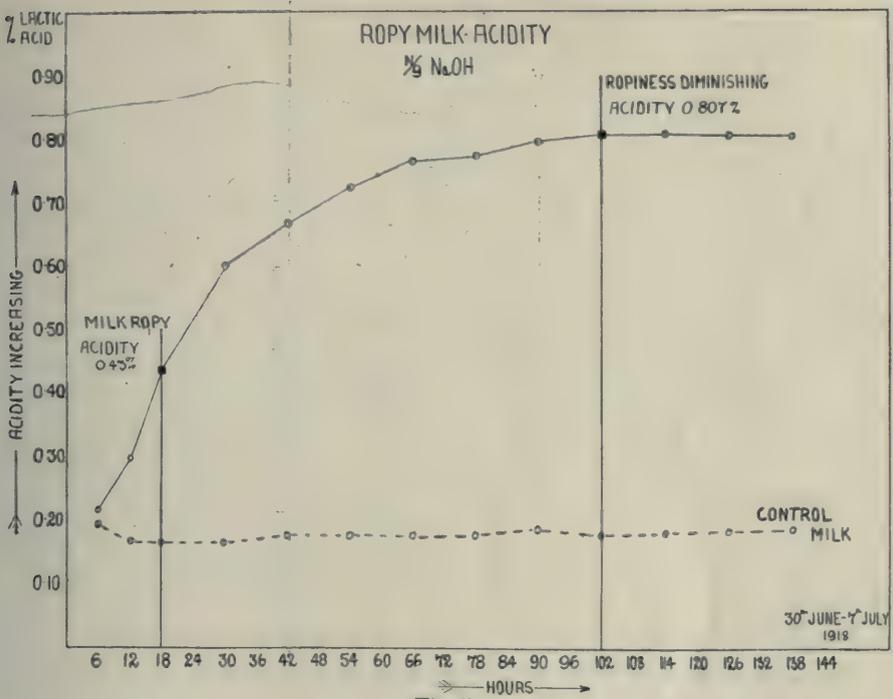


Fig. 5.

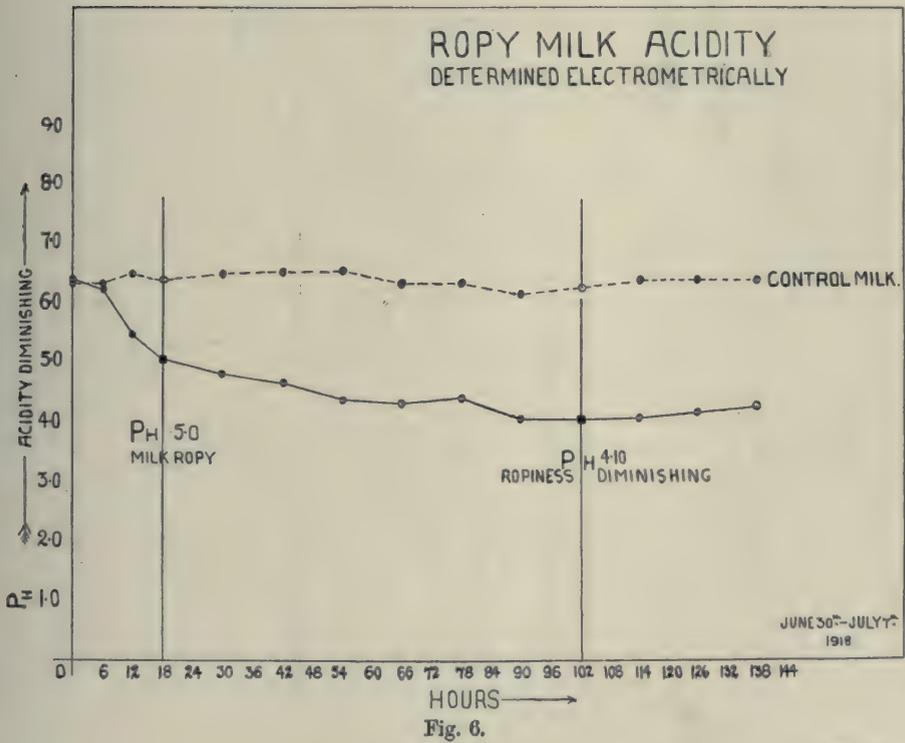


Fig. 6.

ROPY MILK.—ACIDITY.  
DETERMINED ELECTROMETRICALLY.

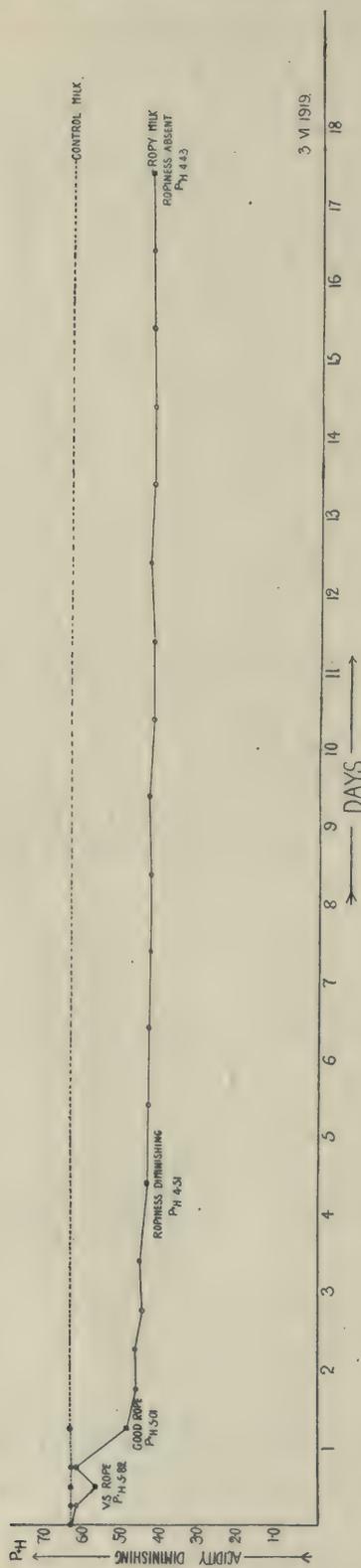


Fig. 7.

that slight ropiness was first found at the 12th hour after inoculation when the  $P_H$  was 5.82. At the 30th hour good ropiness had developed and the  $P_H$  was 5.01. Satisfactory ropiness continued for three days at the end of which time the  $P_H$  was 4.51. The ropiness then gradually diminished until, 17 days after the inoculation, it had entirely disappeared, but throughout this period the acidity remained practically unchanged.

#### CONCLUSIONS.

The experiments show that these strains produced ropiness in milk, associated with a minimum acidity, as determined by  $N/9$  NaOH, of 0.43 % lactic acid, and that the ropiness continued for varying periods of time, accompanied by an acidity which never rose above 1.0 % (except on one occasion). In every case, the ropiness eventually diminished and when studied for sufficiently long periods entirely disappeared, but these changes were not accompanied by any material alteration in the acidity.

The results obtained by the NaOH titration method were confirmed by electrometric determinations, from which it appeared that ropiness was found in milk with a  $P_H$  range of 5.82 to 4.10. With diminishing ropiness no appreciable alteration in the  $P_H$  value could be detected, although in one case the experiment was continued for 17 days by which time ropiness had disappeared.

## XXXVII. THE OCCURRENCE AND NATURE OF THE PLANT GROWTH-PROMOTING SUBSTANCES IN VARIOUS ORGANIC MANURIAL COMPOSTS.

BY FLORENCE ANNIE MOCKERIDGE.

*From the Department of Botany, King's College, London.*

*(Received March 29th, 1920.)*

THE generally accepted idea of the nutrition of an ordinary autotrophic plant is that it requires only a supply of inorganic food materials, consisting of minerals, carbon dioxide and water, from which it is able to elaborate the whole of its organic substance. This conception of plant nutrition, which forms one of the main physiological distinctions between plant and animal, has been modified during recent years by the work of Bottomley, in which he has been assisted by the writer. Experimenting on the growth of green plants of *Lemna minor* in water culture, he has found [Bottomley, 1917, 1] that the addition to the inorganic nutrients of minimal quantities of certain organic substances is absolutely essential if the plants are to grow healthily and normally for any length of time. These organic substances, which have been termed "auximones" by Bottomley, are apparently similar in function, though not necessarily so in nature, to the "vitamines," the accessory growth substances, which play such an important part in animal nutrition.

The use of water-cultures affords practically the only means of absolutely controlling the food materials supplied to a plant, and water plants are obviously the best suited to such growth, since they normally grow with their roots submerged. This fact, together with their rapid multiplication by budding, afforded the reason for the choice of *Lemna minor* for the experiment; and the result of the experiments led to the suggestion that perhaps this plant was peculiar in requiring some organic addition to its nutrient solution. A number of other water-plants have since been tested in the same way, including *Lemna major*, *Salvinia natans*, *Azolla filiculoides*, and *Limnobium stoloniferum*, and these have all been found to grow healthily and multiply rapidly in the presence of a small quantity of organic material, while in inorganic materials alone they gradually died off [Bottomley, 1920, 2], and this was the case no matter what the nutrient solution employed [Bottomley, 1920, 1]. The well-known fact that ordinary plants will grow well in water culture is explained by the comparatively large quantity of

organic food introduced in their seed, and it was shown some time ago [Bottomley, 1914] that wheat seedlings with their endosperm removed at a very early stage responded very much more quickly to the addition of these organic materials than plants which were not so treated. A number of experiments, which will be reported in due course, have more recently been carried out with cuttings; and these also demonstrate the absolute necessity of organic materials for normal healthy growth, and explain the prevalent idea of the unsuitability of cuttings for water culture. All these experiments tend to the conclusion that all green plants require the presence of organic material of a special kind for their maximum growth and development.

An entirely different type of plant, a *Saccharomyces*, has recently been the subject of similar researches on the part of Williams [1919] and Bachmann [1919]. Williams arrives at the conclusion that "a substance of unknown nature...is necessary in addition to the ordinary nutrients for the nutrition of yeast cells," and the effect of this substance is so marked that he suggests that yeast may be used as a biological test for vitamins. He also concludes that this substance is identical with the animal beri-beri vitamin. Bachmann finds that while the various races of yeast vary in their vitamin requirements, yet they all grow better and ferment more readily in a medium containing small amounts of some organic material in addition to their carbohydrate. This necessary substance appears to be a constituent of the yeast cell, and is presumably a synthetic product of the organism, which is therefore apparently stimulated by the products of its own growth.

That all of the animal vitamins are direct products of plant synthesis appears to be probable. It has been ascertained by numerous workers that animals obtain their necessary vitamins from their plant food. It is now established that plants, in their turn, require growth-promoting substances, or auximones; which, in the case of some of the lower plants, are apparently manufactured by themselves, but which in the case of green plants, must be supplied from without. Since these necessary accessory substances are essentially organic in nature, their only possible source in the case of ordinary green plants is to be found in the organic matter of the soil in which they are growing.

Bottomley [1914 and 1917, 1] has found that the auximones are produced very largely during the bacterial decomposition of peat by aerobic organisms, and that they are water-soluble, being extracted by water from peat so treated. The present writer [Mockeridge, 1917] has shown that the auximones thus extracted stimulate not only plant growth, but also the activity of the soil organisms, especially of those concerned in the bacterial treatment of the material; an observation which suggests that the bacteria concerned are stimulated by the products of their own growth, and brings them into line in this respect with the yeasts. Bottomley [1919] has found that the products of the nitrogen-fixing bacteria, *Azotobacter chroococcum* and *Bacillus radicolica*, grown on pure synthetic media, can function as growth-promoting substances

for the higher plants. All of these observations tend to relegate the ultimate production of growth-promoting substances, whether plant or animal, to the soil organisms, and suggest that the moulds and bacteria of the soil, growing upon the organic matter, produce these necessary substances, which are then absorbed by the plants and utilised in their metabolism, and eventually passed on, either in the same form in which they were originally absorbed, or as some other product of plant synthesis, to the animal in its plant food.

If this suggestion be correct, then the greater the bacterial activity in the soil, the greater should be its supply of plant auximones; and the bacterial activity may be concluded to be dependent upon the amount of organic matter present. Consequently, the value of the ordinary organic manurial materials which are necessary for plant culture, and the rôle of which has given rise to so much speculation, may be due, at least partially, to the presence of growth-promoting substances which have been set free by bacterial action; for the artificial treatment of such a material as peat with various soil organisms under controlled conditions of temperature is only a hastening of the natural processes which are being carried out in these manurial composts.

#### CULTURE EXPERIMENTS.

A preliminary experiment was therefore carried out to ascertain whether ordinary organic composts contain water-soluble ingredients which are capable of stimulating plant growth. Samples of both fresh and well-rotted stable manures, leaf-mould, and a good well-manured garden soil were obtained. An estimation of the moisture of each sample was made, and the bulk of each material was shaken with distilled water in a shaking machine for several hours, after which the material was filtered through paper in a Buchner funnel by the aid of a pump, and the residue again shaken with distilled water. This process was repeated until all the water-soluble material had been extracted, when all the filtrates were combined, and the volume of the whole liquid measured. A little chloroform was then added to prevent bacterial action, and the whole was preserved in a stoppered bottle until ready for use.

Each of the composts was extracted in the same way, and a water extract of known strength of each material was thus obtained. The effect of these liquids on the growth of *Lemma major* in water culture was then investigated, and compared with that of bacterised peat.

Twenty flat glass crystallising dishes of 500 cc. capacity were arranged in four series of five dishes each. 250 cc. of Knop's nutrient solution (containing potassium nitrate, potassium dihydrogen phosphate, magnesium sulphate, calcium nitrate, and a trace of ferric chloride) were put into each dish, with the following additions: Series I, numbered from 1 to 5, no addition (control series); Series II, numbered from 6 to 10, the water extract of 1 g. of leaf-mould in every 500 cc.; Series III, numbered from 11 to 15, the water extract of 1 g. of soil in every 50 cc.; and Series IV, numbered from 16 to 20, the water extract of 1 g. of bacterised peat in every 1000 cc. These varying proportions of the different materials were chosen because it was anticipated that their effect would probably be in proportion to their water-soluble organic content, and so the quantities supplied were smaller in proportion as the material appeared to be richer

in organic matter. The dishes were covered to the level of the liquids with paper which was black on the inner surface and white on the outer, to prevent the entry of light through the sides and bottom of the dishes.

Ten similar plants of *Lemna major* were counted out into each dish, and 100 similar plants were counted out for an estimation of their dry weight. The dishes were placed in a well-lighted greenhouse, and a sheet of glass was supported over them in order to exclude dust as much as possible. The solutions were changed twice weekly, to prevent bacterial contamination as much as possible, and the plants were counted once each week. The figures obtained for five weeks were as follows:

Table I.

Series No.	Dish No.	Number of plants					
		At beginning	1st week	2nd week	3rd week	4th week	5th week
I. Knop's Solution	1	10	20	23	23	24	25
	2	10	23	24	26	26	28
	3	10	22	23	26	27	28
	4	10	18	23	27	27	27
	5	10	22	24	24	26	27
	Mean...10		21.0	23.4	25.2	26.0	27.0
II. Knop's Solution + leaf-mould extract	6	10	24	27	34	43	64
	7	10	27	32	42	52	65
	8	10	25	27	34	43	57
	9	10	26	30	41	50	64
	10	10	23	29	41	53	73
	Mean...10		25.0	29.0	38.4	48.2	64.6
III. Knop's Solution + soil extract	11	10	27	36	47	53	67
	12	10	23	32	48	54	71
	13	10	21	29	42	45	61
	14	10	26	39	51	53	75
	15	10	25	36	41	47	58
	Mean...10		24.4	34.4	45.8	50.4	66.4
IV. Knop's Solution + bact. peat extract	16	10	31	43	65	86	142
	17	10	29	40	63	87	132
	18	10	29	44	65	82	130
	19	10	28	38	59	75	118
	20	10	27	44	66	86	141
	Mean...10		28.8	41.8	63.6	83.2	132.6

At the end of the fifth week the plants in the two dishes of each series which were nearest the mean were thoroughly washed, and an estimation was made of their dry weight. The following figures were obtained:

Average weight of 100 plants in all series at beginning of experiment	33.4 mg.
"    "    " Series I at end of experiment	25.3 mg.
"    "    "    II    "    "	37.4 mg.
"    "    "    III  "    "	38.9 mg.
"    "    "    IV   "    "	39.2 mg.

From these figures it is clear that both the extract of soil and that of leaf-mould have the power of increasing the rate of growth of plants of *Lemna major* in water-culture, and also of maintaining them in health; for

while the control plants which were successively budded off from the original ten gradually diminished in size and became sickly and yellowish, the plants in all the other series remained green, and not only maintained their original weight, but increased upon it. Both the soil and the leaf-mould thus contain substances which can be regarded as growth-promoting, since they enable the plants better to assimilate the food materials supplied to them; although, as may be expected, neither the soil nor the leaf-mould, even in the larger quantities used, contain these substances in the comparatively large proportion in which they occur in the compost in which bacterial action has been hastened.

The effect of an extract of stable manure was tested in a separate series of experiments. Samples of both fresh and well-rotted stable manure were obtained, and after an estimation of their moisture content, the materials were thoroughly extracted with water as described above for the other organic composts. A little chloroform was added to the standardised extracts, and this was driven off on the water-bath at about 80° when the liquids were required for use. In all cases the extracts were standardised on the basis of the dry weight of the compost.

Fifty dishes, each containing 250 cc. of Knop's solution, were arranged in ten series of five dishes each.

To these the following additions were made: Series I, numbered from 1 to 5, no addition (control series); Series II, numbered from 6 to 10, a water extract of 1 g. of bacterised peat per 1000 cc.; Series III, numbered from 11 to 15, the water extract of 1 g. of bacterised peat per 2000 cc.; Series IV, numbered from 16 to 20, the water extract of 1 g. of bacterised peat per 5000 cc.; Series V, VI and VII, numbered from 21 to 25, from 26 to 30, and from 31 to 35 respectively, similar proportions of extracts of fresh stable manure; and Series VIII, IX and X, numbered from 36 to 40, 41 to 45, and 46 to 50 respectively, an extract of well-rotted stable manure in similar quantity. Ten plants of *Lemna major* were counted out into each dish, and 100 similar plants were counted out for an estimation of their dry weight. The whole set was treated precisely as in the preceding experiment, the plants being counted once weekly for five weeks. The following results were obtained:

Table II.

Series No.	Dish No.	Number of plants					
		At beginning	1st week	2nd week	3rd week	4th week	5th week
I. Knop's Solution	1	10	25	31	40	52	58
	2	10	25	32	40	50	53
	3	10	22	28	36	41	43
	4	10	24	27	34	40	45
	5	10	22	30	39	47	49
	Mean...10		23.6	29.6	37.8	46.0	49.6
II. Knop's Solution + extract 1 gm. bact. peat per 1000 cc.	6	10	31	50	72	115	151
	7	10	31	45	62	102	131
	8	10	30	48	75	118	154
	9	10	29	47	70	110	150
	10	10	27	43	60	100	134
	Mean...10		29.6	46.6	67.8	109.0	144.0

Table II *continued.*

Series No.	Dish No.	Number of plants					
		At beginning	1st week	2nd week	3rd week	4th week	5th week
III. Knop's Solution + extract 1 gm. bact. peat per 2000 cc.	11	10	25	41	50	81	91
	12	10	24	40	52	80	91
	13	10	29	42	57	82	100
	14	10	31	43	61	93	105
	15	10	30	43	61	93	108
	Mean...10		27.8	41.8	56.2	85.8	99.0
IV. Knop's Solution + extract 1 gm. bact. peat per 5000 cc.	16	10	24	33	50	73	85
	17	10	28	40	56	74	91
	18	10	24	38	50	72	89
	19	10	26	40	51	73	83
	20	10	25	35	50	68	80
	Mean...10		25.4	37.6	51.4	71.8	85.6
V. Knop's Solution + extract 1 gm. fresh manure per 1000 cc.	21	10	24	33	45	54	61
	22	10	23	34	45	55	60
	23	10	20	36	48	58	64
	24	10	24	32	40	50	57
	25	10	24	34	43	51	57
	Mean...10		23.0	33.8	44.1	53.6	59.8
VI. Knop's Solution + extract 1 gm. fresh manure per 2000 cc.	26	10	26	36	44	55	61
	27	10	22	29	37	49	56
	28	10	21	31	38	49	54
	29	10	22	30	41	52	56
	30	10	24	30	40	54	57
	Mean...10		23.0	31.2	40.0	51.8	56.8
VII. Knop's Solution + extract 1 gm. fresh manure per 5000 cc.	31	10	25	35	41	54	56
	32	10	22	29	34	47	52
	33	10	23	30	36	50	55
	34	10	21	31	36	46	50
	35	10	22	33	38	45	49
	Mean...10		22.6	31.6	37.0	48.4	52.4
VIII. Knop's Solution + extract 1 gm. rotted manure per 1000 cc.	36	10	25	42	51	74	82
	37	10	26	36	45	71	81
	38	10	29	41	52	74	84
	39	10	26	40	54	71	77
	40	10	25	37	45	66	72
	Mean...10		26.2	39.2	49.4	71.2	79.2
IX. Knop's Solution + extract 1 gm. rotted manure per 2000 cc.	41	10	25	37	45	60	76
	42	10	28	38	52	70	75
	43	10	27	38	44	62	72
	44	10	28	41	50	70	78
	45	10	26	36	44	61	72
	Mean...10		26.8	38.0	47.0	64.6	74.6
X. Knop's Solution + extract 1 gm. rotted manure per 5000 cc.	46	10	25	38	49	58	64
	47	10	26	38	50	67	74
	48	10	26	35	40	58	61
	49	10	29	38	40	57	61
	50	10	28	41	46	60	63
	Mean...10		26.8	38.0	45.0	60.0	64.6

At the conclusion of the experiment an estimation was made of the dry weight of the contents of the two dishes which were nearest to the mean in each series, and the dry weight of 100 plants in each series was calculated from the figures so obtained. These, compared with the original weights, gave the following results:

Weight of 100 plants in each series at beginning of experiment	35.8 mg.
"    "    Series I at end of experiment	26.4 "
"    "    "    II    "    "	52.8 "
"    "    "    III   "    "	50.4 "
"    "    "    IV   "    "	51.3 "
"    "    "    V    "    "	36.6 "
"    "    "    VI   "    "	36.8 "
"    "    "    VII  "    "	35.7 "
"    "    "    VIII "    "	44.9 "
"    "    "    IX   "    "	45.3 "
"    "    "    X    "    "	43.7 "

The control plants, as in all previous experiments, had thus not only multiplied far less rapidly, but had also failed to maintain their normal size, as indicated by their weight; while in all the series containing the added extracts, in addition to showing a more rapid rate of multiplication, the plants had maintained their normal health, and even increased in size. It is noteworthy that in all the three proportions in which the extracts were used, the well-rotted stable manure was more effective than the fresh manure, thus indicating that production of growth-promoting substances is more or less proportional to the bacterial decomposition of the material.

The experiments thus far recorded point to the conclusion that all the materials examined, viz. fresh and well-rotted stable manure, leaf-mould, and well-manured soil, as well as bacterised peat, contain water-soluble substances which are capable of functioning as plant-growth-promoting substances, or auximones, and the greater the bacterial decomposition of the organic matter of the compost, the greater is the quantity of these growth-promoting substances.

These organic additions appear to have a most marked effect on the nuclei of the plants so treated, and it was thought to be probable that the active agent might be some essential constituent of the nucleus. Bottomley [1917, 2] has shown that raw peat contains nucleic acid derivatives, particularly a dinucleotide, and more recently [1919] he has also shown that these nucleic acid derivatives have a marked stimulating effect on plant growth. The bacterised peat contains a very small quantity of nucleic acid, or dinucleotide, if any at all, but a proportionately larger quantity of purine and pyrimidine bases, and so it appears probable that it is the derivatives of the nucleic acid, rather than the acid itself, which has the auximonic effect.

An examination was accordingly made of the various organic composts with which the above experiments were carried out, to ascertain whether they contained either nucleic acid or its constituent groupings, which, indi-

vidually or collectively, might function as growth-promoting substances. At the same time a typical sample of the *Sphagnum* which gives rise to the peat was also examined for the occurrence of these substances; and not only was the *Sphagnum* so examined, but also a comparison was made between a sample of peat from the very surface of the deposit, and a sample from a depth of about 12 feet, in order to ascertain the effect, if any, on the nucleic acid constituents, of the changes which had resulted in the conversion of the *Sphagnum* into peat.

#### EXTRACTION EXPERIMENTS.

##### *Methods of extraction.*

Various methods of isolation were tried. The usual method of extracting with caustic soda and acidifying the extract with hydrochloric acid to remove the humic acids always resulted in a loss of the desired substances, which were apparently adsorbed in the humic acid precipitate, for this always contained phosphoric acid and showed an abnormally high nitrogen content. Schreiner and Lathrop's modification [1912], by which the alkaline extract was acidified with acetic instead of hydrochloric acid, to avoid the precipitation of any of the nucleic acid, gave slightly better results; but the extraction with caustic soda was very unsatisfactory, on account of the difficulty of filtration with the large quantities of material employed. Where this method was employed at all, a further modification was introduced which obviated this difficulty to some extent. The material was well shaken with a 1 % solution of caustic soda in a shaking machine for several hours, to ensure a thorough extraction, and the material was then, before filtration, acidified with the required acid (hydrochloric or acetic or both). This rendered filtration much more easy, and obviated the necessity of further precipitating and removing the humic acids. Whenever caustic soda was used, this method was followed out.

The use of sodium carbonate as the extractive was an improvement upon the caustic soda, for the mixture filtered much more readily, but in any case the extraction of the humic acid with the nucleic acid was very inconvenient, and it became desirable, if possible, to employ a method which avoided the solution of the humic acid.

The employment of sodium chloride as the extractive, according to the method of Clarke and Schryver [1917], was also tested upon all the materials examined, but the amount of material extracted in this way was much smaller than that obtained by the alkaline extraction, and this method was therefore discontinued.

After much experiment sodium bicarbonate was finally found to be the best extractive to employ for the separation of the nucleic acid constituents, for this reagent separates as large a quantity of the required substances as does the caustic soda, without dissolving the humic acids at all, so that the trouble of removing the latter, and the loss of nitrogen bases by adsorption

in the precipitate, are avoided. The method generally employed for extracting all of the composts investigated was therefore as follows. The material was air-dried and ground as finely as possible. A weighed quantity, which varied with the density of the material used, was put into a three-litre bottle, and generally sufficient was used to about half-fill the bottle, which was then almost filled with a 1 % solution of sodium bicarbonate. The bottle was tightly corked, and shaken on a shaking machine for several (four to six) hours. The contents were then filtered through paper in a Buchner funnel by the aid of a pump, and the residue again extracted with a fresh quantity of bicarbonate solution. This was repeated a third, and sometimes a fourth, time, if the third extraction showed much coloration. A fresh weighed quantity of the material was similarly treated, and this was repeated until from five to ten kilos of the compost had been extracted. The combined extracts were acidified with acetic acid, to precipitate any protein, and the slight precipitate was allowed to settle. The supernatant liquid was decanted off, just neutralised with sodium carbonate solution, and concentrated *in vacuo* to a comparatively small volume. The liquid was then again filtered by the aid of a pump, and poured into a mixture of about five times its own volume of absolute alcohol acidified with hydrochloric acid. A flocculent precipitate appeared which was more or less copious depending on the compost under examination, and this was allowed to settle for about 24 hours. The supernatant liquid was then decanted off through a filter, and more absolute alcohol added to the precipitate, which was well stirred and allowed to settle once more. The alcohol was again decanted off through a filter, and this washing was repeated once more. The precipitate was then dried in a vacuum desiccator, and the filtrate and washings were added together and preserved for examination.

This precipitate was white in appearance, or, in some cases, slightly yellowish. From the method of its preparation, and its appearance, the substance was presumed to be nucleic acid, or a derivative of it, and in each case it was examined for the typical constituent groupings of this acid. For the sake of convenience it will be referred to as the "nucleic acid precipitate."

#### A. Examination of the "Nucleic Acid Precipitate."

The material was first tested for phosphoric acid by incinerating a small portion and applying the ammonium molybdate test to the ash. The powder was then subjected in each case to two methods of hydrolysis, (a) a method of mild hydrolysis, in which the substance was heated at 100° with 10 % sulphuric acid under a reflux condenser for about two or three hours; and (b) a method of stronger hydrolysis, in which sulphuric acid was used at a temperature of 140° for about six hours. In all cases this second method was found to be more effective. Both methods always resulted in the production of a brown deposit of humic acids, indicating the presence of a carbohydrate radicle.

*Separation of Purine and Pyrimidine Derivatives (I).*

Two methods were then employed for the separation of purine and pyrimidine derivatives, these methods being slight modifications of those recommended by Jones [1914]. The first method involved the use of a silver salt. The hot liquid, after hydrolysis, was diluted with hot distilled water and filtered to remove the deposit of humic acids. A hot saturated solution of barium hydroxide was added until all the sulphuric and phosphoric acids had been precipitated, together with some of the colouring matter, and the liquid was alkaline to litmus. After removal of the precipitate by filtration, the excess of baryta was removed by carbon dioxide, and the pale straw-coloured liquid was concentrated *in vacuo* to a comparatively small volume. A little nitric acid was then added, followed by a saturated solution of silver nitrate, so long as a precipitate formed. This precipitate of silver-purine was filtered off, washed on the filter with very dilute nitric acid, and further examined as described below.

The filtrate and washings were thoroughly cooled, and a solution of silver nitrate further added until the addition of a drop of cold barium hydroxide solution resulted in the production of a yellow precipitate. The liquid was then made slightly alkaline to litmus with a cold saturated solution of barium hydroxide, and the silver-pyrimidine precipitate filtered off for examination.

*(a) Examination of the Silver-Purine Precipitate.*

The silver-purine precipitate was suspended in hot water, decomposed with dilute hydrochloric acid, and the precipitate of silver chloride removed. The liquid was then made strongly alkaline with ammonia at the boiling point, boiled briskly for a few minutes to precipitate guanine, and filtered, the precipitate being washed with dilute ammonia. The filtrate and washings were again brought to the boiling point, and a hot saturated solution of picric acid added. This precipitated adenine in the form of clusters of small needle crystals, which could be separated by filtration.

The guanine and the adenine picrate, where they occurred, were purified and used for the preparation of other salts, by which their identity was confirmed.

*(b) Examination of the Silver-Pyrimidine Precipitate.*

The precipitate of silver-pyrimidine was suspended in hot water and decomposed with sulphuretted hydrogen, the precipitate of silver sulphide being removed by filtration. A little barium in the filtrate was removed by sulphuric acid, and the liquid concentrated *in vacuo*. A hot saturated solution of picric acid was added in order to precipitate any cytosine present as the crystalline picrate. After standing for 24 hours, the cytosine picrate was filtered off, and purified by recrystallisation from hot water.

The filtrate was shaken with sulphuric acid and ether in order to remove

the picric acid, and the sulphuric acid was in turn removed with baryta. After concentration, the filtrate was allowed to stand, in order to allow of the separation of any uracil present.

*Separation of Purine and Pyrimidine Derivatives (II).*

The second method employed for the examination of the hydrolysed product concerned chiefly the purine bases. The hot liquid was diluted and filtered free of humic acids as before, and then concentrated ammonia was added at the boiling point until the guanine was completely precipitated together with the phosphate. The whole was boiled briskly for a few minutes and the precipitate then filtered off and washed with dilute ammonia. The precipitate was freed from phosphate by treating it with warm dilute caustic soda, which dissolved the guanine while the phosphate remained insoluble. The liquid was then filtered free from the latter, and the guanine precipitated from solution by means of dilute acetic acid, and used for the preparation of various salts for purposes of identification.

The filtrate and washings from the ammoniacal precipitate were added together and acidified with sulphuric acid. From this liquid the adenine was precipitated as the copper compound in the usual way by boiling the liquid, to which copper sulphate had been added, with a saturated solution of sodium bisulphite, which was added until the precipitate formed began to show a yellowish appearance. The whole was then boiled rapidly for a few minutes, and the precipitate of copper-purine filtered off, washed well with boiling water, suspended in hot water and decomposed with sulphuretted hydrogen. The filtrate from the copper sulphide was concentrated and used for the preparation of various salts of adenine.

The filtrate from the copper-purine compound, after removal of the copper with sulphuretted hydrogen and of the sulphuric acid with baryta, was either treated with silver nitrate and baryta for the precipitation of pyrimidine derivatives as above, or these bases were separated by the use of a mercuric salt. A solution of mercuric sulphate was added to the liquid, which was acidified with sulphuric acid, and the whole allowed to stand for 24 hours. The precipitate was filtered off, decomposed with sulphuretted hydrogen, and the process continued as in the examination of the pyrimidine-silver precipitate after removal of the silver.

*(c) Examination for Presence of Carbohydrate Radicle.*

The so-called "nucleic acid precipitate" was in every case tested by Molisch's reaction for carbohydrates. After mild acid hydrolysis, and also after hydrolysis by ammonia, the products were tested for reducing sugars by Fehling's solution, and for pentoses by the phloroglucinol and orcinol tests.

B. *Examination of the Filtrate after Removal of the*  
 "Nucleic Acid Precipitate."

The filtrate, after removal of the "nucleic acid precipitate," was just neutralised with ammonia, and the alcohol distilled off *in vacuo*. The aqueous fluid was tested for the presence of phosphoric acid by both magnesia mixture and ammonium molybdate. The presence of carbohydrates was also investigated by means of the tests described above.

A preliminary examination of the remainder of the filtrate showed that in order to separate the bases, acid hydrolysis was unnecessary, for they were readily separated from the fluid by the employment of the silver, copper and mercuric salts. All of these materials were used for the further examination of the filtrate for purine and pyrimidine substances, the methods employed being those already described.

RESULTS OBTAINED WITH THE DIFFERENT ORGANIC MATERIALS.

(a) *Sphagnum Moss*. A fairly copious precipitate of so-called "nucleic acid" was obtained by both the caustic soda and the sodium bicarbonate extraction. This precipitate gave a good reaction for phosphoric acid when incinerated, and gave positive results with Molisch's test for carbohydrates. After both mild acid and alkaline hydrolysis, the products reduced Fehling's solution and responded to the orcinol and phloroglucinol tests for pentoses.

Both of the purine bases which occur in yeast nucleic acid, adenine and guanine, were isolated from the hydrolysed material, the former in greater proportion than the latter. Adenine was recognised by (1) the characteristic form of its picrate, which occurred in clusters of fine needles, giving on re-crystallisation elongated prisms which melted with decomposition at about 275°; (2) the hydrochloride, occurring as flat deliquescent prisms; (3) the dichromate, as six-sided plates; (4) the double salt with gold chloride, which appeared as elongated orange-coloured prisms. This base also responded to Kossel's test for purines, gave a gelatinous precipitate with ammoniacal silver nitrate, and a red colour, unchanged by heating, with ferric chloride.

Guanine was recognised by the formation of (1) the hydrochloride, as long needle crystals; (2) the dichromate, forming orange coloured prisms with truncated ends; (3) the picrate, a woolly mass of long thread-like needles when first formed, drying to a compact felt-like mass which became orange-red on heating, and decomposed without melting at about 190°; (4) the sulphate, forming needle crystals which separated out rapidly on cooling a hot solution in sulphuric acid. The solubility of this substance in hydrochloric acid and in ammonia was also characteristic; besides which, a small portion evaporated with a drop of concentrated nitric acid on porcelain left a muddy yellow spot which gave a brownish-red colour with sodium hydroxide, indicating the presence of guanine.

On examining for pyrimidine bases, both cytosine and uracil were isolated, the latter being greater in quantity than the former. Cytosine was recognised by the formation of (1) the hydrochloride, in the form of needle crystals, (2) the picrate, as almost insoluble needles, (3) the chloroplatinate, in the form of six-sided plates. The free base was also obtained in the form of plates by treating the solution of the chloride with ammonia, and this gave the colour reaction of Wheeler and Johnson, which is characteristic of pyrimidines.

The uracil was identified by its method of preparation, by its failure to form an insoluble picrate, and by the clusters of needle crystals formed from a sulphuric acid solution, which gave the colour reaction of Wheeler and Johnson.

The filtrate from this "nucleic acid precipitate" also gave the well-marked reactions for phosphoric acid and for carbohydrates (pentoses), which, of course, would not necessarily arise from nuclear material; besides which each of the purine and pyrimidine substances, adenine, guanine, cytosine and uracil, was present in fair quantity, and was recognised in each case by the preparation of the salts and the application of the tests mentioned above.

The *Sphagnum* moss, therefore, evidently contains some true nucleic acid, which is precipitated by acid alcohol from an alkaline extract; but in view of the larger proportion in which both adenine and uracil occur, as compared with guanine and cytosine, in the hydrolysed material, it is evident that the precipitate does not consist wholly of nucleic acid, but contains also an admixture of the adenine-uracil dinucleotide which occurs in peat [Bottomley, 1917, 2]. Besides these, the *Sphagnum* also contains the free purine and pyrimidine bases from nucleic acid; so that, broadly, it may be said that the nucleic acid constituents of *Sphagnum* consist of some true nucleic acid, a smaller quantity of adenine-uracil dinucleotide, and some free purine and pyrimidine derivatives resulting from the disintegration of the nucleic acid.

(b) *Surface Peat*. A peat taken from the very surface of the deposit, and still retaining something of the colour of the original *Sphagnum*, was similarly investigated. This yielded a smaller quantity of "nucleic acid" precipitate than did the *Sphagnum*, but on hydrolysis the material showed relatively much more adenine and uracil, and less guanine and cytosine than did the *Sphagnum* material. Carbohydrate (pentose) and phosphoric acid were also present in the products of hydrolysis. Evidently, therefore, it contained a smaller proportion of nucleic acid as compared with adenine-uracil dinucleotide than did the former.

The filtrate from the "nucleic acid precipitate" also contained carbohydrate, phosphoric acid, and both purine and pyrimidine bases, viz. adenine, guanine, cytosine and uracil, and these occurred in relatively larger proportion, as compared with the "nucleic acid precipitate," than in the *Sphagnum* moss. The purine and the pyrimidine substances were identified by the same products and tests as indicated above.

These results indicate that in the surface peat disintegration of the original

nucleic acid has already taken place to a certain extent, the products being a larger proportion of dinucleotide and free bases as compared with the original *Sphagnum*.

(c) *Deep Peat*. A peat taken from about 12 feet below the surface of the deposit was investigated, and a certain quantity, which was less than in either of the other cases, of "nucleic acid precipitate" obtained. On hydrolysis, this was found to contain carbohydrates and phosphoric acid, and of the nitrogen bases adenine and uracil, but no guanine or cytosine. It therefore contained no true nucleic acid at all, but consisted entirely of adenine-uracil dinucleotide. This is in agreement with the results of Bottomley.

The filtrate from this precipitate contained also carbohydrate and phosphoric acid, and a relatively large quantity of nitrogen bases. Adenine, guanine, cytosine and uracil were all isolated and identified as above, but the purine bases occurred in much larger quantity than the pyrimidines. This may be due to loss by leaching during the long period of the peat formation, or it may be due to chemical or bacterial changes during the decay of the plant substance, which changes have attacked the pyrimidine substances to a greater extent than the purines.

During the process of peat formation, it is evident that the original nucleic acid has undergone extensive disintegration, probably first of all into dinucleotides, of which a comparatively small quantity remain, and finally into the free constituent bases, which therefore occur in relatively larger quantity in the deeper peat than in either the original *Sphagnum* or the surface peat, but of which a proportion of the pyrimidine bases has disappeared.

(d) *Bacterised Peat*. An examination of bacterised peat was made by both the caustic soda and the sodium bicarbonate methods. Only a very small quantity of "nucleic acid precipitate" was obtained, but this was found to be in all probability a true nucleic acid, yielding all four nitrogen bases in approximately equal proportion. In view of the absence of nucleic acid from the deep raw peat which is used in manufacture, this small quantity is evidently a synthetic product formed during bacterisation, though at present its origin is obscure.

The filtrate from this deposit contained a fair quantity of all of the four nitrogen bases, though here again the adenine and guanine far exceeded in quantity the cytosine and uracil, as in the case of raw peat.

Thus it appears that the further bacterial action has resulted in the breaking down of the adenine-uracil dinucleotide into its constituent groupings, and these are very largely present in a water extract of the material, which does not contain any of the nucleic acid extracted by alkalis.

(e) *Leaf-mould*. A sample of good and fairly pure leaf-mould was extracted by caustic soda, sodium carbonate and sodium bicarbonate, and all three methods yielded similar results. A small quantity of material was obtained by the acid-alcohol precipitation, and this yielded on hydrolysis, adenine, guanine, cytosine and uracil, besides carbohydrates and phosphoric

acid. The guanine and cytosine, however, were present only in very small quantity as compared with the adenine and uracil, so that the material evidently contained a comparatively small proportion of true nucleic acid, the remainder being presumably adenine-uracil dinucleotide. The filtrate from this "nucleic acid precipitate" contained all four nitrogen bases in relative abundance, besides more carbohydrate and phosphoric acid. The leaf-mould, therefore, contains a fair quantity of nucleic acid derivatives, by far the greater proportion being in the form of the free bases, while only a small fraction exists as true nucleic acid, with a larger quantity of dinucleotide. In the sample investigated the disintegration of the original nucleic acid into its constituent groupings had apparently been almost complete.

(f) *Fresh Stable Manure.* This material was extracted by both caustic soda and sodium bicarbonate, and in each case a small quantity of the precipitate with acid-alcohol was obtained. This material consisted almost entirely of nucleic acid, for it gave on hydrolysis adenine, guanine, cytosine and uracil in practically equal proportions, so far as could be judged. The filtrate contained also comparatively large amounts of all these four bases, together with small quantities of xanthine and hypoxanthine.

The nucleic acid in this compost had probably been derived from the plant material constituting the animal's food, and the greater proportion had evidently been decomposed into the free bases in its passage through the animal's body. Since the animals are practically entirely herbivorous, the whole of these nucleic acid derivatives evidently owe their origin ultimately to the elements contained in their plant food. But the purine and pyrimidine bases have by no means necessarily been derived wholly, or even very largely, directly from the plant nucleic acid. On the contrary, a large proportion may be presumed to have arisen as products of metabolism in the animal's body, being elaborated from the plant materials supplied.

(g) *Well-rotted Stable Manure.* This was extracted in just the same way as for the fresh manure, and only a very slight "nucleic acid precipitate" was obtained. On hydrolysis this material yielded adenine and uracil as the only nitrogen bases, so that evidently no true nucleic acid was present, the precipitate being dinucleotide.

In the filtrate from this material were found all the free bases as indicated above for the fresh manure, but they occurred in rather larger quantity. It was noticeable here that the adenine and guanine were relatively the most abundant, occurring in greater quantity than the pyrimidine derivatives, which had evidently either been leached out, or had been transformed by the action of enzymes or micro-organisms. The filtrate also showed the presence of carbohydrate and phosphoric acid. The alkaline extract was much darker in colour, giving a much more copious precipitate of humic acids on acidification, which had, no doubt, arisen by bacterial action on the cellulose and soluble carbohydrates in the animal's food.

In the rotted stable manure, therefore, the decomposition of the nucleic

acid has proceeded to such an extent as to resolve it completely into its constituent bases.

(h) *Soil*. A sample of the same good fertile soil as that which was used in the *Lemna* experiments, and which had been well-manured for several years, was also extracted with caustic soda, sodium carbonate and sodium bicarbonate. A small quantity of the "nucleic acid precipitate" was obtained by each method, and on hydrolysis yielded in each case adenine, guanine, cytosine and uracil, the first and last being in greater proportion than the other two. Phosphoric acid and carbohydrate were also found amongst the products of hydrolysis, so that the precipitate evidently contained some nucleic acid together with adenine-uracil dinucleotide.

From the filtrate from this precipitate were isolated all of the four nitrogen bases from plant nucleic acid, viz. adenine, guanine, cytosine and uracil, together with xanthine and hypoxanthine and a little carbohydrate. All of these substances, however, occurred in relatively much smaller quantity than in any of the other materials examined, while as might have been expected, phosphoric acid occurred in much greater quantity. Among the purine bases, adenine was found to occur in greater quantity, and all of the purine bases were present in greater quantity than the pyrimidines, of which the proportions were extremely small. The occurrence of xanthine, hypoxanthine and adenine among purine bases, has already been reported in soil [Schreiner and Shorey, 1910; Shorey, 1913] and of the pyrimidine derivatives cytosine has also been identified [Schreiner and Shorey, 1910]. The soil under examination here showed the presence of all four of the purine bases mentioned, as well as both of the pyrimidine derivatives, in varying quantities, but it is not to be expected that all soils will necessarily show the presence of the same substances, nor that the composition of any one soil will remain constant for any length of time, in view of the multiplicity of the bacterial and enzymic changes which are continually taking place.

The nucleic acid derivatives which have been found in the various composts examined can be briefly summarised in the following table:

COMPOST	NUCLEIC ACID	DINUCLEOTIDE	FREE NITROGEN BASES
<i>Sphagnum</i>	Fair amount	Small quantity	Fair quantity of adenine, guanine, cytosine and uracil
Surface peat	Less than above	More than above	More than above
Deep peat	None	Less than surface peat	More than surface peat
Bacterised peat	Trace	None	Still more than above
Leaf-mould	Very little	Small quantity	Fairly large amounts of four bases as above
Fresh stable manure	Small quantity	Practically none	Fair amount of adenine, guanine, cytosine and uracil; small amounts of xanthine and hypoxanthine
Well-rotted stable manure	Practically none	Very small quantity	Rather more adenine and guanine than above; less cytosine and uracil; small amounts of xanthine and hypoxanthine
Soil	Small quantity	Small quantity	Small amount of adenine; less guanine, xanthine and hypoxanthine; still less cytosine and uracil

## SUMMARY AND CONCLUSION.

The ordinary organic manures which are generally applied to soils in agricultural and horticultural operations, viz. leaf-mould, fresh and well-rotted stable manures, have been shown to contain, in varying proportions, water-soluble substances which are effective as plant growth-promoting substances, or auximones. Such ingredients are also to be found in well-manured fertile soil, for water extracts of all these materials, when added in certain proportions to inorganic nutrient solutions, promote the healthy growth and development of plants of *Lemna major*, which without such additions rapidly become unhealthy and diminish in weight.

A comparison between fresh and well-rotted stable manure shows that in whatever proportions the materials are used, in all cases the extract of rotted manure is more effective than that of the fresh material, while an extract of peat which has been artificially subjected to bacterial decomposition under controlled conditions is more effective than either. The increase in growth following upon the addition of these materials to the culture solution is such as can not be attributed to the purely nutritive value of the materials added, and all these composts therefore must be assumed to contain growth-promoting substances, or auximones. The amount of these substances varies directly with the extent of the bacterial decomposition of the material, which decomposition is an important factor in determining the efficacy of the compost from a practical standpoint.

The exact nature of the essential substances is still very problematical, but Bottomley [1917, 2] has shown that peat contains derivatives of nucleic acid, and [1919] that these derivatives are effective in increasing the growth of plants when added to culture solutions. Microscopic examination has shown that the various auximones have a marked influence on the size and the contents of the cell, especially on the nucleus, and it seemed not improbable that they might supply some essential nuclear constituent. A detailed examination was therefore made of all of the above materials, viz. well-manured soil, leaf-mould, fresh and well-rotted stable manures, to determine whether they contain derivatives of nucleic acid; and at the same time an examination was made of *Sphagnum* in various stages of its conversion into decomposed peat, in order to ascertain what changes, if any, these materials have undergone during such conversion.

As a result it has been found that all these materials contain appreciable quantities of nucleic acid and its derivatives in various stages of decomposition. Generally speaking, nucleic acid, a dinucleotide, and the free purine and pyrimidine bases have all been found to occur in varying proportions, and the greater the bacterial decomposition of the material concerned, the greater is the resolution of the original nucleic acid into its free bases.

The greater the extent of the decomposition, the more effective is a water extract of the material when added to a culture solution, and the greater is the value of the material from a practical point of view. It would

therefore appear, assuming that the whole effect is due to nucleic acid or its derivatives, that the free bases, individually or collectively, are of more value as growth-promoting substances than the nucleic acid. Schreiner, Reed and Skinner [1907] have found that xanthine and guanine, in concentrations of 40 parts per million or less, increased the growth of wheat seedlings in water culture. Their experiments were made on ordinary seedlings with a fairly large reserve of organic matter in their endosperm, and were of only 10 to 12 days' duration, while in all the experiments carried out by Bottomley and the writer, it has been found that the beneficial effect of the added organic substance is best manifested after 21 days from the commencement of the experiments, when the original supply of auximones in the plant has presumably become either exhausted or so diffuse as to be insufficient for the needs of the plant. Similarly, Schreiner and Skinner [1912] have shown that nucleic acid, hypoxanthine, xanthine and guanine are beneficial under similar conditions, the guanine especially to root development, and a noticeable feature of the experiments recorded above was the marked improvement in the health of the roots. If the experiments of these authors had been carried out over longer periods and with plants which have not a large organic reserve to start with, it is most probable that the added organic materials would have proved even more beneficial. The effect, however, was quite marked, xanthine for instance giving an increase of 21 % in the gross weight of the plants. It is proposed to continue the present work by investigating the action of the free bases, separately and collectively, on the growth of plants which reproduce themselves in a similar manner to that of the *Lemnaceae*.

The value of organic manures is a well-recognised fact, and stress has been laid on their physical effect on the texture of the soil, and on the value of the organic matter as food for soil bacteria, but the fact that the organic materials may be of direct importance to the plant has received very little attention. Livingston [1907] acknowledged this fact when experimenting on the effect on plant growth of extracts of green and stable manures. He added these materials to a slightly modified Knop's solution, and found that "by far the most important factor in the effectiveness was the organic matter," which he found to be of direct value to the plant, although in what lay its effectiveness was not clear. In the experiments recorded here it has been shown that all of the organic manurial composts are of direct value to the plant, and that they all contain nucleic acid or its derivatives, which have been shown by Bottomley and the authors quoted above to be beneficial to plant growth, serving as growth-promoting substances, or auximones. It is evident, therefore, that part, at least, of the beneficial effect of organic manures must be due to the direct value of these substances to the plants. It is not suggested that these nucleic acid derivatives are the only auximones present in the organic composts. In such accumulations of material, the composition of which never remains constant, but is continually undergoing change on account of bacterial

and enzyme action, it is quite probable that there are many such substances capable of functioning as auximones, but the nucleic acid derivatives certainly play an important part. These auximone substances probably occur partly as degradation products of the organic debris, partly as direct products of the synthesis of moulds and bacteria, for it has been shown that auximones do occur as synthetic products of yeast [Williams, 1919, and Bachmann, 1919], and of nitrogen-fixing bacteria [Bottomley, 1919]. Whatever their origin, their liberation in the soil organic matter is evidently due to the action of micro-organisms. These organisms play a part in the auximone cycle not inferior to that of the green plant, which may be regarded as acting as a carrier, absorbing the auximones, which are liberated by the soil organisms, and utilising them in its own metabolism, in the course of which it elaborates the vitamins which in their turn are passed on to the animals that require them.

In conclusion, I wish to express my gratitude to Professor W. B. Bottomley for supervising this work, and to the Department of Scientific and Industrial Research for a grant which enabled it to be carried out.

#### REFERENCES.

- Bachmann (1919). *Journ. Biol. Chem.*, **39**, 235.  
 Bottomley (1914). *Ann. Bot.*, **28**, 531.  
 — (1917, 1). *Proc. Roy. Soc. B*, **89**, 481.  
 — (1917, 2). *Proc. Roy. Soc. B*, **90**, 39.  
 — (1919). *Proc. Roy. Soc. B*, **91**, 83.  
 — (1920, 1). *Ann. Bot.*, **34**.  
 — (1920, 2). *Ann. Bot.*, **34**.  
 Clarke and Schryver (1917). *Biochem. J.*, **11**, 319.  
 Jones (1914). *Nucleic Acids*. (Longmans.)  
 Livingston (1907). U.S. Dept. Agric., Bureau of Soils, *Bull.*, **36**.  
 Mockeridge (1917). *Proc. Roy. Soc. B.*, **89**, 508.  
 Schreiner and Lathrop (1912). U.S. Dept. Agric., Bureau of Soils, *Bull.* **89**.  
 — Reed and Skinner (1907). U.S. Dept. Agric., Bureau of Soils, *Bull.* **47**.  
 — and Shorey (1910). U.S. Dept. Agric., Bureau of Soils, *Bull.* **74**.  
 — and Skinner (1912). U.S. Dept. Agric., Bureau of Soils, *Bull.* **87**.  
 Shorey (1913). U.S. Dept. Agric., Bureau of Soils, *Bull.* **88**.  
 Williams (1919). *Journ. Biol. Chem.*, **38**, 465.

## XXXVIII. RAPID VOLUMETRIC METHODS FOR THE ESTIMATION OF AMINO-ACIDS, ORGANIC ACIDS AND ORGANIC BASES.

BY FREDERICK WILLIAM FOREMAN.

*From the Institute for the Study of Animal Nutrition, School of Agriculture,  
Cambridge University.*

*(Received April 2nd, 1920.)*

IN the course of an investigation of the process of putrefaction, it became necessary to devise methods by which many estimations could be made of such products of enzyme or bacterial action as organic acids, amino-acids and organic bases. Existing methods such for instance as that for determining the organic acids volatile in steam, the estimation of amino-acids by Sørensen's formol titration method and the separation of organic bases by distillation were tried but found wanting either on account of the lengthy nature of the process or the inaccuracy of the results.

It was recognised that volumetric methods which would make possible the rapid and accurate estimation of mixtures of organic acids, amino-acids and organic bases would have such wide applications that it would be worth while devoting much time and trouble to the subject. The investigation has resulted in the discovery of a simple and accurate volumetric method which admits of general application to the analysis of such organic mixtures as result from the degradation of proteins or similar bodies by acids, enzymes or bacteria.

On treating a fluid in which putrefaction has been proceeding with nine volumes of alcohol as described in a previous paper [Foreman and Graham-Smith, 1917] proteins, albumoses, peptones and other substances whose presence might interfere with the estimation of simpler constituents are precipitated. The filtrate which is light yellowish brown in colour contains about 87 % alcohol. The clue which led up to the evolution of a successful method applicable to such alcoholic filtrates was found in an observation by Sutton [1900] that potash or soda will displace ammonia in equivalent quantities from ammonium salts in alcoholic solution at ordinary temperatures, whilst at the same time the ammonia forms no compound with phenolphthalein used as indicator. The acid in an ammonium salt can therefore be titrated with standard alkali in alcoholic solution. Sutton does not state the strength of alcohol necessary to secure this result. The alcoholic filtrates contain amines as well as ammonia together with about 13 % of water. It seemed reasonable

to assume that the amines might behave as ammonia towards phenolphthalein in alcoholic solution, in which case the bases would not interfere with the direct quantitative titration of the organic acids in the alcoholic filtrates provided the presence of a little water did not vitiate the results.

In order to provide a stock of standard material on which the method could be tested, the following procedure was adopted.

The body of a rabbit, including the skin and internal organs with their contents, was minced, and  $2\frac{1}{2}$  times the weight of water added in a Winchester quart bottle. The bottle was loosely plugged with cotton wool and kept at  $25-30^\circ$  for four weeks, in an inclined position so as to expose as great a surface as possible. The contents were lightly shaken once daily, the plug being removed during this operation. At the end of the period the fluid contained the products of advanced aerobic putrefaction. A portion was treated with nine volumes of 97 % alcohol, allowed to stand all night and filtered. The solution containing 87.3 % alcohol was stored in a stoppered bottle and will be referred to frequently in this paper as the "Alcoholic Extract." The following results were obtained on analysis of 50 cc. portions by the methods described in an earlier communication [Foreman and Graham-Smith, 1917].

Volatile bases	24.85 cc. N/10 acid
Formol titration of residual liquid (amino-acids)	2.1 cc. N/10 soda
Organic acids volatile in steam	19.7 cc. N/10 soda

The "Alcoholic Extract" was titrated under the differing conditions described in Table I, using phenolphthalein as indicator. The 97 % alcohol used was slightly acid to phenolphthalein, 100 cc. requiring 0.15 cc. N/10 alkali.

Table I. Preliminary Experiments with the "Alcoholic Extract."

Col. 1	2	3	4	5
Ref. No.	Reagents added to 10 cc. portions of "Alcoholic Extract" before titrating	Aq. N/10 soda required cc.	% alcohol in the mixture after titrating	Readings calculated to a basis of 50 cc. "Extract," after correcting for the original acidity of the alcohol
1	0	4.2	61	20.1
2	20 cc. 97 % alcohol	4.7	81	23.35
3	50 cc. "	4.7	88.5	23.13
4	50 cc. 97 % alcohol and 0.5 cc. ammonia (sp. gr. .88)	4.7	87.8	23.13
5	50 cc. 97 % alcohol and 5 cc. aq. butyric acid solution (= 7.6 cc. N/10 soda to phth.)	12.25 7.6	73.9	22.88
6	100 cc. 97 % alcohol and 5 cc. aq. butyric acid solution	4.65 12.4 7.6	83.0	23.25
7	50 cc. 97 % alcohol	4.8 4.75	88.5	23.38
8	To the titrated liquid of No. 7 added a neutralised mixture of 10 cc. formalin and 50 cc. 97 % alcohol	up to 4.85	84.7	23.88

The following observations were made during the carrying out of the first six experiments described in Table I. Reference will be made later to Exps. Nos. 7 and 8.

1. The light yellowish brown colour of the "Alcoholic Extract" practically disappeared on diluting with alcohol.

2. The endpoints were quite satisfactory.

3. On raising the percentage of alcohol from 61 to 81 the reading was increased, but between 81 and 88.5 % no further increase occurred.

4. The result was greater than previously obtained for the volatile organic acids in the same quantity of "Alcoholic Extract."

5. The presence of even a large amount of free ammonia did not affect the reading although the mixture contained 12 % of water.

6. The butyric acid added titrated quantitatively in the mixture.

The results showed that ammonia liberated by the alkali from the ammonium salts, which the "Alcoholic Extract" contained, did not affect the indicator when 81 to 88 % of alcohol was present. This inference was confirmed by obtaining quantitative results for the acid radicle on titrating a known quantity of pure ammonium chloride in 85 % alcohol.

As the "Alcoholic Extract" contained amines as well as ammonia, the behaviour of primary, secondary and tertiary amines towards phenolphthalein in alcoholic solutions was investigated. Known quantities of the pure hydrochlorides of these bases were titrated in 85 % alcoholic solution and found to require  $N/10$  alkali quantitatively equivalent to the acid radicle in each case. The actual experiments will be found in a later section. These amines therefore resembled ammonia in forming no compound with phenolphthalein in the alcoholic solutions.

The effect of alcohol in different concentrations upon the titration value of the acid radicles when titrated in the presence of the free bases was then studied in detail. For this purpose 97 % alcohol was added in small quantities at a time to known quantities of standard aqueous solutions of ammonium salts and salts of primary, secondary and tertiary amines, and the liquids titrated with  $N/10$  alkali after each addition using phenolphthalein as indicator. The total titration value increased as the content of alcohol in the solutions was augmented, reaching a maximum when 80 to 85 % alcohol was present. In the experimental section, a typical experiment of this nature carried out with trimethylamine hydrochloride is fully described. In this case the reading reached its highest point when the concentration of alcohol had been increased to 78 %.

Having determined that ammonia and amines form no compound with phenolphthalein in the presence of 85 % alcohol, the amount of  $N/10$  alkali required to neutralise a certain volume of the "Alcoholic Extract" under this condition was regarded, at this stage of the investigation, as quantitatively equivalent to the organic acid radicles present. An attempt was therefore made to obtain a direct figure for the amino-acids in the "Alcoholic

Extract" by continuing the titration after adding a neutral alcoholic solution of formalin to the neutralised alcoholic solution. Experiments Nos. 7 and 8 of Table I, were therefore carried out. The additional *N*/10 soda required for neutralisation on adding the alcoholic formalin, however, was only 0.5 cc. per 50 cc. "Alcoholic Extract," whereas a reading of 2.1 cc. *N*/10 soda for the amino-acids in the same volume of "Alcoholic Extract" had been obtained previously by the formol titration of the residual liquid from the distillation of the volatile bases (p. 471). In attempting to find a reason for this deficiency, the behaviour of amino-acids was investigated under the same conditions. A 0.53 % aqueous solution of an amino-acid mixture, consisting of leucine and valine and containing 11.34 % *N*, was therefore treated and titrated as described in Table II.

Table II. Preliminary experiments with amino-acids.

Col. 1	2	3	4
Ref. No.	Treatment of the 0.53 % aq. solution of amino-acids before titrating	Aq. <i>N</i> /10 soda required (corrected) cc.	% alcohol in the mixture after titrating
9 (a)	5 cc. + 50 cc. 97 % alcohol	1.97	85.0
(b)	5 cc. + 100 cc. „	1.97	90.7
10	To the titrated liquid of Exp. 9 (b) added a neutralised mixture of 10 cc. formalin and 50 cc. 97 % alcohol	2.17	87.0

The result of Exp. No. 9 of the above table was most astonishing. Assuming each amino-acid to require one equivalent of *N*/10 soda for neutralisation, 5 cc. of the leucine-valine solution would theoretically require, calculated on the *N* content, 2.15 cc. *N*/10 soda. The figure actually obtained was 1.97 cc., which remained constant on increasing further the concentration of alcohol. These two amino-acids therefore titrated almost quantitatively as ordinary monobasic organic acids in 85 % alcohol using phenolphthalein as indicator, although in aqueous solution they are practically neutral. The result also showed that the reading obtained when the "Alcoholic Extract" was titrated in the presence of 81-88 % alcohol (see Exps. Nos. 2 and 3, Table I) represented amino-acids and organic acids together, and not the organic acids alone, as originally supposed.

The amino-acid solution resembled the "Alcoholic Extract" in requiring a small additional amount of *N*/10 alkali on adding alcoholic formalin to the neutralised alcoholic liquids.

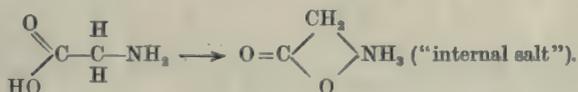
The surprising discovery that leucine and valine titrated in 85 % alcohol as if they were ordinary organic acids led to the supposition that this behaviour might prove common to amino-acids in general. If so, then the property might be utilised as a basis of a new titration method for the estimation of amino-acids. Other amino-acids were therefore submitted to investigation under the same conditions. The results of the experiments will be

given later. For convenience in presentation they may be summarised briefly as follows.

Phenylalanine, tyrosine, cystine, histidine, asparagine quantitatively required one equivalent of soda for neutralisation to phenolphthalein in the presence of 85 % alcohol, while the requirements of lysine and tryptophan were practically quantitative. Arginine was neutral. Glycine, alanine, leucine and valine gave a slight deficiency. The results obtained for aspartic and glutamic acid and proline however were considerably below the calculated value, with a corresponding lack of definiteness in the endpoints.

*Hypothesis for explaining the behaviour of amino-acids in alcoholic solutions.*

Assuming the neutrality of amino-acids in aqueous solutions to be due to the formation of "internal salts" as described by Mann [1906] an explanation of their behaviour in strong alcoholic solution on adding KOH seems possible. In the account given by Mann, glycocoll as a type is represented thus:



Walker [1904] believed that amino-acids in aqueous solution either form "internal salts" or unite in pairs in such a way that the basic radicle of one molecule links on to the acid radicle of another, the original trivalent N atom always becoming pentavalent whichever of these changes the amino-acid undergoes. Therefore the opinion was formed that the behaviour of amino-acids in alcoholic solutions on adding potash is comparable with that of an ammonium salt under the same conditions, the  $\alpha$ -amino-group and ammonia being liberated from the "internal salt" and the ammonium salt respectively, and the potassium salts of the acid radicles formed. With its basic  $\alpha$ -amino-group liberated from the "internal salt" the amino-acid can be regarded as a true substituted ammonia, which would be expected to behave similarly to ammonia and the simple amines in forming no compound with phenolphthalein in alcoholic solutions.

The effect of alcohol in different concentrations upon the basicity of the  $\alpha$ -amino-group of amino-acids, was studied in the manner already described for investigating the basicity of ammonia and the amines (p. 453). The results obtained in two typical cases, viz.: histidine and arginine are given later (see Tables V and VI). Curves constructed from these data follow the same general contour as those obtained in a like manner for ammonium chloride and the hydrochlorides of the amines, the readings reaching the maximum at 80 to 85 % alcohol.

*The abnormal behaviour shown by certain amino-acids titrated in alcoholic solution.*

As already stated, certain amino-acids, more particularly aspartic acid, glutamic acid and proline, gave low results when titrated in their alcoholic solutions, although others behaved in accordance with the "internal salt" hypothesis (p. 455). Further work will be necessary before the deficiencies can be satisfactorily explained. Several possible explanations were considered. The following alternative suggestions both appear to receive support from the available evidence:

(1) That low titration values may be due to the union of a carboxyl group with alcohol.

(2) That condensation probably occurs in the alcoholic solutions.

At a much later stage, the discovery was made that the carboxyl groups of amino-acids will titrate in aqueous acetone in the same manner as they do in aqueous alcohol (see p. 457). Suggestion No. 1 is supported by the fact that aspartic acid, glutamic acid and proline, gave practically quantitative results when titrated in about 84 % acetone using phenolphthalein as indicator.

A comparison of the behaviour of aspartic acid and asparagine, on the other hand, seems to favour suggestion No. 2:



Group (a) of aspartic acid titrated accurately in aqueous solution. On adding the stated amount of alcohol to this neutralised solution, group (b) required only about half the calculated equivalent of alkali to produce the faintest possible pink colour, the endpoint being very indefinite. The solution became faintly opalescent when the alcohol was added. These facts seem to support the suggestion that some kind of loose condensation, between group (b) of one aspartic molecule and the amino-group of another occurred in the alcoholic solution. Group (b) of asparagine however titrated quantitatively in alcoholic solution with a sharp endpoint. Therefore the phenomenon does not occur when no carboxyl group (a) is present. From this point of view it would appear that group (a) is involved in the condensation. Further investigation is proceeding.

*Conditions under which the method becomes of general application.*

The evolution of a successful general titration method for estimating amino-acids, based upon their behaviour in alcoholic solution, involved the necessity of overcoming the difficulty caused by certain amino-acids giving

low results. In endeavouring to achieve this purpose the significance of Exps. No. 8 of Table I and No. 10 of Table II was appreciated. Reference to these tables will show that the titration values of the "Alcoholic Extract" and the leucine-valine mixture, obtained in alcoholic solution, were slightly increased on adding alcoholic formalin, indicating that formaldehyde might be capable of disrupting any loose combination or condensation. A test was therefore made with glutamic acid. On adding alcoholic formalin to the neutralised alcoholic solution, sufficient further alkali was required to bring the total titration value up to the calculated figure. Subsequently every amino-acid giving a low result when titrated in alcohol alone was found to titrate quantitatively on adding alcoholic formalin. An accurate general method was therefore possible, provided those amino-acids which behaved normally in alcohol alone continued to do so in the presence of formaldehyde.

Histidine and tryptophan gave good results when titrated in alcohol alone. As these amino-acids contain an imino-grouping, methylene derivatives capable of neutralising acid when in aqueous solution are formed by the action of formaldehyde. Therefore it was necessary to show that such bases, which presumably would be formed on adding alcoholic formalin to the neutralised alcoholic solution of a complete amino-acid mixture, would be inactive to phenolphthalein, in which case they would not interfere in the general method. A study of the basicity of the basic methylene derivative of diethylamine, obtained by the action of formaldehyde upon the hydrochloride of this amine, showed that bases of this type, in common with ammonia, the amines and the freed amino-groups of amino-acids, do not form ionisable compounds with phenolphthalein if sufficient alcohol is present. The basic methylene derivative ceased to react with the indicator on increasing the concentration of alcohol to 70–75 %. The formation of basic methylene compounds of such amino-acids as histidine and tryptophan would therefore not be likely to vitiate the titration results. This statement was confirmed in actual experiments with these two amino-acids.

*Low readings in alcohol raised on adding acetone.*

Preliminary experiments have shown that acetone has the same effect as alcohol in submerging the basicity of the basic groups of amino-acids towards phenolphthalein. Aspartic acid, glutamic acid and proline, which gave low titration values in alcohol alone, titrated quantitatively in 80–85 % acetone. The behaviour of the other amino-acids has not yet been investigated. As an important differentiation of amino-acids in a mixture may be obtained by first titrating in 85 % alcohol and then continuing the titration after adding alcoholic formalin, the effect of adding acetone as an alternative to alcoholic formalin was tried in the case of aspartic and glutamic acids. These experiments, described later, showed that acetone was equally effective in raising the titration value to the quantitative figure. A thorough investigation of the effect of acetone is proceeding.

*Description of the general method for estimating the carboxyl-groups of amino-acids.*

*Preparation of solution.* A known weight of the amino-acid, or its suitable salt, or of an amino-acid mixture is dissolved in CO<sub>2</sub>-free water and made up to a known volume so that the resulting solution is approximately *N/10* in strength in relation to the carboxyl-groups. In cases such as tyrosine and cystine where the amino-acid is insoluble in water or soluble in too large a volume of water, *N/10* HCl or *N/10* soda or other appropriate means must be used for bringing them into solution so that no separation occurs on adding alcohol. When only a very small quantity of the amino-acid is available it may be weighed accurately into a suitable vessel dissolved in the desired amount of water and the whole of the solution used.

*Stage 1. Titration in water.* A 5 cc. or 10 cc. portion of the solution is titrated with aq. *N/10* soda to phenolphthalein (soda standardised to phenolphthalein).

*Stage 2. Titration in alcohol.* 5 cc. or 10 cc. of the original solution is transferred to a conical flask of about 250 cc. capacity, 10 volumes of 97 % alcohol (rect. sp.) and three drops of phenolphthalein solution added, and the mixture titrated in daylight over a white plate with *N/10* alcoholic potash (standardised to phenolphthalein) until a light pink colour is produced (p. 460). The endpoint is usually obtained quite sharply on adding the last one or two drops of standard alkali (two drops = 0.05 cc.). A correction is made for the original acidity of the alcohol.

*Stage 3. Titration in alcoholic formaldehyde.* After obtaining the reading in Stage 2, the same liquid is used for Stage 3. 12.5 cc. of aqueous formaldehyde solution prepared by diluting colourless formalin, with two volumes of distilled water, and neutralising the mixture to phenolphthalein, is added for each 50 cc. alcohol used in Stage 2, and the titration continued to the same endpoint as before. A mixture of neutralised diluted formalin and alcohol in the same proportions as they have been used is then titrated and the result obtained on completing Stage 3 is corrected accordingly. The acidity of rectified spirit is very slightly increased on adding the neutral formaldehyde solution.

The result obtained in Stage 1 gives useful information when dealing with the dibasic amino-acids, arginine, and salts of amino-acids (see p. 467).

The Stage 2 titration value includes the reading obtained in Stage 1. Several amino-acids are correctly estimated in Stage 2.

The increase in titration value obtained in Stage 3 gives an idea of the character of the amino-acid, and when dealing with an amino-acid mixture affords an indication of the amount of dibasic amino-acids and proline present. The carboxyl-groups of all the amino-acids contained in an amino-acid mixture, except that of arginine, are estimated by the total titration value obtained on completing Stage 3.

To avoid dilution of the alcohol, the use of *N*/10 alcoholic potash instead of *N*/10 soda was adopted. Since four drops of this reagent from an ordinary standard burette measure only 0.1 cc., a further advantage is gained in titrating. The alcoholic potash should be made from absolute alcohol and be free from carbonate. It should be as nearly colourless as possible and carefully guarded against evaporation.

The experiment with the basic methylene derivative of diethylamine showed that acid radicles can be titrated in the presence of such bases, if the concentration of alcohol is not less than 70 to 75 %. The addition of the stated amount of diluted aqueous formalin instead of alcoholic formalin was adopted at this stage, as it was found that sufficient formaldehyde was thus provided and at the same time the concentration of alcohol was not made lower than shown necessary in the experiment referred to. This slight modification proved more convenient.

The titrations should be done promptly in order to minimise any possible effect of CO<sub>2</sub> absorbed from the air.

#### *Wider application of the method.*

It will be evident from a study of the experimental results that the method outlined above is capable of much wider application in the following ways.

1. Aqueous solutions containing salts of ammonia, primary, secondary and tertiary amines and allied bases, either separately or as mixtures, can be investigated quantitatively, by means of Stage 2 of the method. The titration value thus obtained would represent the exact equivalent of the acid radicles present. If the original solution contained free acids as well as these salts, the result would include the equivalent of the free acids. The presence of free organic bases however would not influence the titration value.

2. In the case of mixtures of amino-acids and organic bases, the carboxyl-groups of the amino-acids, not including that of arginine, would be correctly estimated by the total titration value obtained on completing Stage 3 of the method. At the same time, the difference between the total titration value and the result of Stage 2, would give an indication of the content of dibasic amino-acids and proline, if present.

3. Animal products for example, muscle and organ extracts, faeces, urine, milk, blood, etc., vegetable extracts, and fluids containing the products of bacterial growth, may be investigated by the general method after treating in the manner described for preparing the "Alcoholic Extract" (p. 452). In many of these fluids, a mixture of organic acids, amino-acids, and organic bases occurs. The total titration value obtained on completing Stage 3 of the method would represent the total acid radicles present including the carboxyl-groups of the amino-acids, except that of arginine. The increase in titration value obtained by proceeding from Stage 2 and completing Stage 3 would approximately be proportional to the amount of dibasic amino-acids and proline present.

## EXPERIMENTAL SECTION.

The experimental results illustrating the development of the new general method for estimating amino-acids will be given, as far as possible in the same order as they have been referred to in the foregoing section. Since Sørensen's formol titration method had been found to give very deficient results when titrating the acid radicles in salts of secondary amines, and to be unreliable in many cases when used for estimating amino-acids, each solution made up for the purpose of testing the new general method was also submitted to formol titration so that the results obtained by the two methods could be compared. Much difficulty was experienced in the formol titrations owing to the indefinite character of the endpoints (see p. 467). The light pink colour obtained sharply in titrating solutions by the new method was adopted as a standard and attempts were made throughout the formol titrations to obtain a colour of the same intensity. The results given in this section for all the titrations in alcohol or in alcoholic formaldehyde have been corrected for the original acidity of the alcohol or alcoholic formaldehyde.

*The effect of alcohol upon the basicity to phenolphthalein of ammonia, primary, secondary and tertiary amines.*

Standard aqueous solutions of the pure hydrochlorides of these bases, approximately *N*/10 in strength, were made up and 5 cc. or 10 cc. portions from each solution were titrated as described in Table III.

Table III.

Col. 1	2	3	4	5
Ref. No.	Substance	Formol titration <i>N</i> /10 alkali cc.	Titration in 85-90 % alcohol <i>N</i> /10 alkali cc.	Theoretical value of the acid radicle in the portion taken <i>N</i> /10 alkali cc.
1	Ammonium chloride	9.3	9.35	9.35
2	Ammonium sulphate	—	9.95	10.0
3	Monoethylamine hydrochloride	5.9	6.1	6.13
4	Dimethylamine hydrochloride	3.1 to 3.9	6.8	6.72
5	Diethylamine hydrochloride	0.85 to 1.0	9.02	9.13
6	Trimethylamine hydrochloride	0	4.72	4.6

*Remarks.* On adding the alcohol to the aqueous ammonium sulphate the salt separated. It was found that the separation could be prevented by previously dissolving 10 % glycerol in the alcohol. This mixture was neutralised before use. In the case of Nos. 4, 5 and 6 in Col. 4 the endpoints were especially sharp. The endpoints of Nos. 4 and 5 in Col. 3 were very indefinite. The figure for No. 6 in Col. 5 was determined by analysis.

*Discussion.* The results of the titrations in alcohol were practically quantitative in all cases.

Formol titrations of the salts of secondary amines showed that the basic methylene compounds were capable of neutralising the acid radicles to a large extent. The acid radicle of the tertiary amine salt possessed no formol titration value. It is interesting to note that methylene derivatives of secondary amines, *e.g.*  $(C_2H_5)_2.N.CH_2.N.(C_2H_5)_2$  are tertiary amines, not sufficiently strong however to titrate quantitatively to phenolphthalein. This fact limits the usefulness of the formol titration method.

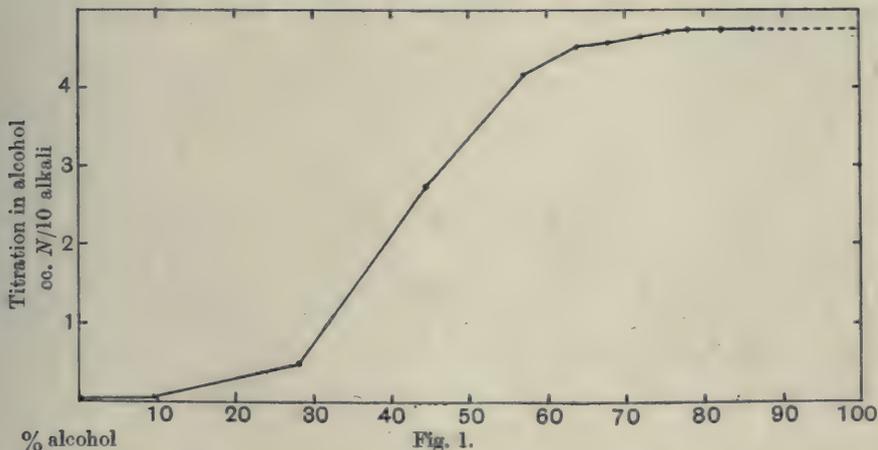
*The effect of alcohol in different concentrations upon the basicity of trimethylamine.*

5 cc. of the standard aqueous solution of the hydrochloride used in Exp. No. 6 of Table III was diluted with 5 cc. water. 97 % alcohol was added in small quantities at a time, and the liquid titrated with *N*/10 alcoholic potash after each addition. As too much alcohol had been added in the third increment a further 10 cc. water was added after taking the third reading. The readings were corrected for the original acidity of the alcohol, and the percentage of alcohol in the solution at the end of each titration was calculated.

The results given in Table IV are shown graphically in Fig. 1.

Table IV.

97 % alcohol cc.	none	1.0	3.5	13.5	23.5	33.5	43.5	53.5	63.5	78.5	103.5	153.5
Readings (corr.) <i>N</i> /10 alcoholic KOH	cc. 0.02	0.07	0.5	2.78	4.19	4.5	4.59	4.67	4.73	4.78	4.78	4.7
% alcohol	0	9.4	28.0	43.8	56.7	63.7	68.7	72.4	75.2	78.3	82.1	86.2



*Discussion.* The reading reached its highest point at 75–78 % alcohol, showing that at this concentration the free base is inactive to phenolphthalein. Similar results have been obtained on titrating ammonium chloride and the hydrochlorides of the other amines in the same way.

*The effect of alcohol upon the basicity of the basic groups of amino-acids towards phenolphthalein.*

The results of the experiments will be found in Table X, p. 466.

*The effect of alcohol in different concentrations upon the basicity of the  $\alpha$ -amino-group of amino-acids.*

*Experiment 1.* 0.524 g. histidine monohydrochloride ( $C_6H_9O_2N_3, HCl \cdot H_2O$ ) was dissolved in water and made up to 50 cc. 10 cc. of the solution were neutralised to phenolphthalein with aq. *N*/10 soda. 5.1 cc. were required. Successive quantities of 97 % alcohol were then added, and the liquid titrated after each addition, exactly as described in investigating the basicity of trimethylamine. The results are given in Table V.

Table V.

97 % alcohol added cc.	1.0	2.0	3.0	4.0	5.0	10.0	15.0	20.0	30.0	40.0	50.0	75.0	100.0	150.0
Readings (corr.) } <i>N</i> /10 alcoholic potash	0.05	0.2	0.5	1.2	1.7	3.38	3.88	4.22	4.63	4.79	4.88	4.97	5.1	5.12
% alcohol	6.5	12.7	19.0	25.0	30.0	46.0	54.3	60.0	67.8	72.8	76.3	81.8	85.0	88.5

*Remarks.* The endpoints were not so distinct in the first five cases.

*Discussion.* In making up the solution the molecular weight of the salt was regarded as representing two equivalents of acid, one for the chloride radicle and the other for the carboxyl-group. The solution was made exactly *N*/10 on this basis. The chloride radicle in the pure salt titrated practically quantitatively in aqueous solution. According to Plimmer [1915] histidine is alkaline in reaction. The result does not support this statement so far as phenolphthalein is concerned.

The results show that the  $\alpha$ -amino-group of histidine, liberated by the potash, behaved as ammonia and the amines to phenolphthalein in alcoholic solutions.

*Experiment 2.* 0.375 g. very pure arginine dinitrate ( $C_6H_{14}O_2N_4, 2HNO_3$ ) was dissolved in water and made up to 50 cc. The solution was thus exactly *N*/20 in relation to the  $2HNO_3$ . 10 cc. were neutralised with aq. *N*/10 soda to phenolphthalein. 2.5 cc. were required. 7.5 cc. water were then added and the experiment continued exactly as described in the case of the histidine hydrochloride. The results are given in Table VI.

Table VI.

97 % alcohol cc.	1	1½	2	3	4	5	10	15	20	30	40	50	100	150	200
Readings (corr.) } <i>N</i> /10 alcoholic potash	0.02	0.07	0.15	0.25	0.54	0.69	1.64	2.08	2.27	2.45	2.47	2.5	2.55	2.55	2.55
% alcohol	4.8	7.1	9.4	13.6	18.0	21.6	35.9	44.9	51.3	60.2	66.1	70.4	81.3	85.8	88.3

*Discussion.* As explained later (p. 468), on adding alcohol to the neutralised aqueous solution, the  $\alpha$ -amino-group of arginine was liberated by the alkali from its internal combination with the carboxyl-group. The basicity of the free  $\alpha$ -amino-group to phenolphthalein was completely submerged when the concentration of alcohol was increased to a sufficient extent.

*The effect of different concentrations of alcohol upon the basicity to phenolphthalein of the basic methylene derivative of diethylamine.*

The basic methylene derivative was made by adding 30 cc. neutralised diluted aqueous formalin (2 vol. water to 1 vol.) to 10 cc. of the standard aqueous solution of diethylamine hydrochloride used in the experiment No. 5 of Table III. The mixture was neutralised with *N*/10 soda to phenolphthalein, 1.0 cc. being required. Alcohol was then added in small quantities at a time and the experiment continued exactly as described in the case of the salts of trimethylamine, histidine and arginine. The results are given in Table VII.

Table VII.

97 % alcohol cc.	none	1.0	2	3	5	10	20	33	40	50	75	100	150	175
Readings (corr.) <i>N</i> /10 alcoholic potash } cc.	1.05	1.2	1.5	2.39	4.09	6.38	7.96	8.53	8.77	8.85	9.05	9.15	9.2	9.22
% alcohol	2.56	5.1	7.9	11.7	18.2	28.5	40.3	49.8	53.7	58.1	66.0	71.2	77.7	79.9

*Discussion.* The acid radicle derived from the original amine salt titrated quantitatively when the concentration of alcohol was increased to 71 %. The basic methylene derivative, in common with ammonia, the amines and the free  $\alpha$ -amino-group of amino-acids forms no ionisable compound with phenolphthalein when sufficient alcohol is present.

*The low values given by certain amino-acids when titrated in alcohol are raised on adding formaldehyde to the neutralised alcoholic solutions.*

The experimental evidence will be given in Table X, p. 466.

*Effect of acetone upon the basicity of the basic groups of amino-acids towards phenolphthalein.*

A thorough investigation of this question is proceeding. Up to the present only four of the amino-acids have been tested. Portions of standard aqueous solutions were first titrated with aq. *N*/10 soda to phenolphthalein. Acetone was then added, and the titrations continued. The results are given in Table VIII. Corrections have been made for the original acidity of the acetone.

*Remarks.* Glycine separates from aqueous acetone when the concentration of acetone reaches about 73 %. In Exp. No. 1, the glycine redissolved as the titration proceeded. Only a faint opalescence resulted on adding the acetone in Nos. 3 and 5. The results of Nos. 4 and 6 were obtained by continuing the titration after adding further acetone to the neutral liquids from Nos. 3

and 5 respectively. A small separation which remained in suspension was produced by the additional acetone in each case. This probably consisted of the sodium salts, as apparently the titration of the carboxyl-group was not disturbed.

In the case of proline about 0.2 cc. further soda was required on adding more acetone.

Table VIII. Preliminary experiments.

Ref. No.	Substance	Titration in aqueous solutions aq. N/10 soda cc.	Further aq. N/10 soda required on adding acetone cc.	Total titration value cc.	% acetone at the end of titration	Theoretical total titration value of portion taken cc.
1	Glycine	0.05	5.2	5.25	83.3	5.0
2	Proline	0.15	5.1	5.25	76.3	5.28
3	Aspartic acid	3.75	3.7	7.45	75.1	7.5
4	" "	3.75	3.8	7.55	84.8	7.5
5	Glutamic acid hydrochloride	9.9	4.75	14.65	71.8	15.0
6	" "	9.9	5.0	14.9	83.4	15.0

*Discussion.* In the case of glutamic acid a higher concentration of acetone than 71.8 % was required. The results show that the carboxyl-groups of these amino-acids titrate practically quantitatively if sufficient acetone is present. The general effect of acetone resembles that of alcohol.

*The low titration values given by certain amino-acids in alcohol are raised on adding acetone to the neutralised alcoholic solutions.*

Up to the present only aspartic and glutamic acids have been investigated. Portions of standard aqueous solutions were treated with the requisite amount of alcohol and titrated with aq. N/10 soda to phenolphthalein. Acetone was then added, and the titrations continued. The results are given in Table IX.

Table IX. Preliminary experiments.

Col. 1	2	3	4	5
Substance	Titration in alcohol, aq. N/10 soda cc.	Total titration value on adding acetone, N/10 soda cc.	% acetone at the end of titration	Theoretical total titration value of the portion taken cc.
Aspartic acid	6.3	7.6	28.1	7.5
Glutamic acid hydrochloride	12.65	14.85	45.0	15.0

In these two experiments the endpoint colour of the indicator showed a tendency to fade when obtaining the figures of col. 3. This phase extended over a range of about 0.2 cc. The fading was probably due to the effect of CO<sub>2</sub> absorbed from the air. This point needs further investigation.

The results show that to whatever cause the low titration values in alcohol are attributed (see p. 456) the addition of acetone or formaldehyde brings

about a disturbance of the equilibrium so that the total titration values are practically quantitative. It is evident that the action of these two reagents requires thorough investigation.

*Estimation of carboxyl-groups of amino-acids by the new method.*

Accurate *N*/10 solutions of the amino-acids were made up, the quantity necessary for the purpose in each case being calculated on the basis of the number of carboxyl-groups in the molecule. In the investigation of the amino-acid salts, the acid radicles as well as the carboxyl-groups were taken into account in the calculations. When dealing with mixtures, the calculation was based on the nitrogen content. A description of the specimens used and the modified procedure adopted for amino-acids of low solubility are given below:

1. Glycine pure.
2. Leucine 10.8 % N.
3. Phenylalanine, very pure, prepared from the pure hydrochloride.
4. Tyrosine, very pure. Three times crystallised.

0.0906 g. was dissolved in 7.5 cc. aq. *N*/10 HCl by dipping the vessel into hot water for a few seconds. After cooling, the requisite amount of alcohol was added, and the titration done promptly. Allowance was afterwards made for the acid used.

5. Cystine, very pure. Colourless hexagonal plates under microscope.

0.0601 g. was dissolved in 6 cc. cold aq. *N*/10 alkali. The right amount of alcohol was then added and the excess of alkali determined by titrating with *N*/10 HCl.

6. Tryptophan, very pure. Colourless glistening platelets.

0.102 g. was dissolved in 5 cc. water at about 85°, cooled, the alcohol added and titrated promptly.

7. Proline. Completely soluble in cold absolute alcohol.

Crystallised in desiccator. M.P. 216°. Contained only traces of impurity represented by a very small content of amino-nitrogen.

8. Asparagine. Large crystals.

9. Aspartic acid. Contained traces of insoluble impurity.

0.3328 g. dissolved in about 40 cc. hot water, cooled and made up to 50 cc.

10. Glutamic acid hydrochloride. Twice crystallised.

11. Histidine monohydrochloride.  $C_6H_9O_2N_3$ , HCl.H<sub>2</sub>O. Very pure.

M.P. 255–256°.

12. Arginine dinitrate  $C_6H_{14}O_2N_4$ , 2HNO<sub>3</sub>. Very pure.

Specimen completely freed from all traces of free HNO<sub>3</sub> by standing over potash in a desiccator for a long time, then thoroughly dried over CaCl<sub>2</sub>. M.P. 145° (uncorr.) decomposing 150–160°.

13. Lysine picrate, pure recrystallised.

0.0938 g. dissolved in 10 cc. water by dipping vessel into hot water for a few seconds. Alcohol then added and the mixture titrated at once.

The results are given in Table X.

Table X.

Ref. No.	Col. 1 Substance	2 Titration in aqueous HCHO. Solutions not neutralised previously. aq N/10 soda cc.	New General Method			6 Theoretical total titration value of the portion taken N/10 alkali cc.
			3	4	5	
			Stage 1 Titration in water aq N/10 soda cc.	Stage 2 Titration in alcohol N/10 alc. potash cc.	Stage 3 Titration in alcoholic formaldehyde N/10 alc. potash cc.	
<i>Amino-acids:</i>						
1	Glycine	5.05	0.05	4.82	5.15	5.0
2	Leucine	4.3	—	4.7	5.1	5.05
3	Phenylalanine	4.65	—	5.0	5.02	5.0
4	Tyrosine	4.3	—	5.0	5.12	5.0
5	Cystine	4.87	—	5.0	5.05	5.0
6	Tryptophan	—	—	4.92	5.15	5.0
7	Proline	0.82	0.07	2.07	2.6	2.64
8	Asparagine	5.0	0.55	5.02	5.3	5.0
9	Aspartic acid	4.3	2.47	4.2	4.95	5.0
<i>Salts of Amino-acids:</i>						
10	Glutamic acid hydrochloride	6.9	4.9	6.4	7.45	7.5
11	Histidine monohydrochloride	3.75	2.55	5.0	5.05	5.0
12	Arginine dinitrate	4.9	2.5	5.05	5.15	5.0
13	Lysine picrate	4.5	—	4.9	5.05	5.0
<i>Mixtures of Amino-acids:</i>						
14	Glycine, alanine, leucines and valine	6.3	0.2	7.05	7.5	7.5
15	The monobasic amino-acids from the hydrolysis of caseinogen	2.32	—	2.75	3.0	?
16	Mixture of the same quantity of the solution used for No. 10 with half the quantity used for No. 15	—	—	8.12	9.05	9.0

*Remarks.* The titration values obtained in Stage 2 include those obtained in Stage 1.

In obtaining the results for Nos. 7, 9 and 10 in Stage 2, the faintest possible pink colour appeared at 1.75 cc., 3.8 cc. and 6.2 cc. respectively. No. 10 gave 6.6 cc. in Stage 2 when titrated hot.

The titrations of lysine picrate were difficult owing to the light brownish yellow colour of the solutions. Several readings were taken in the presence of an independent observer in the following manner: after each reading the liquid was made acid by adding a known quantity of N/10 HCl and again titrated with N/10 alkali. After allowing for the acid added in each case, the following results were obtained in succession:

Stage 2: 4.9, 4.95.

Stage 3: 5.05, 5.1, 5.05, 5.05. Colour too pronounced at 5.25 cc. No other pure lysine salt was available.

Picric acid was found to titrate quantitatively as a monobasic acid in

both Stages 1 and 2. Potassium picrate separated from the alcoholic solution on standing.

The mixture used in No. 15 consisted of the amino-acids recovered after removing alcohol-insoluble calcium salts from the hydrolytic products of caseinogen.

The results given in Table X may be summarised as follows:

*Formol titration method.*

Glycine and asparagine ...	... .	quantitative results.
Cystine ...	... ..	only 2 % low.
Arginine (net result) ...	... ..	practically neutral.

In all other cases considerable deficiencies were shown.

*New method.*

Stage 1. Quantitative results were obtained for the following carboxyl-groups and acid radicles:

Dibasic amino-acids ...	... ..	One of the carboxyl-groups.
Histidine monohydrochloride ...	... ..	The chloride radicle.
Arginine dinitrate ...	... ..	One of the two nitrate radicles.

Stage 2. Phenylalanine, tyrosine, cystine, histidine, and asparagine gave quantitative titration values:

Tryptophan and lysine, practically quantitative.

Arginine (net result), quantitatively neutral.

Glycine, 3.5 % low.

Leucine and the mixture of glycine, alanine, leucines and valine, 6-7 % low.

Proline, 20-30 % low.

Aspartic and glutamic acids, 30-45 % low in relation to the value of one carboxyl-group.

Stage 3. Practically quantitative results in all cases.

*Discussion. Formol titration method.* In carrying out the titrations in aqueous formaldehyde, 30 cc. diluted formalin (1 vol. to 2 vols. water) neutralised immediately before use were used in each experiment. It was found necessary to dilute the formalin before it could be neutralised precisely to phenolphthalein. In a few cases the titration values were slightly increased on adding further neutral formaldehyde. When this happened the excess was made greater until the reading reached a maximum. No. 14 gave the highest increase obtained in this way, viz. by 0.35 to 6.65 cc. Tyrosine gave an increase by 0.2 cc. to 4.5 cc. In the other cases (including glycine) the increase was only about 0.1 cc.

Phenolphthalein was found to indicate very indefinitely in the formol titrations. Sørensen [1907] appears to have encountered the same difficulty, as he advocated the addition of 0.2 *N* alkali until a deep red colour developed, to be matched with a deep red colour prepared under known conditions in a control solution. The formol results given in col. 2 of Table X were obtained by titrating, until beyond all doubt the solutions were light pink in colour

(p. 460). If the opinion be held that the solutions are still acid, although a light pink colour has developed, then the indicator is unsuitable, and the results are unsatisfactory.

Abnormalities in the formol titration method are fully discussed by Jodidi [1918], who states that accurate results are given when the amino-acid molecule contains "amino- and carboxyl-groups *only*," and that if other basic groups, such as the imino-group, are present, the method is unsuitable. The results obtained in this present investigation confirm the observations of Jodidi save in regard to the monobasic monamino-acids, which all gave low titration values with the exception of glycine. Jodidi attributes the adverse influence of the imino-group to the effect of the basic methylene derivatives produced by the action of formaldehyde.

*The new method.* With the exception of aspartic acid, glutamic acid and proline, the endpoints of the titrations in alcohol (Stage 2) were quite satisfactory. Especially was this the case with those amino-acids which gave quantitative results. All the titrations in Stage 3 of the method gave satisfactory endpoints. The sharpness with which the light pink endpoint appears is well shown on titrating a little pure phenylalanine or trimethylamine hydrochloride in 90 % alcohol. If desired the endpoint so obtained may be used as a standard.

*Arginine.* In making up the standard solution of arginine dinitrate, the molecular weight of the salt was regarded as representing two equivalents in relation to the  $2\text{HNO}_3$ . The solution was made exactly  $N/20$  on this basis. 10 cc. required 2.5 cc.  $N/10$  alkali when titrated in aqueous solution, and double this quantity when titrated in alcoholic solution. The salt therefore behaved as a monobasic acid in water and as a dibasic acid in alcohol. This behaviour can only be explained by assuming that the guanidine nucleus is combined with one  $\text{HNO}_3$  in the aqueous solution of the dinitrate, and that the other  $\text{HNO}_3$  combines with an equivalent of alkali during the titration. In the neutralised aqueous solution therefore the  $\alpha$ -amino-group and the carboxyl-group form an "internal salt." On adding the requisite amount of alcohol to the neutralised aqueous solution, the alkali equivalent of the carboxyl-group is required as in the case of other amino-acids. Consequently, the guanidine nucleus reacts as a univalent base to phenolphthalein in both aqueous and alcoholic solutions. This is the only nitrogenous base or basic group of those investigated up to the present, possessing the power of reacting with the indicator in alcoholic solution. Other guanidine derivatives and guanidine have been investigated under the same conditions. The results show that the reaction to phenolphthalein in alcoholic solution is a common property when the molecule contains a NH grouping attached to a carbon atom by a double bond. An account of this investigation will be given in another communication.

The neutrality of arginine to phenolphthalein in alcoholic solution distinguishes it from other amino-acids. The utilisation of this character as the

basis of a new method for estimating arginine in the hydrolytic products of proteins is under investigation.

Since the completion of the experimental work described in this paper, I found that Victor Birekner [June, 1919], when titrating grain extracts, noted that the acidity to phenolphthalein increased on adding alcohol to the aqueous solutions. The phenomenon was attributed to amino-compounds in the extracts. Birekner's main conclusions were summarised by him as follows: "Amino-acids which in aqueous solution are nearly neutral to phenolphthalein react distinctly acid in the presence of alcohol. This fact should be taken into account when making acidimetric titrations in alcoholic liquids containing amino-compounds such as various animal and vegetable extracts." The amino-acids Birekner tested were those which do not give quantitative results even in much higher concentrations of alcohol than he employed.

The fact that amino-acids become acid in alcoholic solutions was discovered quite independently and by an entirely different route.

#### *Wider application of the method.*

Further evidence of the quantitative character of the estimation of total acid radicles including the carboxyl-groups of amino-acids, by the new method, was obtained by titrating known mixtures of glycine and butyric acid. The results corresponded with the amounts known to be present.

The "Alcoholic Extract" which had been stored for several months, since the experiments of Table I were carried out, was again investigated by the method. 50 cc. required 23.37 cc. *N*/10 alkali in Stage 2 and 23.75 cc. in Stage 3. By comparing these figures with those given in Table I it will be noted that such preparations as the "Alcoholic Extract" can be preserved for a considerable time without incurring appreciable loss or change.

A definite quantity of the solution of the mixture of amino-acids used in Exp. No. 15 of Table X was added to a portion of the "Alcoholic Extract." The titration values obtained in Stages 2 and 3 were found to include the effect of the additional carboxyl-groups quantitatively.

It is evident that by the use of this method, data may be accumulated quickly in the investigation of many important problems, amongst which the following may be mentioned.

(1) The order of breakdown of the constituents of animal matter by the organisms of putrefaction, with a view to obtaining clues as to the best means of detecting the early stages of putrefaction in meat or fish, so that the quality of these materials may be gauged.

(2) The breakdown, separately in artificial media, of proteins, fats and carbohydrates by enzymes and bacteria. The action of pathogenic or other organisms in pure culture upon various sugars and amino-acids. Decarboxylation of amino-acids.

(3) Rapid estimations of organic acids in faeces with the object of throwing

light upon the work of organisms in the intestines as affected by unsuitability of rations or disproportion in the essential food constituents.

(4) Comparison of foods by experiments designed to imitate digestion.

(5) Investigation of extracts of agricultural products such as silage.

(6) Rate of hydrolysis of proteins, and the investigation of the order in which the amino-acids are split off.

(7) Investigation of the purity of specimens of amino-acids or their salts. It will be noted that the amino-acids are not destroyed during their investigation by means of Stage 2 of the method, and can therefore be recovered. When only small amounts of material are available this fact is of obvious importance.

Further investigations are proceeding in these directions.

#### *The rapid estimations of volatile bases and amino-acids.*

In the older method for estimating volatile bases and amino-acids in "Alcoholic Extracts" [Foreman and Graham-Smith, 1917], it was necessary to distil the bases at a low temperature in order to avoid the decomposing effect of the excess of alkali upon the amino-acids. A considerable amount of time and attention was therefore required in carrying out the distillations.

The total titration value of an aliquot portion of the "Alcoholic Extract," determined by completing Stage 3 of the new method, affords an exact measure of the amount of alkali necessary for liberating the bases from their salts. If no more than this amount be added a rapid method of distillation at a higher temperature can be adopted because decomposition of amino-acids is reduced to a minimum. The residual liquid becomes aqueous as the alcohol is removed and the alkali salts of the amino-acids hydrolyse, so that an amount of free alkali equivalent to the amino-acids is found in the aqueous solution.

An improvement in the titration of the volatile bases distillate is rendered possible by finding that a sharp endpoint can be obtained in all concentrations of alcohol when alizarin is used as an indicator. The endpoint is so sharp that one drop  $N/10$  alkali in excess produces a very decided colour change. As the distillates can be titrated without adding water, a further advantage is gained by using alizarin instead of methyl orange.

The method used was as follows:

50 cc. "Alcoholic Extract" was placed in the flask of an ordinary steam distilling apparatus and the correct amount of  $N/10$  alcoholic potash added. This amount had been ascertained in another portion by completing Stage 3 of the new method. A rapid current of steam derived from distilled water which had been freed from  $\text{CO}_2$  by prolonged boiling, was passed through the solution until a foam nearly filling the flask developed. Air was admitted into the flask in which the steam was generated in time to prevent any of the foam from passing into the condenser. The foam usually appeared in four to five minutes. The steam inlet tube was then raised above the surface of the

liquid and the apparatus swept by a further current of steam for one minute. The bases with the alcohol were carried from the condenser to the receiver by a tube which dipped into a known quantity of  $N/10$  acid. The excess of acid in the alcoholic contents of the receiver was determined by titrating with  $N/10$  soda (standardised to alizarin) using alizarin as indicator. The amount of acid equivalent to the volatile bases was estimated by difference. The distillates need no dilution with water before titrating. The indicator can be added in the form of a weak aqueous emulsion, or in 0.5 % alcoholic solution.

The flask containing the residual liquid, which appeared to be quite free from alcohol, was cooled under the tap, the steam inlet tube washed with water and removed, and the solution titrated without delay with  $N/10$  acid to phenolphthalein. Even if a very small amount of alcohol were present, its influence upon the titration value in the aqueous solution would be inappreciable (see Tables V and VI). The content of free alkali in the residual liquid represents approximately the difference between the total titration value of the carboxyl-groups of the amino-acids and the effect of those groups which titrate to phenolphthalein in aqueous solution.

By this method, the bases from three successive 50 cc. portions of the "Alcoholic Extract" neutralised 24.15 cc., 24.05 cc. and 24.15 cc.  $N/10$  acid. The figure obtained by the older method several months earlier, for the same quantity of extract was 0.7 cc. higher. It was possible, however, for small losses to occur during the interval through exposing the extract to the air whilst removing aliquot portions. A further experiment showed that the whole of the volatile bases can be estimated in a single distillation. In one case 50 cc. alcohol were added to the residual liquid and the resulting solution steam distilled, as before, into a fresh quantity of  $N/10$  acid. Only 0.1 cc. further acid was neutralised, making a total of 24.15 cc. for the two distillations.

The residual aqueous liquids consistently required 2.05 cc.  $N/10$  acid for neutralisation to phenolphthalein. The figure obtained by the formol titration for the amino-acids several months earlier was 2.1 cc.

The effect of the process upon the composition of added amino-acids was tested. The complex amino-acid solution used in Exp. No. 15 of Table X was used for this purpose. A quantity possessing a total titration value of 3.0 cc. was added to 50 cc. "Alcoholic Extract," and steam distilled according to the method. The titration of the residual liquid gave a result 0.65 cc. too low, showing that a certain amount of change occurred during the short exposure of the added amino-acids to a temperature of  $100^{\circ}$ . It should be noted that the concentration of free alkali in the residual liquid is greater when more amino-acids are present. In this experiment the volatile bases result was the same as obtained previously, viz. 24.05 cc. It appears therefore that whatever change in the composition of the amino-acids occurred, volatile bases were not produced. Consequently the rapid method, which can be

completed in seven or eight minutes, seems to be very reliable so far as the volatile bases are concerned. Further attempts are being made to obtain an accurate separate figure for the amino-acids.

#### SUMMARY AND CONCLUSIONS.

1. Ammonia, primary, secondary and tertiary amines, and basic methylene derivatives of secondary amines, do not form ionisable compounds with phenolphthalein in alcoholic solutions containing water, if the concentration of alcohol is sufficiently high.

2. In aqueous alcoholic solutions of the salts of these bases the acid radicles can be titrated accurately with *N*/10 alkali, using phenolphthalein as indicator, if more than about 80 % alcohol is present.

3. When aqueous-alcoholic solutions of certain amino-acids containing about 85 % alcohol are titrated with standard alcoholic potash, the amino- or imino-groups liberated from their "internal salt" combinations, resemble ammonia and the amines in showing no basicity to phenolphthalein, and the carboxyl-groups are accurately estimated.

4. Other amino-acids, more particularly dibasic amino-acids and proline, give low results when titrated in alcohol under these conditions, possibly owing to loose combination of alcohol with a carboxyl-group, or loose condensation. The subsequent addition of formaldehyde or acetone, however, results in a disturbance of the equilibrium, so that the carboxyl-groups titrate quantitatively.

5. The effect of acetone upon the basicity of the basic groups of amino-acids to phenolphthalein is similar to that of alcohol. The amino-acids tested up to the present have all given practically quantitative results when titrated with *N*/10 alkali in aqueous acetone containing 80–85 % acetone.

6. The guanidine nucleus of arginine behaves differently from other nitrogenous bases or basic groups by titrating quantitatively as a univalent base in alcohol as well as in water. An explanation of this unique behaviour will be given in a further communication. Arginine is neutral to phenolphthalein in alcoholic solutions if sufficient alcohol is present, the carboxyl-group and the guanidine nucleus exactly neutralising one another.

7. A simple general method for accurately estimating the carboxyl-groups of amino-acids, based upon the foregoing conclusions, is described.

8. It has been shown further that the method is capable of much wider application. The total acids (including the carboxyl-groups of amino-acids) whether in the free state or combined with organic bases, can be accurately estimated in alcoholic preparations made from aqueous fluids such as those which contain the products of bacterial growth.

As the titration value is unaffected by the free bases their removal is

unnecessary. Consequently the solutions need no heating, and there is no risk of decomposition.

9. A rapid method for estimating volatile bases is also described.

Finally I wish to thank Mr C. E. Bryant for assistance in carrying out the experiments and for acting as an independent observer of the endpoints of the titrations.

#### REFERENCES.

- Birekner (1919). *J. Biol. Chem.*, **38** [No. 2, June], 245.  
Foreman and Graham-Smith (1917). *J. Hygiene*, **16**, 144.  
Jodidi (1918). *J. Amer. Chem. Soc.*, **40**, 1031.  
Mann (1906). *Chemistry of the Proteids*, p. 211.  
Plimmer (1915). *Practical Organic and Bio-Chemistry*, p. 285.  
Sørensen (1907). *Biochem. Zeitsch.*, **7**, 79.  
Sutton (1900). *Volumetric Analysis*, 8th edition, p. 38.  
Walker (1904). *Zeitsch. physikal. Chem.*, **40**, 82.

## XXXIX. ADSORPTIVE STRATIFICATION IN GELS. IV.

By SAMUEL CLEMENT BRADFORD.

(Received April 7th, 1920.)

(With Plates VII and VIII.)

DIRECT proof has already been given [Bradford, 1920] of the fundamental rôle of adsorption in the formation of banded precipitates. Unfortunately, owing to its extremely complex nature, mathematical analysis of the phenomenon appears impossible. The adsorbing surface at each band is affected by the mass of the precipitate and by the various factors of von Weimarn's formula, which vary, in an intricate way, with the concentrations of the reaction components. Moreover, notwithstanding statements to the contrary, the diffusion of the hypertonic reagent appears, at times, to be considerably affected by the mass and structure of the precipitate through which it has to percolate. In this connection it is sufficient to compare the extreme slowness of the diffusion of  $N/5$   $\text{Pb}(\text{NO}_3)_2$  solution into  $N/20$   $\text{K}_2\text{CrO}_4$  agar gel, with the rate of diffusion into  $N/200$   $\text{K}_2\text{CrO}_4$  agar gel of the same solution, which reaches the base of the tube in 14 days. As in the case quoted, band formation does not seem to occur unless the precipitate allows of ready diffusion of the hypertonic reagent. This may be one reason why very colloidal precipitates do not form good bands. But this effect may also be due to the sudden separation of too large a mass of precipitate at one time, owing to excessive supersaturation. The complexity of the phenomenon, generally, is further increased by the influence of the reaction components on the formation of the precipitate, which was first pointed out by Bechhold [1905], and is confirmed in a remarkable way by the experiments to be described. It appears hopeless, therefore, at present, to make exact deductions which can be tested quantitatively.

The precipitate, produced by the interaction of an aqueous reagent on a gel containing a suitable solute, will create a solid boundary surface between the reagent and the solute, at which, by Gibb's law, the concentrations of both dissolved substances will usually increase. If the aqueous reagent is hypertonic to the gel, its concentration at the solid surface will be in excess of that of the solute in the gel, and the reagent will be able to diffuse into the gel. With a hypotonic aqueous reagent the adsorption of the gel solute will usually be greater, so that the aqueous reagent will be precipitated, as it collects at the solid surface, and will be unable to pass into the gel. This is Pringsheim's law. The result, however, depends on the formation of a precipitate. Hitherto, it does not always appear to have been realised that, in

the absence of a precipitate, diffusion can take place in both directions. Coloured reagents make this apparent. When strong ammonium hydroxide is poured on a gel containing copper sulphate the deep blue colour gradually diffuses both ways, until, after 48 hours, the solution and the gel have become the same colour throughout.

Since the separation of the strata depends on adsorption of the gel solute from the region between, it follows that the concentration of the hypotonic reagent must be of primary importance. The distance between the bands should usually be affected more by variations in the concentration of the gel solute than of that of the aqueous reagent. Moreover, adsorption experiments with coloured reagents indicate that the concentration of the gel solute in the direction away from the precipitate may increase very slowly at first, and then quite suddenly, as the maximum concentration of the gel is approached. This would tend to eliminate the influence of the valency of the reaction components in the metastable product, since the critical value, at which precipitation takes place, would be reached at approximately the same point where the sudden rise in concentration occurs. For the same reason this narrow region is the only place where any kind of supersaturation should happen.

These conclusions are subject to modification on account of the inhibiting, or precipitating, action of the various ions present, but they are fairly well borne out by experiment. Four tubes containing: (1) *N*/80 CaCl<sub>2</sub> agar gel, (2) *N*/50 CaCl<sub>2</sub> agar, (3) *N*/70 Na<sub>2</sub>CO<sub>3</sub> agar and (4) *N*/20 Na<sub>2</sub>CO<sub>3</sub> agar, treated with: (1) and (2) 2*N*/1 Na<sub>2</sub>CO<sub>3</sub> and (3) and (4) *N*/10 CaCl<sub>2</sub> solutions gave bands at distances (cm.) apart as follows:

(1)	(2)	(3)	(4)
0.57	0.35	0.65	0.15
0.73	0.45	0.65	0.22
1.00	0.55	0.85	0.225
		0.95	0.225
			0.275
			0.225
			0.50
			0.40
			0.55

For a given pair of reagents the figures are roughly inversely proportional to the concentration of the gel.

In another set the tubes contained: (1) and (2) *N*/30 KI agar and (3) and (4) *N*/60 KI agar. These were treated with: (1) and (3) *N*/1 Pb(NO<sub>3</sub>)<sub>2</sub> solution and (2) and (4) *N*/2 Pb(NO<sub>3</sub>)<sub>2</sub> solution. In this case halving the strength of the hypertonic reagent increased the distances of the bands only very slightly, while a corresponding change in the hypotonic reagent caused an approximate doubling of the distance apart of the strata. The inversely proportional effect comes out although the ionic product contains the square of the concentration of the hypotonic reagent.

In a third series the tubes contained: (1) and (3)  $N/100$   $K_2CO_3$  agar and (2) and (4)  $N/200$   $K_2CO_3$  agar. On these were poured 10 cc. each of: (1) and (2)  $N/5$   $Pb(NO_3)_2$  and (3) and (4)  $N/10$   $Pb(NO_3)_2$  solutions. The following curves, Fig. 1, were drawn freely through points obtained by plotting the distances apart of the bands, as ordinates, against the distances below the surface of the gel of the mid-points between the bands, as abscissae.

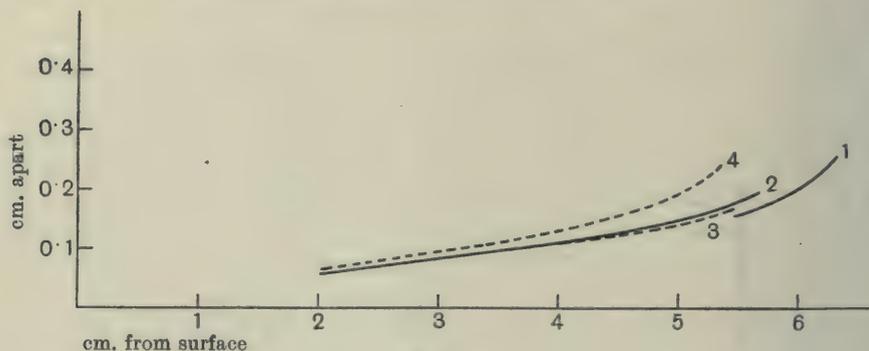


Fig. 1.

The very slight difference caused by halving the strength of the hypertonic reagent is apparent, as compared with the much greater effect of a similar change in the gel solute.

Similar reasoning shows that different effects should be obtained according to which reagent of a pair is dissolved in the gel, or to the actual solute chosen to provide a given reaction component. For example bands of lead carbonate should depend on whether the lead salt or the carbonate is dissolved in the gel, and on the particular soluble lead salt and carbonate used. 1% agar gels containing: (1)  $N/100$   $K_2CO_3$ , (2)  $N/100$   $Pb(NO_3)_2$  and (3)  $N/100$   $Na_2CO_3$  were treated with: (1) and (3)  $N/10$   $Pb(NO_3)_2$  and (2)  $N/10$   $K_2CO_3$  solutions. The remarkable differences in the bands are shown in Figs. 1, 2 and 3, Pl. VII. The very fine lamination in (2) does not show clearly in the figure. With sodium carbonate as hypotonic reagent the bands are noticeably less regular than with the potassium salt.

It has already been shown [Bradford, 1918] that, since adsorption must depend directly on the specific surface of the precipitate, the formation of bands is determined by the value of  $N$  in von Weimarn's formula

$$N = K \cdot \frac{P}{L}.$$

Recently, this expression has been criticised by Buchner and Kalf [1920]. Their experiments show that, in a given case, the dispersity of the precipitate is proportional to  $P/L$ , that is

$$N \propto \frac{P}{L}.$$

They contend, however, that the factor  $K$  in von Weimarn's formula is not directly proportional to the viscosity of the reaction medium and to the

degrees of aggregation, chemical (= complexity of structure) and physical, of the reaction components. Thus they obtained precipitates in the gel state with values of  $P/L$  varying from 75, 140 and 360 in the case of  $BaF_2$ ,  $CaSO_4$  and  $PbI_2$ , to about  $10^4$ ,  $10^6$ ,  $10^7$  and  $10^8$  for  $BaSO_4$ ,  $AgCl$ ,  $AgBr$  and  $AgI$  respectively, and they argue that, while chemical complexity has no exact meaning, the physical aggregation and viscosity cannot be so great as to account for these differing values.

That the chemical complexity of the molecule cannot be ignored is shown by glancing through the pages of Beilstein where its undoubted influence on the form in which a substance occurs may easily be traced. There is, however, an important factor which influences the value of  $P$ , which does not seem to have been taken into account hitherto. This is the amount of mixing of the reagents which happens before a precipitate is formed. It can easily be shown that in the case of a substance such as  $PbI_2$ , for example, where

$$H = Pb^{++} \times I'^2,$$

the maximum value of  $P$  occurs when two volumes of the iodide solution mix with one volume of the solution of lead salt. In a given case, the degree of mixing before precipitation would certainly be affected by the metastable limit as modified by the various reaction components. The extraordinary effect of  $H$  on the form of the precipitate is shown by the experiments with the silver chromates which follow. Thus it will be seen that the value of  $P$  may easily differ greatly from that calculated on the assumption of immediate mixing of equivalent volumes of the reacting solutions. It seems probable that the remarkably different values of  $P/L$  found by Buchner and Kalfi may be due, very largely, to variations in the corresponding metastable limits.

It must further be noted that  $L$  is not the normal solubility of the given substance, but its solubility under the actual conditions of the experiment. Again, the permanency of form of the precipitate may be influenced by the presence of the reaction components, which may tend to produce double salts, or otherwise to peptise the precipitate.

However, these considerations emphasise the difficulty of evaluating the factors in von Weimarn's formula, and it might be preferable to write it

$$N = U \cdot \frac{P}{L},$$

where  $U$  is a function, as yet undetermined, of the viscosity of the medium and of the size and structure of the particles in solution, whether due to physical aggregation or chemical complexity. That is to say the formula is, as yet, an incomplete expression of the truth. It is, nevertheless, most useful as a working rule.

It will be seen, therefore, that banded precipitates can be produced only when the specific surface of the precipitate is great enough to cause the exhaustion of the solute from the neighbouring region of gel. Decrease in

$N$  leads to diminution in adsorbing surface until stratification ceases. On the other hand, increase in  $N$  may be accompanied by increase in density of the precipitate and slower diffusion of the precipitant through the column of precipitate, until the precipitant cannot permeate the precipitate fast enough to traverse the exhausted region of gel immediately beyond before this is replenished with solute by diffusion from the gel. This is often noticed with lead salts, which, frequently, do not form strata unless the hypotonic reagent is very dilute, usually about  $N/200$ . When the gel solute is too concentrated, a dense, continuous band of precipitate grows until all the solute has been exhausted from the gel. This generally happens long before the column has reached the base of the tube, as in Fig. 1 of a previous paper [Bradford, 1920] which shows a column of lead chromate nearly complete.

Since the solute in the gel must be sufficiently dilute to allow of its exhaustion from the neighbourhood of the precipitate, with corresponding limitation of the value of  $P$ , it is clear that stratification can take place only when  $L$  is very small, or when  $K$  is large enough to compensate for a somewhat larger value of  $L$ . This is why only the more insoluble precipitates readily yield banded structures, and these only within comparatively narrow limits of concentration.

Although it has been argued that the adsorption theory leaves the gel out of account altogether [Hatschek, 1919], it will be seen that the above considerations are sufficient to explain the extraordinary differences in the form of a given precipitate when produced in different gels. The reaction medium, by its viscosity and protective action, profoundly modifies the values of  $K$  and  $P$ , and, thereby, the specific surface of the precipitate. The nature of the gel is, therefore, of fundamental importance. Various examples of the effect of the gel on the structure of the precipitate have already been discussed [Bradford, 1918]. Two of the most striking cases, and, perhaps, the most well-known, are those of the chromate and dichromate of silver, which form beautiful brownish red bands in ordinary gelatin gels and yield in agar only macroscopic black crystals. It was pointed out, that the absence of stratification in agar must be due to the small specific surface of the precipitate, and that, by increasing this, it should be possible to produce bands in agar similar to those in gelatin. This has been completely realised by experiment. It further transpires that the original Liesegang phenomenon in ordinary gelatin is due to the presence of impurities.

Fig. 6, Pl. VIII, shows the result of treating  $N/20$   $K_2Cr_2O_7$  1% agar gel with  $N/2$   $AgNO_3$  solution. It is a good illustration of the effect of slow diffusion in producing large crystals of a substance which occurs as a finely divided precipitate when the reacting solutions are quickly mixed. The formation of macroscopic crystals of silver dichromate shows that the number of crystallisation centres,  $N$ , is much too small. The only criterion for the production of bands is a sufficient increase in  $N$ . The application of this principle provides a crucial test for the adsorption theory.

A difficulty in the experimental work is the narrowness of, frequently, both the concentration limits, between which good bands are obtained. For this reason it is possible to make a series of experiments with substances, capable of forming beautiful strata, without obtaining any.

The first experiments were directed towards increase of specific surface by variation in the concentration of the reaction components, or the solubility of the precipitate. The following table shows the result of diminishing the strength of both reagents.

Reagent in the 1 % agar gel	Aqueous reagent	Result
$N/50 \text{ K}_2\text{Cr}_2\text{O}_7$	$N/10 \text{ AgNO}_3$	Red surface layer, clear space 0.25 mm., then small crystals with suggestion of stratification.
$N/75 \text{ K}_2\text{Cr}_2\text{O}_7$	$N/10 \text{ AgNO}_3$	Red surface layer, small crystals arranged distinctly in bands at 1, 4 and 10 mm. below surface.
$N/100 \text{ K}_2\text{Cr}_2\text{O}_7$	$N/20 \text{ AgNO}_3$	Red surface layer, band of microscopic black crystals at 5 mm., part of a red band at 14 mm.
$N/200 \text{ K}_2\text{Cr}_2\text{O}_7$	$N/20 \text{ AgNO}_3$	Red surface layer, band of very small crystals at 1 mm., wide dark red turbidity from 12.5 to 27 mm.
$N/300 \text{ K}_2\text{Cr}_2\text{O}_7$	$N/20 \text{ AgNO}_3$	Red surface layer, band of very tiny crystals at 1 mm., and larger particles from 3 mm.

As the concentrations are reduced, the particles of precipitate grow smaller, with corresponding increase of specific surface, and the tendency to produce bands begins to make itself manifest. The crystals were, however, still much too large. The addition of alcohol further diminished the size of the particles and increased the formation of bands.  $N/20 \text{ K}_2\text{Cr}_2\text{O}_7$  agar gel containing 30 % of absolute alcohol, treated with  $N/10 \text{ AgNO}_3$  solution, gave a precipitate in tiny feather-like crystals in distinct layers at 0.5, 2.0 and 3.5 mm. below the red surface layer.

Since the aqueous phase in gelatin gels contains about 0.3 % of gelatin, the effect of adding small quantities of gelatin and gum arabic was tried in a great many experiments. Many of these gave short columns of red precipitate instead of merely surface layers, but after about 1 cm. the colour of the column changed to black with the development of distinct crystals or spherites. With the aid of a lens, however, the red columns were frequently seen to be laminated. By increasing the concentration of the silver nitrate solution to  $N/2$ , two separate red bands were obtained in  $N/75 \text{ K}_2\text{Cr}_2\text{O}_7 \frac{1}{2}$  % agar gel containing 0.5 % of gelatin. Numbers of very beautiful black spherites of  $\text{Ag}_2\text{Cr}_2\text{O}_7$  with a greenish lustre were formed below the bands, reaching 0.5 mm. in diameter at the base of the tube. With  $2N/1 \text{ AgNO}_3$  solution and  $N/175 \text{ K}_2\text{Cr}_2\text{O}_7$  1% agar gel containing 0.06 % gelatin, five bands were formed, the first two red, those below composed of microscopic black spherites. These experiments appeared to show that the red precipitate is not due entirely to the effect of the gelatin. While the column of precipitate remained red it showed very similar banding to the commencement of a column in gelatin. The difficulty appeared to be that the specific surface diminished, as the reagents became more dilute by diffusion, until stratification ceased.

It seemed desirable to find some other way of increasing  $N$ . Remembering Bechhold's experiments on the effect of ions on the precipitate, Liesegang's [1914] practice of adding citric acid to washed gelatin was considered. It seemed probable that the trivalent anion of citric acid might tend to prevent the precipitation of negatively charged silver chromate particles, thus increasing the supersaturation and giving a much greater value of  $P$ . A series of experiments from this point of view showed that there is a definite concentration of citric acid, or of soluble citrate, for each concentration of chromate, or of dichromate, in the gel, which gives perfectly formed strata with clear interspaces.

Figs. 7 to 12, Pl. VIII, show the effect of adding increasing amounts of citric acid. As the particles grow smaller, the banding gradually appears. At first, while the crystals are still large enough to appear black, only a few layers are formed close together as seen in Figs. 7 and 8. With more citric acid a number of skeletal bands of red precipitate appear, which are much wider apart, but which break up into black spherites lower down the tube, Fig. 9. With the optimum amount of citric acid the red bands are complete and extend right down the tube, Fig. 10. Further increase of citric acid yields a more colloidal precipitate, at first irregularly stratified, Fig. 11, but ultimately separating in large flocks with little trace of stratification, as in Fig. 12. In Figs. 7 to 10 the effect was obtained by decreasing the concentration of potassium chromate while keeping the citric acid constant at  $N/300$ . The tubes contained respectively  $N/88$ ,  $N/107$ ,  $N/125$  and  $N/150$   $K_2CrO_4$ . Fig. 11 had  $N/140$   $K_2CrO_4$  with  $N/250$  citric acid, and Fig. 12  $N/100$  chromate with  $N/130$  citric acid. The optimum concentration of citric acid for  $N/150$   $K_2CrO_4$  is  $N/300$ . A similar series was obtained with  $N/100$   $K_2CrO_4$  agar gel by varying the amount of trivalent anion. The optimum concentration was found to be  $N/200$ . With  $N/75$   $K_2CrO_4$ ,  $N/125$  citric acid gave the best results.

The agar gel was made by soaking shredded agar for 24 hours in sufficient water to make a 1.25 % gel, afterwards boiling till dissolved and filtering through a Buchner funnel with washed linen. The gel was stored in wide-mouth bottles containing a trace of camphor. The test-tubes used were roughly graduated at 3 cc. and 15 cc. The required amounts of standard solutions of the reagents were added to make the gel of the desired strength, and the liquid made up to the 3 cc. mark with water. The hot agar sol was then filtered into the tube up to the 15 cc. mark, the whole mixed and allowed to set by placing in cold water. This gave a 1 % agar gel. To each tube 3 cc. of  $2N/1$  silver nitrate solution were added, very carefully, from a pipette with its nose just above the top of the gel. Without great care, or if the tube was afterwards slightly shaken, the silver solution was liable to run down between the gel and the tube. The standard solutions of  $K_2CrO_4$  and citric acid were made from recrystallised materials, the former by weighing and the latter by titration with carbonate-free alkali and phenolphthalein. It was remarkable that with ordinary stock reagents very different relative amounts of chromate

and citric acid were required. This was assumed to be due to traces of impurities.

The bands produced with citric acid and potassium chromate in agar are much wider apart than in gelatin, and, as they are almost free from secondary white precipitate, the effect is very beautiful. Moreover, they begin to form immediately the silver solution is poured on the gel, which makes them particularly suitable for demonstration. The same recipes give good results in plate preparations and in filter paper.

The effect of citric acid in gelatin was attributed by Liesegang to the hydrogen ion. This does not appear to be the case, since equally beautiful results are obtained by the use of potassium citrate. The bands are now very close together, almost exactly like those in gelatin, but perhaps closer. After about 10 mm. they become granular and blurred, suddenly changing into more colloidal, thicker and wider bands at the base of the tube, just as silver chromate does in gelatin. The optimum concentration for  $N/150$   $K_2CrO_4$  was  $N/85$  potassium citrate. By substituting citric acid for part of the potassium salt, the bands became wider apart and lost the blurred granular and more colloidal regions as in Fig. 5, Pl. VII. Wonderfully beautiful bands are produced with potassium citrate and potassium dichromate, which resemble, very closely, those formed in washed gelatin with citric acid. Fig. 4, Pl. VII, shows a 1 % agar gel with  $N/100$   $K_2Cr_2O_7$  and  $N/55$  potassium citrate and  $2N/1$   $AgNO_3$  poured on top. It must be noted, however, that these reagents were not specially pure, or less citrate might have been necessary.

Bearing in mind that the chromates of silver do not form bands in washed gelatin, it seems evident that the different effects obtained with these salts in commercial gelatin and in agar gels are due, rather to mineral impurities contained in the gelatin, than to differences in the action of the gel-substances themselves. This raises the question as to whether the great protective action of commercial gelatin is a property of the pure substance.

The action of the citrate ion in these experiments is artificially to increase the supersaturation of the silver chromate before precipitation takes place, giving a larger  $P$  and greater specific surface of the precipitate. By actual cataphoresis, in the presence of gelatin and the reaction components, Mr Hatschek very kindly confirmed the hypothesis that silver chromate particles are negatively charged. This supports the view that the increased supersaturation is due to the trivalent anion. The inhibiting effect of citric acid was demonstrated by experiments on the mutual precipitation of dilute aqueous solutions of  $K_2CrO_4$  and  $AgNO_3$  of varying strengths in the presence of different amounts of citric acid. One reagent was added slowly, drop by drop, with vigorous stirring, to the other, mixed with citric acid, until sudden precipitation of the whole excess of silver chromate took place. A number of curves have been obtained showing the relation between the metastable limit and the concentration of citric acid, for different strengths of the reacting solutions. The experiments show that citric acid enormously increases the critical value of the ionic product for silver chromate at which spontaneous precipitation

takes place. Taken in conjunction with Bechhold's observations on the influence of ammonium nitrate in the same reaction, it seems extremely probable that aqueous supersaturated solutions are affected by electrolytes in the same way as colloid solutions. The experiments are being continued.

These investigations must not be taken as supporting the "supersaturation" theory of band formation. It has been shown that the reason why the silver chromates do not form bands in agar or washed gelatin is merely that the particles of precipitate are too large. Precipitates with small specific surface never form bands. The experiments show that, as the size of the particles diminishes, the tendency to stratification appears. The use of soluble citrates is merely a convenient way of increasing the dispersity of the precipitate. To leave no doubt on this point it seemed desirable to continue the experiments in which the specific surface was increased by diminishing the solubility of the precipitate. This time potassium chromate was used, as silver chromate is less soluble than the dichromate. It was immediately found that  $N/100$   $K_2CrO_4$  0.7 % agar gel, containing 30 % of absolute alcohol, treated with  $2N/1$   $AgNO_3$  solution gave a good series of narrow bands of Indian red silver chromate, which were very similar to those in ordinary gelatin, but so close together as to require a lens to see them clearly. In this case the precipitate was distinctly granular, like those of lead chromate and iodide. It is clear, therefore, that increased supersaturation is not necessary for the formation of bands. The success of these experiments affords seemingly conclusive evidence for the adsorption theory.

#### SUMMARY.

Further evidence has been adduced to show that the formation of banded precipitates is due to adsorption of solute from the region between the bands.

Only precipitates with great specific surface form bands.

The specific surface of the precipitate is influenced by the reaction medium, by the presence of ions and particularly of trivalent ions.

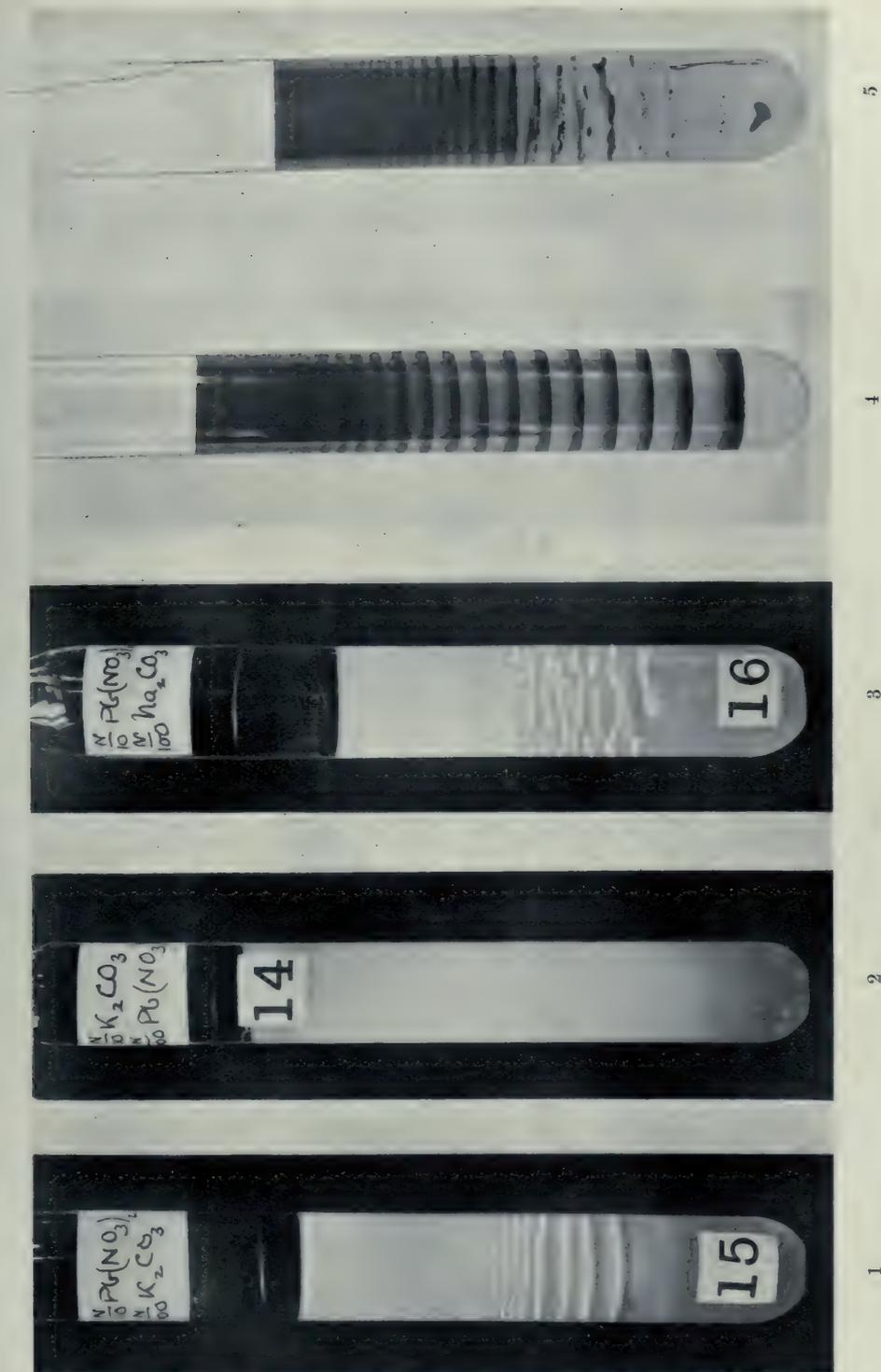
By varying its specific surface, a given substance can be obtained in the banded form, or not, as desired.

The occurrence, or non-occurrence, of bands of the same substance in different gels is due to the influence of the reaction medium on the dispersity of the precipitate.

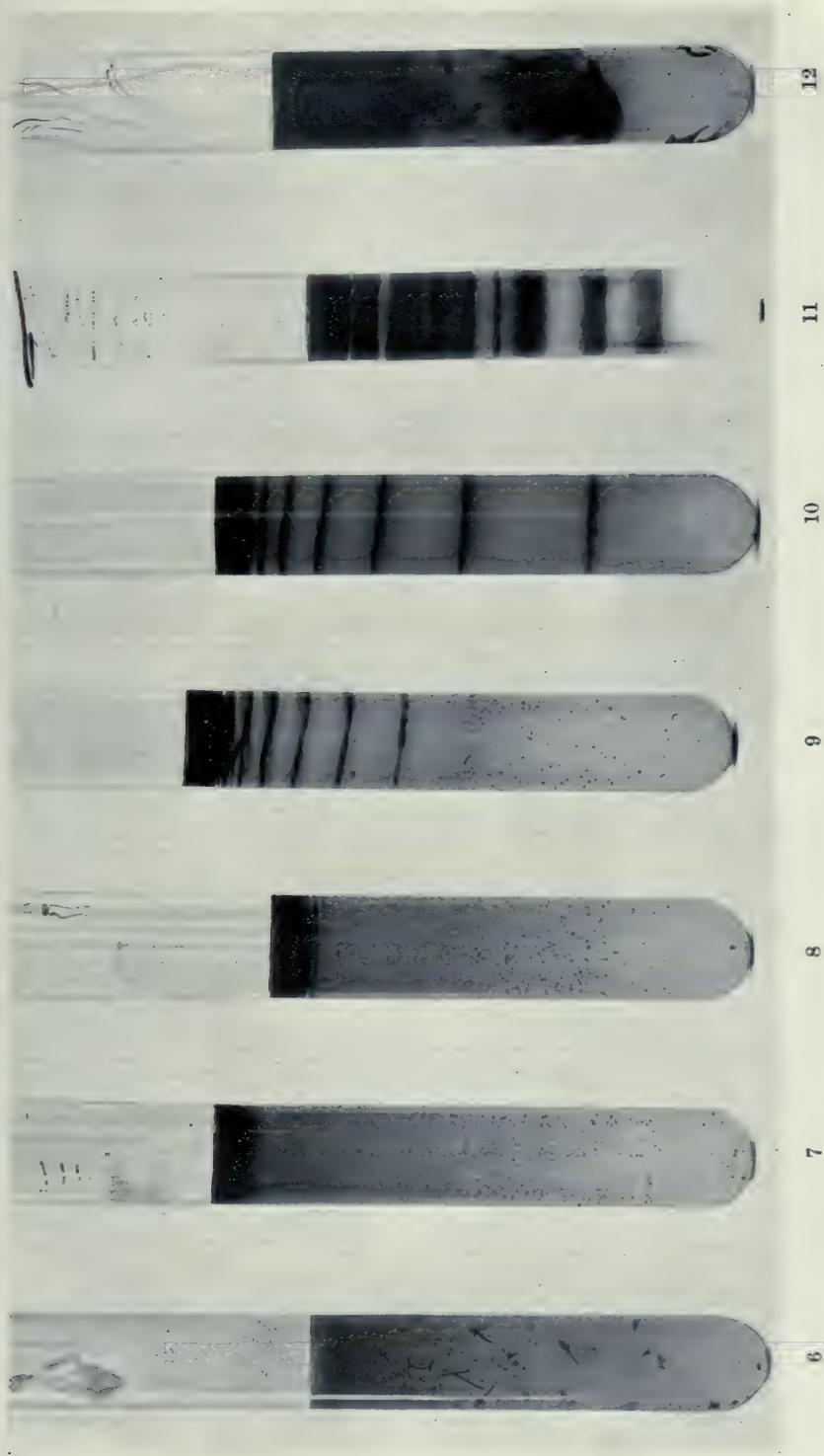
These principles have been tested by application to the well-known cases of silver chromate and dichromate which form bands in gelatin gels and not in agar. The non-occurrence of bands in the latter medium is due to insufficient specific surface of the precipitate. By increasing the dispersity, beautiful bands of both substances have been obtained in agar gels.

#### REFERENCES.

- Bechhold (1905). *Zeitsch. physikal. Chem.*, **52**, 185.  
Bradford (1918). *Biochem. J.*, **11**, 14.  
— (1920). *Biochem. J.*, **14**, 29.  
Buchner and Kalf (1920). *Rec. Trav. Chim.*, **39**, 135.  
Hatschek (1919). *Rep. Brit. Assoc.*, 1918, *Colloid Chemistry*, 21.  
Liesegang (1914). *Zeitsch. physikal. Chem.*, **88**, 1.









## XL. THE NATURE OF YEAST FAT.

BY IDA SMEDLEY MACLEAN AND ETHEL MARY THOMAS.

(Received April 23rd, 1920.)

COMPARATIVELY little is known of the nature of the fat present in simple cellular organisms and of the processes by which it is formed. It is possible that a study of such organisms, where the conditions of growth are easier to control, may throw more light on the method by which fat is formed than investigations of the more complex plants and animals. Since yeast is obtainable in quantity, it was chosen as the most suitable material for such an investigation; as a preliminary it was necessary to identify with certainty the constituents of yeast fat.

### CHARACTERISTICS OF YEAST FAT.

The fat examined was obtained from the following sources:

- (1) Baker's yeast supplied from the manufacturers.
- (2) A pure culture of a brewery yeast grown for us under definite conditions by Dr Thaysen at the bacteriological laboratory of the Royal Naval Cordite Factory.
- (3) Specimens of brewery yeast.

### *Method of Extraction of the Fat.*

The pressed yeast was spread in a thin layer and dried in the hot room at 37° for 48 hours<sup>1</sup>. It was then soaked over night in alcohol and shaken for eight hours at ordinary temperature. The second and third extractions were similarly carried out, a mixture of alcohol and light petroleum (B.P. 60–80°) being used as the solvent. After the third extraction, 25 g. of the dry residue were extracted for eight hours in a Soxhlet apparatus with ether. The amount of fat obtained in the final extraction was always very small and was used only for calculating the total amount of fat. The alcohol and petroleum were evaporated on a water bath under diminished pressure and the residue dissolved in light petroleum and filtered. After evaporation of the solvent, the residue was dissolved in alcohol and excess of acetone added to precipitate the lipins. The solvent was distilled from the filtered solution; the iodine and saponification values were determined and the fat converted to the methyl esters, or saponified for the preparation of the sterols.

<sup>1</sup> Recently, the preliminary drying at 37° has been discarded since a considerable proportion of the fat seems to disappear during the process.

The crude yeast fat prepared by extraction of the alcohol-soluble residue with ether or with light petroleum consists of a brown liquid from which crystals of a sterol separate on standing. It has a high iodine value and a low saponification value attributable to the large proportion of unsaponifiable matter present which may be from 25 to 45 %.

#### *Iodine Value.*

The iodine value was determined by Wijs' method after precipitating the lipins by acetone. Twelve specimens grown from a pure culture of brewery yeast by Dr Thaysen gave values lying between 147 and 175.5, the average being 161.2. Four specimens of brewery yeast obtained locally gave an average value of 164.4. Six specimens of baker's yeast gave values from 121.2 to 150.8, the average being 135.6.

The iodine value therefore varies within wide limits. We found that this was dependent chiefly on the amount of sterol present in the crude fat. The yeast sterol has a very high iodine value and gives quite erratic results when the latter is determined by Wijs' method (see p. 490).

#### *Saponification Value.*

This is always low and shows wide variation, depending on the proportion of sterol present. Nine specimens of brewery yeast obtained from Dr Thaysen gave an average value of 162.3, the numbers lying between 138 and 184; four specimens of brewery yeast obtained from a brewery gave an average value of 164. The values from baker's yeast were higher, three specimens from one firm giving values from 196 to 199, while other specimens gave values of 187 and 151.

We endeavoured to determine the proportion of sterol in a specimen of crude fat obtained from baker's yeast, and in the unsaponifiable matter derived from it, by precipitation with digitonin solution [Windaus, 1909]. The result shows that the greater proportion of the sterol seems to be present not in the free state but as fatty acid ester.

In one experiment, the proportion of unsaponifiable matter was determined by extracting the solution with ether after it had been saponified and drying the residue in vacuo at 100°.

0.5456 g. fat gave 0.2175 g. unsaponifiable matter.

The unsaponifiable residue from a similar quantity of fat was dissolved in 50 cc. 95 % alcohol and a solution of 0.5 g. digitonin in 50 cc. of 90 % alcohol added. The resulting precipitate was filtered through a Gooch crucible, washed with 95 % alcohol and dried at 110°.

Weight of digitonin cholesteride	0.3763 g.
Solubility allowance	0.014
	0.39 g.
	% sterol = 17.23 %.

0.266 g. fat dissolved in 90 % alcohol and precipitated with 25 cc. of a 1 % solution of digitonin in 95 % alcohol gave 0.051 g. or allowing for solubility = 0.058 g.

% unsaponifiable matter	= 39.8 %	} 17.2 %.
% combined sterol	= 12.3 %	
% free sterol	= 4.9 %	

Yeast sterol very rapidly undergoes alteration; some decomposition appears to take place during the saponification process and it seems probable that the figures obtained for the sterol are too low. It is also probable that the solubilities of the yeast sterol and the cholesterol compounds with digitonin are not identical; the figures given can therefore only be regarded as approximate, and as representing the minimum amount of sterol in the sample.

### CONSTITUENTS OF YEAST FAT.

#### *The Fatty Acids.*

Nägeli and Loew [1878] first investigated yeast fat and described its fatty acid as consisting mainly of oleic acid. Gérard and Darexy [1897] found in it butyric, palmitic and stearic acids. Hinsberg and Roos [1903] isolated from it three fatty acids, (1) an acid identical or isomeric with oleic, (2) an unsaturated acid having the formula  $C_{12}H_{22}O_2$ , and (3) a saturated pentadecic acid to which they ascribed the formula  $C_{15}H_{30}O_2$ . These observers noted the ready oxidisability of the acids, in one instance the bulk of the acid having been converted to a neutral substance after standing for three weeks. The following year [1904] they succeeded in isolating and identifying a specimen of palmitic acid from the alleged pentadecic acid.

Neville [1913], apparently in ignorance of the later work of Hinsberg and Roos, confirmed the presence of the acid  $C_{15}H_{30}O_2$  which he was unable to separate into palmitic and a lower acid. He also isolated a substance melting at  $77^\circ$  which he regarded as arachidic acid; from a study of the oxidation products of the unsaturated acids he deduced the presence of an acid isomeric with linolic acid and of the  $C_{16}$  and  $C_{18}$  members of the oleic series.

We used as material for this part of the investigation, baker's yeast containing 2 % of fat calculated on the dry yeast. This had an iodine value of 121.2 and a saponification value of 187.5.

#### *Preparation of the Methyl Esters.*

Haller's method, *i.e.* heating with a 2 % solution of HCl in methyl alcohol, was first tried but is unsuitable when dealing with a very unsaturated fat such as that of yeast. The fat is little soluble in methyl alcohol: prolonged heating is necessary to bring about alcoholysis and during this time a heavy brown insoluble layer forms. These results resemble those obtained by Haller [1905] when working with linseed oil. Bull's method [1906] was therefore adopted.

100 g. of fat were dissolved in light petroleum and shaken with 26.18 cc. of a 4.25*N* solution of sodium methoxide in methyl alcohol. After standing two hours, water and light petroleum were added and the upper layer separated, washed with sodium carbonate solution and dried with CaCl<sub>2</sub>. After distilling off the solvent, the methyl esters were twice distilled under 20 mm. pressure with a six-pear distilling column. The following fractions were obtained:

B.P.			
145-155°	...	...	1.1 g.
155-180°	...	...	0.2 g.
180-195°	...	...	2.3 g.
195-200°	...	...	9.0 g.
200-205°	...	...	0.5 g.
205-210°	}	...	21.0 g.
210-215°			
215-220°	...	...	6.0 g.

There remained a considerable brown residue in the flask after the first distillation which decomposed on further heating.

#### *Identification of Saturated Acids.*

The fraction 195-200° partly solidified on standing; the solid ester was separated, pressed out, hydrolysed and the acid recrystallised from alcohol. After nine recrystallisations an acid melting at 61-62° was obtained, the melting point not being raised by further recrystallisation.

Analysis showed this to contain 75.00 % C and 12.28 % H. Calculated for palmitic acid 75.00 % C and 12.5 % H. The fraction boiling from 145 to 155° had an iodine value of 61; it was hydrolysed and the acid recrystallised, a solid acid separated which after recrystallisation melted unsharply at 38°. Methyl laurate boils at 148 under 18 mm. pressure; lauric acid melts at 43.6°. It seems probable therefore that this acid is lauric acid, but there was not sufficient material to confirm this further. Where only a small proportion of the C<sub>12</sub> acid is present, it is difficult to remove the linoleic and oleic acids completely by distillation, unless large quantities of the acids are available. It may perhaps be regarded as confirmatory evidence that the melting point of the pentadecic acid given by Hinsberg and Roos [1903] as 56° agrees well with that for a mixture of 20 % lauric and 80 % palmitic acid, 57.4° [Heintz, 1854]; the analytical figures they give also agree with those required by such a mixture (cp. Table I). A comparison of the results obtained by the various observers is shown in Table I.

Table I. Saturated fatty acid isolated from yeast fat.

Observer's name	M.P.	% C.	% H.	M.P. Methyl Ester
Hinsberg and Roos (1903)	56°	74.5	12.3	
" " (1904)	62°	74.85	12.67	
Neville (1913)	59°	74.59	12.38	26
Gérard and Darexy (1897)	60-61°			
MacLean and Thomas	61-62°	75.00	12.28	
Palmitic acid	62°	75.00	12.5	28
Pentadecic acid	53°	74.38	12.39	
Mixture 20 % lauric and 80 % palmitic acids	57.4°	74.4	12.4	

The presence of palmitic acid is therefore definitely established and Neville's pentadecic acid is probably a mixture of palmitic with a small amount of lauric acid.

In agreement with Neville's work, from the highest boiling fraction of methyl esters we isolated after hydrolysis a small amount of an acid melting at 77°: this was regarded by Neville as arachidic acid.

#### *Identification of Unsaturated Acids.*

	B.P.	Iodine value
Fraction of yeast fat methyl esters	200–205° (20 mm.)	96
" " "	205–215° "	130
" " "	215–220° "	123
Methyl oleate	212–213° (15 mm.)	85.8
Methyl linolate	207–208° "	172.7

It follows from the boiling points and iodine values of the distilled methyl esters that the bulk of the yeast fatty acids consists of a mixture of linoleic and oleic acids. Bromination of the free acids gave a mixture of bromides totally soluble in ether. From this, a solid bromide was obtained, insoluble in light petroleum, which after being twice recrystallised from alcohol melted at 113–114°. It was therefore identified as tetrabromo-linoleic acid. The solution in light petroleum contained liquid dibromo-oleic acid.

Hinsberg and Roos [1903] isolated a small amount of an acid which from its analytical numbers they regarded as an unsaturated dodecenic acid. It was separated from the ether-soluble lead salts, which, as has been pointed out by Lewkowitsch [1913], contain also small amounts of the  $C_{12}$  and lower saturated fatty acids if these are also present in the mixture. The iodine value of the acid was not determined and the evidence for the existence of a naturally occurring dodecenic acid cannot yet be regarded as convincing. One of us [Smedley, 1912] noted on distilling the esters of the butter fatty acids a rise in the iodine value of the fraction corresponding to the  $C_{12}$  acids and suggested as a possible explanation the presence of a dodecenic acid in butter fat. Crowther and Hynd [1917] distilled a mixture of oleic acid with a mixture of saturated fatty acids and found under these circumstances a curve similar to that obtained with the butter fat esters, giving a perceptible increase of iodine value for the  $C_{12}$  fraction. The presence of an unsaturated  $C_{12}$  acid in yeast, while therefore not definitely excluded, must be regarded as doubtful.

#### LECITHIN.

The presence of lecithin in yeast fat was first demonstrated by Sedlmayer [1903] who described it as dipalmitocholine-glycerophosphoric acid. He obtained 4 % crude lecithin calculated on the dried yeast, and estimated the amount of pure lecithin as 2 %. Since the whole of the ether-soluble fraction, consisting mainly of sterol and fat, was regarded as the crude lecithin, these figures are too high. We found that the fraction precipitable from ether or alcohol solution by the addition of acetone gave an average value of 8.5 % of the total fat or about 0.17 of the weight of dried yeast.

## THE STEROL OF YEAST.

Gérard [1895] isolated from yeast a plant cholesterol melting at 135–136° and regarded it as belonging to the same group as the ergosterol obtained by Tanret [1889] from ergot. The latter substance however melted at 154°. Gérard regards ergosterol as typical of a group of sterols characteristic of the cryptogams and differentiated by their reaction with  $\text{CHCl}_3$  and concentrated  $\text{H}_2\text{SO}_4$  from the phytosterols and cholesterol characteristic of the higher plants and animals respectively. The latter substances when concentrated sulphuric acid is added to their chloroform solutions, impart a red colour to the chloroform solution, the acid layer becoming fluorescent. With ergosterol however under similar conditions the chloroform solution remains colourless or shows a slight green fluorescence, the acid taking on a deep red colour.

Hinsberg and Roos [1903] isolated two sterols one melting at 159° and one at 148–149°; the melting point of the latter they were unable to raise by recrystallisation. They state that the sterol of m.p. 159° gives a red colour with chloroform and concentrated acid but do not say which layer contains the red colour. They found that their sterol was not identical with the caulosterol (m.p. 158–159°) isolated by Barbieri and Schulz [1882]. Analysis of the sterol after drying over sulphuric acid gave them results agreeing with the formula  $\text{C}_{26}\text{H}_{44}\text{O}$ . Neville [1913] who next investigated this substance, isolated only a sterol melting at 148–149°.

*Preparation of Yeast Sterol.*

The fat from baker's yeast and from the brewer's yeast supplied by Dr Thaysen, extracted as described above, was saponified with alcoholic potash, the solution neutralised and extracted with ether, the ether-soluble fraction being recrystallised from alcohol and finally from ether; crystals of the sterol also separated on allowing the fat to stand in the cold.

By repeated recrystallisation of the sterol from ether, we obtained a substance melting at 154°, which appears to be identical with the ergosterol isolated by Tanret from ergot [1889, 1908].

Our yeast sterol when first isolated melted at 135–136° and many recrystallisations were necessary to raise the melting point to 154°. The melting point of ergosterol is given by Tanret as 154° "à l'état brut" and 165° on the Maquenne block. Solutions of yeast sterol become yellow and decompose slightly on heating or even on standing at ordinary temperature, a yellow oily substance being formed. It is therefore difficult to obtain in the pure state by crystallisation, and the discrepancies in the melting point between the results of the specimens of the yeast sterol described by different observers are probably due to this cause. On adding concentrated sulphuric acid to a solution of yeast sterol in chloroform, the acid layer is coloured red. The close agreement in melting point and specific

Table II. Properties of sterols isolated from Cryptogams.

Name	Formula ascribed	Source	% C. (anhydrous)	% H (anhydrous)	Melting-point	Rotation in $\text{CHCl}_3$ in ether	m.p. acetate	Rotation acetate [ $\alpha$ ] <sub>D</sub>	Observer
Ergosterol	$\text{C}_{28}\text{H}_{40}\text{O}, \text{H}_2\text{O}$	Ergot	{ 84.6 85.0 84.64	{ 11.06 11.20 11.25	154°	-114°	169-175°	-80°	Tanret [1889]
"	$\text{C}_{27}\text{H}_{38}\text{O}, \text{H}_2\text{O}$	"			154° à l'état brut 165° sur le Ma- quette bloc	-126°	180.5°	-91.8°	" [1908]
"	$\text{C}_{34}\text{H}_{40}\text{O}, \text{H}_2\text{O}$	"			150°	-89.5° at 15°	165		Ottolenghi [1906]
Yeast sterol		Yeast (brewery)			135-136° (a) 159° (b) 148-149°	-105°			Gérard [1895]
"	$\text{C}_{26}\text{H}_{41}\text{O}$	"	83.35	11.9	156-157°				Hinsberg and Roos [1903]
"		"			145-147°				Meisenheimer [1915]
"		Yeast			154°				Neville [1913]
"		Yeast (a) Baker's (b) Brewery			154°				MacLean and Thomas
Mycosterol	$\text{C}_{30}\text{H}_{48}\text{O}_2$	<i>Collybia shiitake</i>	(not dehydrated) 82.00	10.81	159-160°	-117° at 18°	170.5	-87.3°	
"	"	<i>Armillaria elodes</i>	81.87	11.35	159-160°	-129.4°	169°		Ikeguchi [1919]
"	"	<i>Hudnum asparatum</i>	82.02	10.95	159-160°	-129.2° -129.5°			" "
Fungisterol	$\text{C}_{28}\text{H}_{40}\text{O}, \text{H}_2\text{O}$	Ergot			144° sur le Maquette bloc	-22.4°	158.5°	-15.9° (ether)	Tanret [1908]

1 Solvent not stated.

rotation, of both yeast sterol and its acetate with the corresponding compounds of ergosterol is shown in Table II. Tanret showed that in ergot the ergosterol is accompanied by a sterol of formula  $C_{25}H_{40}O$ ,  $H_2O$ , m.p.  $144^\circ$  (determined on the Maquenne block) to which he gave the name of fungisterol. It is possible that this is also present in yeast but we were unable to detect it.

In one specimen of yeast obtained from a local brewery no ergosterol could be detected but from the unsaponifiable matter a sterol melting at  $97-98^\circ$  and giving a specific rotation of  $+10.1^\circ$  in chloroform solution was isolated. The presence of a positive rotation and the low melting point suggest a resemblance to coprosterol (m.p.  $99^\circ$  and specific rotation  $+24^\circ$ ) which is obtained by reduction of cholesterol in the intestines. It is possible that in this particular specimen reduction of the ergosterol had taken place giving a coprosterol-like compound. We hope to be able to obtain further information on this point.

#### *The Iodine Value of the Sterols.*

As stated above in the discussion of the iodine value of yeast fat, very high iodine values were obtained on treating yeast sterol with Wijs' reagent. The fact that the iodine value of cholesterol when determined by Wijs' method varies with the time of the reaction and with other conditions has been noted by Werner [1911] and by Lewkowitsch [1913], who found iodine values up to 145.

We examined the iodine value of cholesterol and of yeast sterol by the methods both of Hübl and of Wijs. With Wijs' solution the reaction is not confined to addition of halogen to the ethylene linkages and high values result. The acetates of the sterols behave similarly so that the alcohol group does not appear to be concerned in these changes. The presence of an appreciable quantity of sterols in any fat leads therefore to an abnormal value when determined by Wijs' reagent, and in such cases the iodine value cannot be taken as a measure of the number of ethylene linkages present in the molecule.

With Hübl's reagent the action with the sterol proceeds slowly and gradually, and falls off very much after 24 hours. Lewkowitsch found for the iodine value of cholesterol by Hübl's method the values 68.09 and 67.43. Theory for one unsaturated linkage in the molecule requires 62.8 corresponding to the formula  $C_{27}H_{46}O$ ,  $H_2O$ . We found values of from 65 to 75 which correspond approximately with this theoretical requirement. Werner found by Hübl's method for phytosterol values from 41 to 76, according to the time of the reaction; after half-an-hour by Wijs' method 135.

We then tested a sample of brassicasterol supplied to us by Professor Windaus and found for this with Hübl's reagent an iodine value of 118; theory for two double bonds demands 122. Windaus and Welsch [1909] have shown that brassicasteryl acetate adds on four atoms of bromine and contains

therefore two double bonds, a result with which the Hübl iodine value is in good agreement. Yeast sterol and its acetate under similar conditions give iodine values corresponding to the presence of three double bonds in the molecule. This result is in agreement with the formula ascribed to ergosterol by Tanret,  $C_{27}H_{42}O$ ,  $H_2O$ , a formula differing from that of cholesterol only in that it contains four hydrogen atoms less.

The evidence so far available (see Table III) therefore indicates that yeast sterol differs from the cholesterol and phytosterols yet described in that it contains three double bonds in the molecule.

Table III. Iodine Values of Sterols.

Substance and formula	I value (Wij's)	Time of reaction hrs.	I value (Hübl)	Time of reaction hrs.
<i>Cholesterol</i> $C_{27}H_{46}O$ , $H_2O$				
[I value calc. if one double bond is present = 62·8]	141·6	6	58·65	6
	152·7	18	70·21	18
	165·1	22·5	64·88	22·5
	165·8	22·5		
	153·2	24	73·2	24
	158·4	25		
	165·4	48	71·8	48
<i>Cholesteryl acetate</i>				
[I value calc. if one double bond is present = 59·8]	120·2	6	27·94	6
	127·8	18	48·26	18
	131·1	24	49·8	20
	144·1	48	52·1	24
			62·6	48
<i>Brassicasterol</i> $C_{28}H_{46}O$ , $H_2O$				
[I value calc. if two double bonds are present = 122·0]			118	22·5
<i>Sterol from yeast</i> , $C_{27}H_{42}O$ , $H_2O$				
[I value calc. if three double bonds are present = 190·5]	337·8	23	177·1	21
<i>Steryl Acetate from yeast</i>				
[I value calc. if three double bonds are present = 179·1]	245·2	23	175·1	

*Probable identity of Mycoesterol with Ergosterol.*

Recently Ikeguchi [1919] isolated a sterol from various fungi, *Armillaria edodes*, *Collybia shiitake*, *Hydnum asparatum* and *Lycoperdon gemmatum*, to which he gave the name of mycoesterol. Its properties closely resemble those of ergosterol. It gives similar colour reactions when treated with chloroform and concentrated sulphuric acid, the red colour is in the acid layer, and its melting point, rotation and the melting point of its acetate are all in close agreement with those of ergosterol (see Table II). The difference in the analytical numbers may be due to the fact that Ikeguchi apparently analysed the crystals after recrystallising them from alcohol, when they contain one molecule of water of crystallisation, whereas other observers state that they dried to a constant weight over sulphuric acid.

Ikeguchi's numbers agree fairly with those required for the hydrated sterol. The percentage of carbon is low but this is possibly due to slight loss of water of crystallisation. Ikeguchi shows that only one acetyl group is introduced into the molecule on acetylation, and that a carbonyl group is

not present. He does not however consider the possibility of the second oxygen atom being present as water of crystallisation.

The only property of mycoesterol which is not in agreement with our observations on yeast sterol is its behaviour on bromination. When bromine in acetic acid was added to an ether solution of mycoesterol the crystals recovered agreed in melting point and in colour reactions with the original substance, and the conclusion is drawn that no double bond is present in the molecule. When a similar experiment was performed with yeast sterol, the bromine solution was rapidly decolorised and a black oily product was obtained on evaporating the solution, which solidified on standing, but we had not sufficient material to isolate a pure substance from this. In view however of the close agreement of mycoesterol with ergosterol, further investigation of its behaviour on bromination seems desirable.

We are at present continuing our investigations on the constitution of yeast sterol.

#### SUMMARY AND CONCLUSIONS.

1. Palmitic, oleic and linoleic acids have now been identified with certainty in yeast fat.

2. The pentadecic acid previously described consists of a mixture of palmitic and lauric acids. Evidence for the presence of lauric acid has been obtained.

3. The presence of an acid melting at  $77^{\circ}$  has been confirmed, the melting point of which agrees with that of arachidic acid.

4. No confirmation of the presence of the dodecenic acid described by Hinsberg and Roos was obtained and the possibility of this being a mixture of lauric with oleic and linoleic acids has not yet been excluded.

5. A sterol is present partly in the free state and partly as fatty acid esters; this may constitute 20 % of the total yeast fat.

6. The sterol present in yeast appears to be identical with the ergosterol isolated from ergot by Tanret.

7. The variation in melting point described by different observers seems to be due to differences in the purity of the substance, but no conclusive evidence of the presence of a second sterol in yeast has yet been obtained.

8. It seems probable that the mycoesterol isolated by Ikeguchi from certain fungi is also identical with ergosterol and that ergosterol is characteristic of the whole group of cryptogams just as cholesterol is of the animal and phytosterol is of the higher plant kingdom.

9. Yeast sterol is differentiated from the sterols of the higher plants and animals by the presence of three double bonds in its molecule.

Our thanks are due to Dr Thaysen who provided us with some of the material used in this investigation. The work was carried out for the Food Investigation Board of the Department of Scientific and Industrial Research and we gratefully acknowledge the grants provided for this purpose.

## REFERENCES.

- Barbieri and Schulz (1882). *J. pr. Chem.* (2), **25**, 159.  
Bull (1906). *Ber.*, **39**, 3570.  
Crowther and Hynd (1917). *Biochem. J.*, **11**, 139.  
Gérard (1895). *J. Pharm. Chim.* (6), **1**, 601.  
— and Darexy (1897). *J. Pharm. Chim.* (6), **5**, 275.  
Haller (1905). *Compt. Rend.*, **143**, 657.  
Heintz (1854). *Annalen*, **92**, 295.  
Hinsberg and Roos (1903). *Zeitsch. physiol. Chem.*, **38**, 1.  
— (1904). *Zeitsch. physiol. Chem.*, **42**, 189.  
Ikeguchi (1919). *J. Biol. Chem.*, **40**, 175.  
Lewkowitsch (1913). *Chemical Technology of Oils, Fats and Waxes* (Macmillan).  
Meisenheimer (1915). *Wochenschr. f. Brauerei*, **32**, 325.  
Nägeli and Loew (1878). *J. pr. Chem.*, **17**, 403.  
Neville (1913). *Biochem. J.*, **7**, 347.  
Ottolenghi (1906). *Chem. Centr.*, 1906, **1**, 541.  
Sedlmayer (1903). *Zeitsch. f. das ges. Brauwesen*, **26**, 381.  
Smedley (1912). *Biochem. J.*, **6**, 451.  
Tanret (1889). *Compt. Rend.*, **108**, 98.  
— (1908). *Compt. Rend.*, **147**, 75.  
Werner (1911). *Inaug. diss.*, Berlin.  
Windaus (1909). *Ber.*, **42**, 238.  
— and Welsch (1909). *Ber.*, **42**, 612.

# XLI. THE EXTRACTION OF THE FAT-SOLUBLE FACTOR OF CABBAGE AND CARROT BY SOLVENTS.

BY SYLVESTER SOLOMON ZILVA.

*From the Biochemical Department, Lister Institute.*

*(Received April 29th, 1920.)*

THE study of the fat-soluble accessory factor in fats has so far not contributed anything very definite to our knowledge of the chemistry of this principle. Observations so far made on active fats favour the view that the activity is not due to the fat itself but to some substance or substances which are in association with it. The fat-soluble factor is however not easily separated from it by differential solvents. All other conceivable methods of fractionation are mostly of a drastic nature not suitable for the manipulation of a physiologically active principle of doubtful stability.

It is now well established that some vegetables form a rich source for a substance physiologically resembling the fat-soluble accessory factor in fats but as in the case of the fats little is known of its chemical character. We are even yet unaware whether it is present in the vegetable cell in solution or in suspension. According to McCollum, Simmonds and Pitz [1916, 1] the fat-soluble factor is not extracted from plants with the fats by ether, chloroform, benzene or acetone. Hot alcohol however according to these authors [1916, 2] removes the factor from maize kernel.

This communication describes some experiments in which active fractions were obtained from fresh vegetables by extraction with absolute alcohol and eventually with ether.

Originally it was intended to study the fat-soluble factor in cabbage but owing to some technical difficulties the investigation was extended to carrots. It has been found by various workers that cabbage is rich in the fat-soluble factor. Preliminary experiments carried out in connection with this research have shown that small quantities of this vegetable in fresh condition were sufficient to promote growth in rats fed on a diet lacking the factor. The aim was to treat the minced cabbage at first with a solvent which would fulfil the dual function of disorganising the plant cells and of extracting the accessory factor. Absolute alcohol was chosen and found to be suitable for the purpose. However the extract thus obtained was not relished by the rats who mostly either refused the food containing it or consumed insufficient

of it. It was therefore found necessary to apply this method of extraction to another vegetable, namely carrots, the extract of which was likely to prove more palatable to the animals. This vegetable although not very rich in the fat-soluble accessory factor was shown by Denton and Kohlman [1918] and Steenbock and Gross [1919] to contain appreciable quantities of it. As anticipated it was found possible to extract by means of alcohol from the carrots an active fraction which the animals took well. This extract afforded the opportunity of further fractionation, and it was found that ether extracted the active principle from it leaving the best part of the extraneous matter behind. As the carrot also contains the antineuritic and antiscorbutic factors it was conjectured from theoretical considerations that the alcoholic extraction would also remove these two factors. Experiments instituted with that purpose have indeed shown that the extract contained the antineuritic and to a smaller extent the antiscorbutic factor. These two principles were however not present in the extract in such high proportions as the fat-soluble factor.

#### EXPERIMENTAL.

Fresh green cabbage was thoroughly minced and triturated with sand. Absolute alcohol was then added (500 cc. of absolute alcohol for every 100 g. of cabbage) and the mixture allowed to digest in a cool dark place for about 12-18 hours. It was then filtered through a fluted filter and the residue on the filter paper pressed out and filtered. The combined filtrates were green and transparent and possessed the usual appearance of an alcoholic solution of chlorophyll. The alcoholic extract was then evaporated *in vacuo* at 35°. As the solution became concentrated chlorophyll separated out leaving behind a light brown liquid. The addition of alcohol to this solution which was practically free from alcohol produced a small white precipitate which consisted most probably of protein. On further concentration a greenish brown sweet syrup containing reducing sugars was obtained. This syrup possessed the characteristic taste of boiled cabbage. An equivalent of 25 g. of fresh cabbage was added daily to a diet which was complete in every respect but which lacked the fat-soluble factor. The composition of this BC diet was described in a previous communication [Zilva, 1919]. Of the various rats fed on this diet only few consumed the food well enough to demonstrate the growth-promoting power of the extract. Fig. 1 gives the weight curves of two representative rats. The male animal consumed the best part of his food, on the average about 18 g. per day, with the result that it grew well and reached a satisfactory weight within the 11 weeks during which it was kept on the diet. The female animal consumed only about 10-12 g. of the food per day. Such an amount is hardly quantitatively adequate to promote normal growth even if the diet were qualitatively well balanced. It will be seen that although the animal grew, the rate of growth was below the normal. The experiments with the cabbage extract, however, served as a

preliminary indication and demonstrated that it was possible to extract the fat-soluble factor by the method described.

In order to obviate the irregularity in the food intake of the rats carrots were next employed as a source for the accessory factor. These vegetables were treated in the same way as the cabbage. The filtrate obtained in this case was brilliantly clear and yellow in colour. On concentration *in vacuo* at 35° a very sweet and pleasant-tasting syrup was obtained. The syrup could be dissolved in water producing a turbid reddish yellow solution. An equivalent of 25 g. of fresh carrots was given to the animals in their daily diet at first. This preparation the animals consumed very well and as will be seen from Figs. 2 and 3 they developed well on it. Fig. 2 represents the

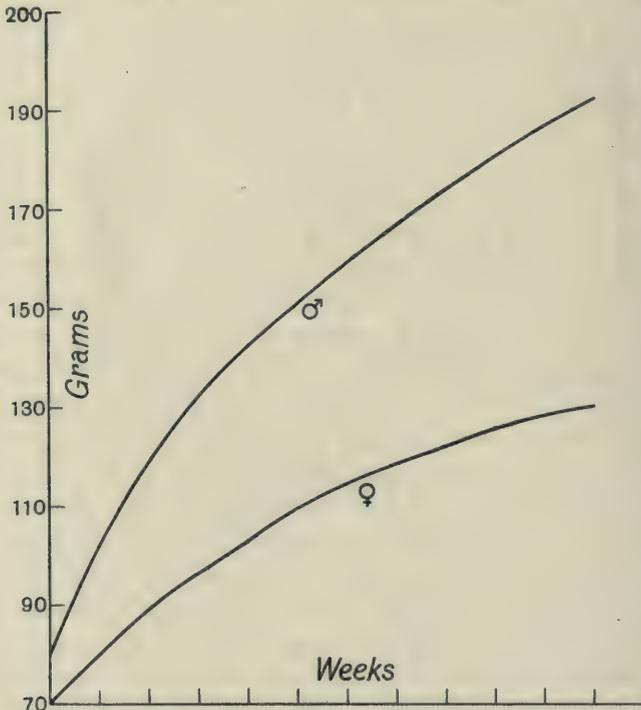


Fig. 1. Weight curve of rats fed on a diet containing the fat-soluble factor in the form of an alcoholic extract of cabbage.

weight curves of three animals which received the extract after they had subsisted for three weeks on a diet lacking the fat-soluble factor. They immediately resumed normal growth instead of ceasing to grow as was the case with the control animal. Fig. 3 shows how, on administration of an equivalent of 25 g. of fresh carrots to an animal which had existed on a diet free from the fat-soluble accessory factor for ten weeks and which was rapidly losing in weight, normal growth was induced.

It was next desirable to ascertain the minimum dose of the extract that would induce normal growth. Doses equivalent to 15 g., 5 g., 1 g. of fresh

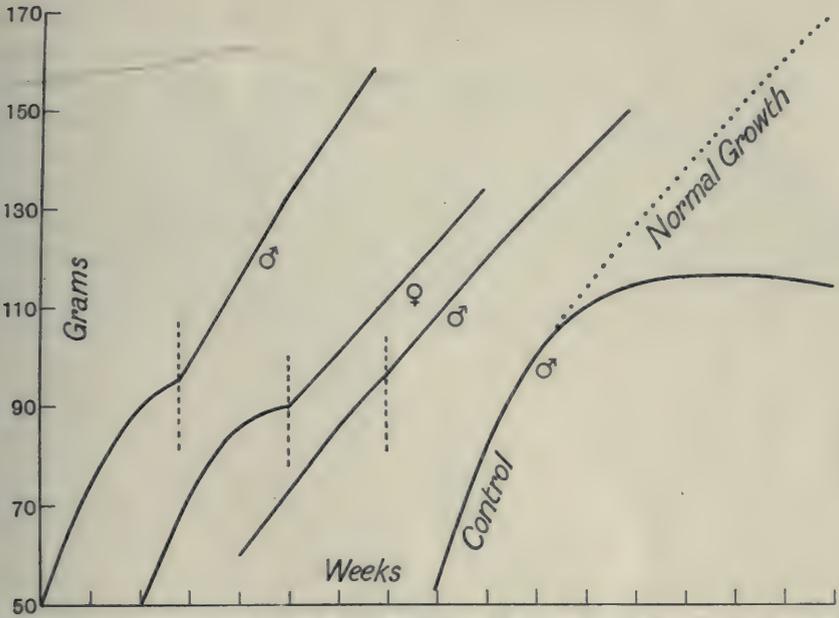


Fig. 2. Weight curve of rats fed on a diet containing the fat-soluble factor in the form of an alcoholic extract from carrots. The perpendicular dotted lines denote the commencement of the treatment.

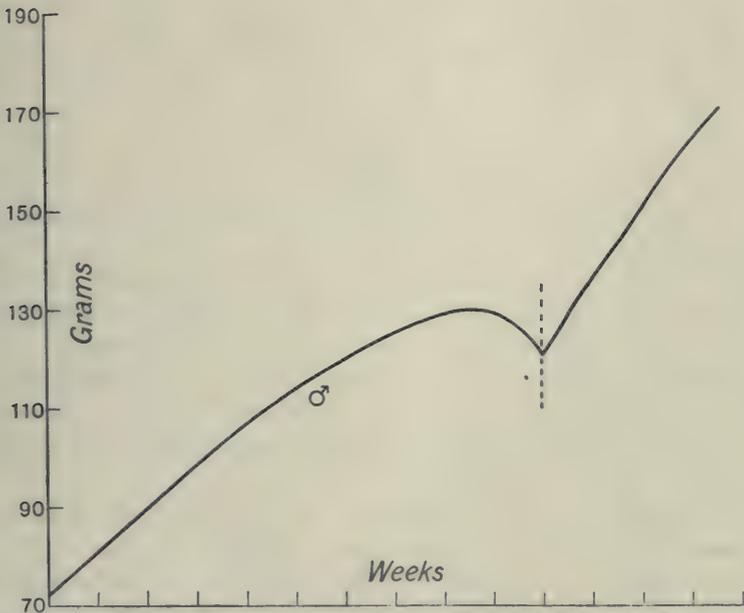


Fig. 3. The perpendicular dotted line denotes the commencement of the treatment.

carrots were given to three rats. In every case growth was induced (Fig. 4). The addition of the equivalent of 15 g. of carrots produced almost normal growth. The extracts from 5 g. and 1 g. produced less intense growth. An animal which had ceased growing for several months owing to the deficiency of the fat-soluble factor also resumed growth on receiving a dose equivalent to 1 g. of carrot. The growth curve in this case commenced flattening out after about ten days. The diet containing the 1 g. was consumed entirely. About 80 %–90 % of the other two doses was consumed daily by the rats. It may be pointed out in conjunction with the above figures that Steenbock and Gross [1919] found that 5 % of dried carrot was insufficient to produce normal growth while a diet containing 15 % sufficed for the purpose.

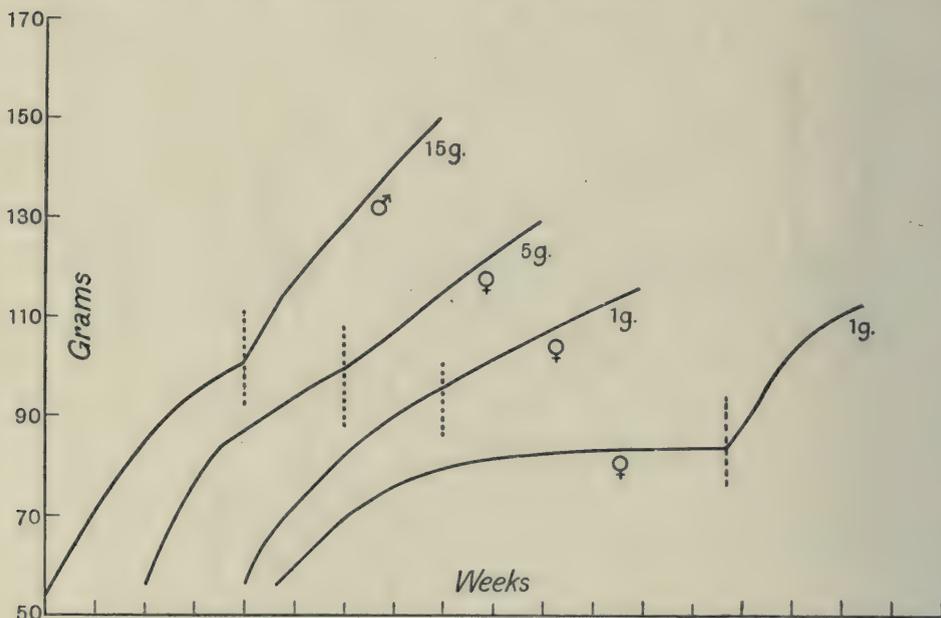


Fig. 4. The perpendicular dotted lines denote the commencement of the treatment.

Absolute alcohol extracts in the form of the syrup about 7.5 % of dry matter from fresh carrots which comprises about one-half of the total solids. On drying the syrup *in vacuo* at a low temperature and keeping the residue in a desiccator it was found that after a fortnight an equivalent of 25 g. of carrots was active both in inducing good growth and in curing xerophthalmia in rats. Any further particulars of the keeping properties of the carrot extract in dry condition have not yet been worked out.

The antineuritic factor was tested out on rats. Equivalent of 25 g. per day were added to an AC diet [Zilva, 1919]. It will be seen from Fig. 5 that this dose was sufficient to induce development a little below the normal rate. As these animals only consumed about 70 % of their daily diet it may be assumed that the dose was about the minimum necessary for normal growth.

Fig. 5 also shows the weight curve of a rat which was deteriorating on account of an antineuritic deficiency but which on receiving the extract equivalent to 25 g. of fresh carrots resumed growth.

The tests for the antiscorbutic factor showed that the content of this principle in the carrot extract was very low. Three guinea-pigs kept on a diet of oats and bran and a daily ration of 40 cc. of autoclaved whole milk received after the tenth day a daily dose of the extract equivalent to 25 g., 10 g. and 5 g. of fresh carrots respectively. The animals which received the 10 g. and 5 g. doses developed scurvy, declined in weight and eventually died in

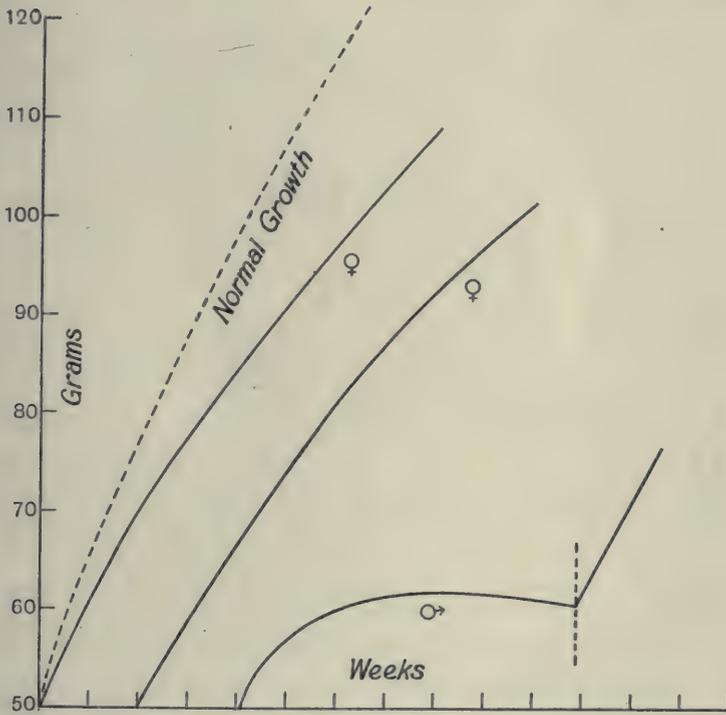


Fig. 5. The perpendicular dotted line denotes the commencement of the treatment.

the ordinary way as they would have done on a purely scorbutic diet (Fig. 6). No delay in the onset of the symptoms or in the fatal termination could be recorded. The guinea-pig which received daily the extract from 25 g. of fresh carrots showed a decided delay in the onset of the scorbutic symptoms. Unfortunately this animal succumbed to some other complaint on the 23rd day. At the post mortem examination mild signs of scurvy were observed which were not acute enough to be responsible for the death of the animal. The extract from 25 g. of carrots contains therefore enough antiscorbutic to delay the onset of the disease<sup>1</sup>.

<sup>1</sup> [Note added on 9th of June.] A daily administration of an alcoholic extract equivalent to 50 g. of fresh carrots protected a guinea-pig subsisting on a scorbutic diet for 42 days.

The alcoholic extract from carrots was next concentrated until all the alcohol was driven off and the aqueous solution became concentrated. This solution was extracted by shaking it with ether in a separating funnel. The ethereal solution at first was yellow but on repeated extraction no more colouring matter could be removed from the solution which nevertheless still retained a fairly intense yellow colour. A solution equivalent to 250 g. of fresh carrots extracted with ether as described yielded a residue weighing 0.4935 g. The residue, which was dark brown, on drying for two hours at  $110^{\circ}$  remained of oily consistency. On extracting it with sodium carbonate and acidifying the solution no precipitate was obtained which would indicate that it did not contain any free higher fatty acids. The residue was next saponified by heating with alcoholic potash under a reflux condenser for two hours, the alcohol driven off, and the aqueous solution filtered. On adding acid to the filtrate a solid substance resembling the higher fatty acids separated out and gradually accumulated on the surface of the liquid. There is little doubt then that the ethereal extract contained some oil.

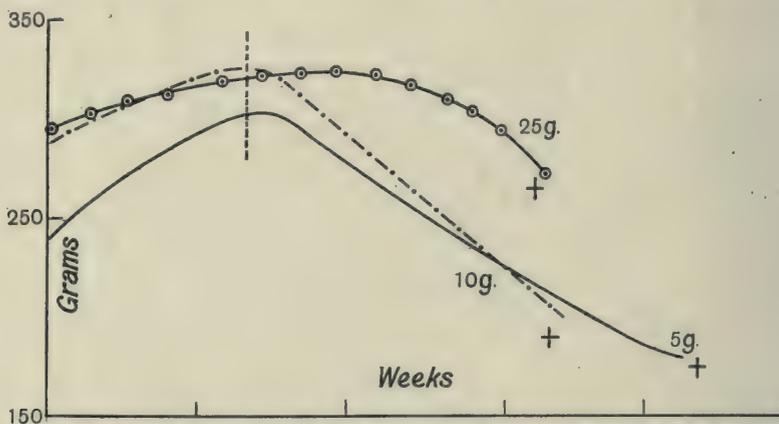


Fig. 6. The perpendicular dotted line denotes the commencement of the treatment.

In order to test the activity of this fraction autoclaved olive oil was added to the ethereal solution and the ether evaporated. A highly coloured and opaque oil was left behind. Both the colour and the opacity were dependent on the strength of the extract. Through the medium of the oil the ethereal extract, equivalent to 25 g. of carrots, was incorporated in the daily diet which was free from the fat-soluble factor and as will be seen from Fig. 7, which represents the weight curve of two rats treated with this fraction, growth was promoted immediately the treatment was commenced.

While these experiments were in progress Osborne and Mendel [1920] published a short note in which they state that they have extracted with ether from spinach leaves and young clover dried at  $60^{\circ}$  an oily residue which was active in small quantities. Although they have obtained their fraction from different plants and directly from the dried vegetable their work corroborates the fact that the fat-soluble factor in plants is soluble in ether. Further

experiments with various solvents carried out preferably on the alcoholic fraction ought to throw some light on the subject whether the activity of the ethereal fraction is due to the small amount of the oil or to some other substance associated with it.

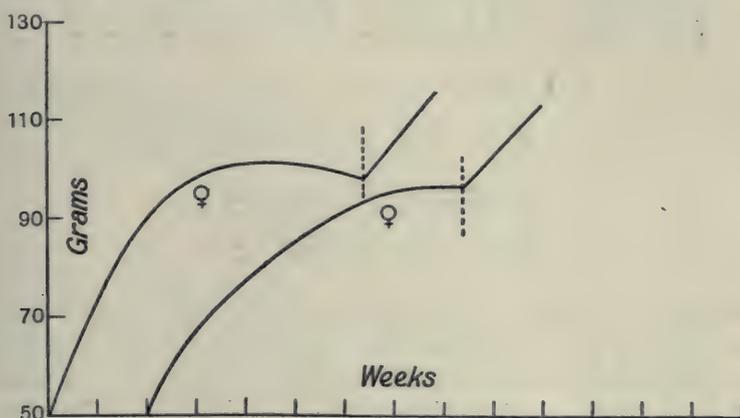


Fig. 7. The perpendicular dotted lines denote the commencement of the treatment.

#### SUMMARY.

1. Alcohol extracts the fat-soluble factor from cabbage and carrots.
2. The alcoholic extract equivalent to 10–12 g. of fresh carrots given daily is sufficient to promote normal growth in rats subsisting on a diet lacking the fat-soluble factor.
3. The alcoholic extract from carrots also contains the antineuritic and to a smaller extent the antiscorbutic factors.
4. An ethereal extract from the alcoholic fraction equivalent to 25 g. of fresh carrots has been found to promote recovery and renew growth in rats declining in weight on account of a fat-soluble factor deficiency.

Part of the expense of this research was defrayed from a grant made by the Medical Research Council, to whom my thanks are due.

#### REFERENCES.

- Denton and Kohlman (1918). *J. Biol. Chem.*, **36**, 249.  
 McCollum, Simmonds and Pitz (1916, 1). *Amer. J. Physiol.*, 1916, **41**, 333.  
 — (1916, 2). *J. Biol. Chem.*, **28**, 153.  
 Osborne and Mendel (1920). *J. Biol. Chem.*, **41**. Proc. Amer. Soc. Biol. Chemists vii.  
 Steenbock and Gross (1919). *J. Biol. Chem.*, **40**, 501.  
 Zilva (1919). *Biochem. J.*, **13**, 164.

## XLII. A CONTRIBUTION TO THE STUDY OF KERATOMALACIA AMONG RATS.

BY MARJORY STEPHENSON AND ANNE BARBARA CLARK.

*From the Biochemical Laboratory, Cambridge.*

*(Received May 11th, 1920.)*

*(With Plates IX and X.)*

THE association of acute mal-nutrition amongst children with a purulent condition of the eyes has long been familiar in medical practice. Mori [1904] associated a condition which he called "xerosis of the conjunctiva and keratomalacia," frequent amongst famine-stricken children, with fat starvation, and noted the curative effect of chicken liver and fish oils. He also observed that the disease occurred among populations using vegetable oils but was absent in sea-board districts where fish oils were used.

More recently Bloch [1917] associated an outbreak of corneal ulceration amongst children with a diet of highly separated milk, and noted the curative effect of whole milk and cod liver oil.

In 1917 McCollum and Simmonds [1917, 1], working with rats on deficiency diets, definitely associated a purulent condition of the eye—which they termed "xerophthalmia"—with the absence from the diet of the fat-soluble vitamine. In the above and a subsequent paper [McCollum and Simmonds, 1917, 2], these authors claim that xerophthalmia is an undoubted deficiency disease. They write: "There are then two deficiency diseases in the sense in which Funk employed this term. One of these is polyneuritis.... The other we believe is the syndrome described above in which the two most prominent features are emaciation and xerophthalmia" [1917, 1].

In order to make good the claim of keratomalacia, a term which we prefer to xerophthalmia, to rank as a deficiency disease in the strict sense of the term (due, in this case to the absence of the fat-soluble vitamine) it is necessary to establish the following points in respect of it:

1. That the determining cause is not bacterial infection.
2. That in one species, at least, the symptoms invariably result from a prolonged course of diet deficient in the fat-soluble factor.
3. That identical symptoms never occur as a result of any form of mal-nutrition in which the fat-soluble factor is adequately supplied in the diet.
4. That the symptoms invariably disappear when the factor is replaced in the diet (except in cases where death occurs before any general improvement can be manifested).

5. That the symptoms are not susceptible of cure by any treatment other than the supply of the fat-soluble factor.

As a contribution to this subject we have attempted to deal with Nos. 1, 2 and 4 of the above points, Nos. 3 and 5 falling outside the scope of our investigation.

The material dealt with in this communication, therefore, falls under two main headings.

A. Dietetic and Statistical.

B. (1) Histological. (2) Bacteriological.

### A. DIETETIC AND STATISTICAL.

#### a. Incidence of the disease.

As has already been noted (p. 502) in order to weigh the claims of keratomalacia among rats to rank as a deficiency disease it is necessary to study statistically the cases which occur when these animals are placed on a diet deficient in the fat-soluble factor. For this purpose the results of eight experiments, including in all 46 rats, are here given. The rats were all young (between 40 and 50 g. in weight) at the beginning of the experiment. The diet consisted of the usual combination of purified caseinogen, starch, sugar, and vegetable fat (usually palm kernel oil), and McCollum's salt mixture [McCollum and Simmonds, 1917, 1], to which were added traces of sodium fluoride, potassium iodide, and manganese sulphate. The water-soluble factor was supplied by a fat-free alcoholic extract of yeast and the anti-scurvy factor by 0.5 cc. of lemon juice per rat per day. In Table I the results of these experiments are recorded; Table II summarises the results of the eight experiments. The duration of the experiment is divided into periods of ten days; in each of these periods are recorded:

1. The total number of deaths occurring in the period.
2. The number of deaths with eye disease occurring in the period.
3. The total number of survivors at the end of the period.
4. The survivors with eye disease at the end of the period.
5. The cases of eye disease occurring during the period.

Fig. 1 represents the deaths occurring in the successive periods of the experiment; the blackened portion indicates the number of those deaths preceded by eye disease. It is seen from this that no deaths occurring before the 50th day were preceded by eye disease; after that period an increasing proportion of deaths were preceded by symptoms of keratomalacia, but *in no period* are *all* the deaths so preceded.

Fig. 2 represents the number of rats surviving at the end of any one period; the blackened portion represents those survivors having eye disease. It is here seen that the incidence of the disease increased with the duration of the experiment, but that at *no period* are *all* the surviving rats afflicted with the symptoms.

Table I.

No. of rats on experiment	Day of experiment on which deaths occurred	Day of experiment on which eye disease first noted	No. of rats which had eye disease	No. of rats which had no eye disease	No. of survivors
I. 5	29	No eye disease	1	4	0
	52	" "			
	79	" "			
	80	64th day			
	80	No eye disease			
II. 5	11	" "	2	3	0
	11	" "			
	40	" "			
	85	77th day			
	86	77th day			
III. 5	65	61st day	4	1	0
	65	61st day			
	65	No eye disease			
	80	77th day			
	86	77th day			
IV. 5	15	No eye disease	2	3	0
	15	" "			
	31	" "			
	82	77th day			
	86	56th day			
V. 6	47	No eye disease	4	2	0
	54	50th day			
	57	57th day			
	78	No eye disease			
	79	47th day			
104	82nd day				
VI. 5	26	No eye disease	0	5	0
	38	" "			
	38	" "			
	38	" "			
	38	" "			
VII. 9	15	" "	0	9	0
	27	" "			
	30	" "			
	30	" "			
	31	" "			
	31	" "			
	31	" "			
	41	" "			
	43	" "			
VIII. 6	26	" "	0	6	1
	26	" "			
	56	" "			
	64	" "			
	88	" "			
	(killed)				
Total	46	—	13	33	1

Table II. Summary of eight experiments.

	Day											
	0	10	20	30	40	50	60	70	80	90	100	110
	to	to	to	to	to	to	to	to	to	to	to	to
	9	19	29	39	49	59	69	79	89	99	109	119
Total deaths ... ..	0	5	5	10	4	4	4	3	9	0	1	0
Deaths with eye disease ...	0	0	0	0	0	2	2	1	7	0	1	0
Total survivors (end of period)	46	41	36	26	22	18	14	11	2	2	1	1
Survivors with eye disease (end of period) ... ..	—	—	—	—	1	2	3	7	1	1	0	0
Cases of eye disease occurring	0	0	0	0	1	3	3	5	1	0	0	0

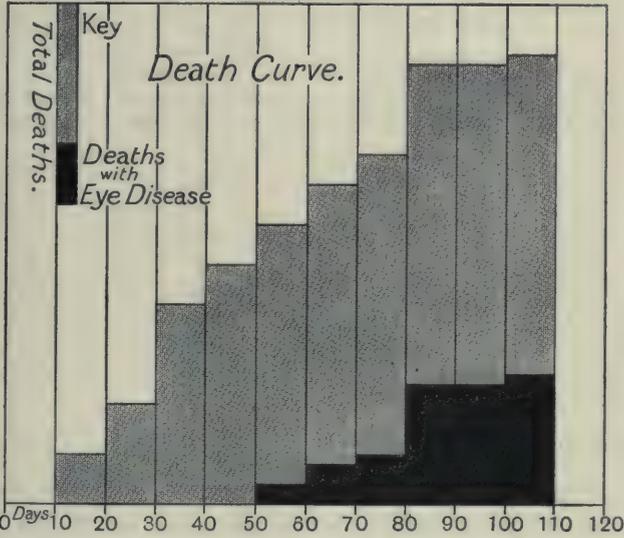


Fig. 1.

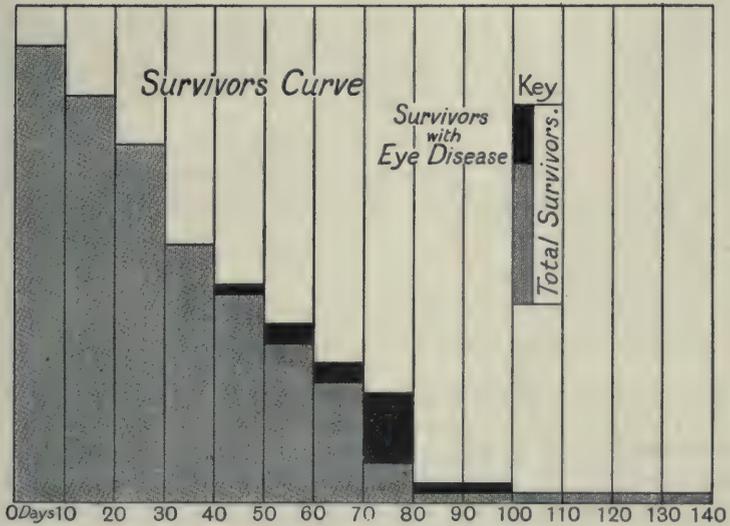


Fig. 2.

These facts taken in conjunction suggest: (1) That a diet deficient in the fat-soluble factor, whilst strongly predisposing the subject to eye disease, does not invariably result in it<sup>1</sup>. (2) That the occurrence of the disease is not coincident with the cessation of growth, but begins at a later stage.

### *β. Cure of the Eye Disease.*

In ten cases of eye disease (distinct from any appearing in the previous tables) cure was attempted; the results are tabulated below (Table III).

Table III.

Day of experiment on which disease was first noted	Day of experiment on which cure was attempted (i.e. fat-soluble factor added to the diet)	Day of experiment on which cure was complete	Day of experiment on which rat died
72nd	82nd	86th	96th
54th	82nd	93rd	106th
72nd	82nd	107th	survived
50th	54th	60th	62nd
54th	54th	67th	101st
50th	54th	60th	survived
57th	60th	64th	"
37th	39th	46th	"
37th	39th	46th	"
36th	39th	43rd	"

These experiments were made in connection with a separate investigation; the material carrying the fat-soluble factor was a light petroleum extract of dried carrot which was incorporated with the palm kernel oil. This material was shown in control experiments (including 21 rats in all) to be carrying the fat-soluble factor.

It is seen that *in every case* the eye symptoms cleared up though the time taken to effect the cure varied considerably; it is noteworthy however that four out of the ten rats failed to survive.

Thus of 46 rats on a diet deficient in the fat-soluble factor 96 % failed to survive 90 days, and 28 % contracted eye disease. Of ten rats which had contracted disease and were given the fat-soluble factor 100 % were cured of the disease, but only 60 % restored to health, 40 % dying after the eye symptoms had cleared up.

From these two sets of experiments ( $\alpha$  and  $\beta$ ) an apparent paradox emerges; absence of the fat-soluble factor almost invariably causes death (preceded by

<sup>1</sup> It is our opinion that the non-appearance of "xerophthalmia" on diets deficient in the fat-soluble factor recorded from this laboratory [Bulley, 1919] was due to the fact that for a period during the war the caseinogen used for the experimental animals was insufficiently extracted with alcohol. We have since found that a small amount of lipoid material, somewhat rich in the factor, was present in this caseinogen and in our belief protected the rats from eye disease. This, and the comparative rarity of the disease in the human subject, suggests that keratomalacia only appears when the diet is very highly deficient in the fat-soluble factor.

cessation of growth) and *sometimes* produces eye disease; replacement of the fat-soluble factor *invariably* cures the eye disease but sometimes (on the same rats) fails to restore growth and save life.

The first explanation of this apparent paradox that suggests itself is that the concentration of the fat-soluble factor in the tissues necessary to protect the eye is smaller than that necessary to ensure normal growth; this would account both for the later incidence of the disease and its more certain cure. It does not however afford a satisfactory explanation of all the facts. Supposing the concentration of the fat-soluble vitamine (in the tissues) necessary to life be  $x$ , while that necessary to protect from eye disease be  $y$ . In the case of the rats dying before they contract eye disease  $x$  is greater than  $y$ , whilst in those rats which develop eye disease before death  $x$  is less than  $y$ . Such a difference in the requirement of young animals of the same species is surprising. The facts may mean no more than that such a variation in individual resistance exists as is seen clinically when one element in a symptom complex is more prominent in one case, and some other element in another. But under the controlled conditions of experimental studies such variations within the same species are not usually seen.

An alternative hypothesis which suggests itself is that two factors are involved; one responsible for life and growth and another necessary for the nutritive integrity of a special tissue such as the cornea. Rats, which at the start of the experiment contain a relatively larger supply of the life-growth factor develop eye disease before death, whilst those which start with relatively more of the corneal-protecting factor exhaust their life-growth factor (*i.e.* die) before developing eye disease. Such a hypothesis presupposes that these two factors are present in varying proportions in different natural food-stuffs, and that the relative amounts of each accumulated in the rat at the beginning of the experiment are conditioned by its previous range of diet. The final consideration of these points must be postponed until they can be considered in relation to the histological and bacteriological results.

## B. HISTOLOGICAL AND BACTERIOLOGICAL.

We have been unable to find in the literature any detailed description of keratomalacia among rats, and have therefore attempted an investigation of this disease. In planning this work the questions we had in view were as follows:

1. What microscopic degenerative changes take place in the eye during the course of this disease?
2. Are these changes directly and solely caused by bacterial invasion, or is this infection preceded by histological changes in the tissue brought about by a prolonged course of a diet deficient in the fat-soluble factor?
3. Is one particular organism predominant in the bacterial infection?

## B (1). HISTOLOGICAL.

Preparations have been made of the eyes of:

- A. Normal rats on mixed diet.
- B. Rats which though on a diet deficient in the fat-soluble factor showed none of the symptoms of eye disease.
- C. Rats in which symptoms of disease were apparent in one eye but not in both.
- D. Rats in which both eyes were in an advanced stage of the disease.
- E. Rats in which a cure had been effected and sight restored by change of diet, *i.e.* by the addition of the fat-soluble factor.
- F. Rats in which change of diet had cleared up the purulent symptoms and effected the healing and regeneration of tissue, but in which sight had not been restored.

*Method.*

The eyes were placed in 10 % formalin for 24 hours, washed in water, passed through the alcohols and xylene, and embedded in paraffin. Sections were stained by each of the following:

1. Ehrlich's haematoxylin and eosin.
2. Iron haematoxylin and van Gieson.
3. Gram-Weigert stain for bacteria.

*Results.*

Figs. 9-18 will be found on Plates IX and X.

A. *Normal rats on mixed diet.*

*Case 1.* Fig. 17 shows a section of a normal eye; Fig. 9 shows a section of a normal cornea.

B. *Rats which though on a diet deficient in the fat-soluble factor showed none of the external symptoms of eye disease.*

*Case 2. Rat No. 53 ♀.*

*Diet.* Laboratory synthetic diet with palm kernel oil extract of yeast and lemon juice.

Growth curve, see Fig. 3.

Note that the growth and life of this rat were unusually prolonged and that no symptoms of eye disease were shown at any time. The rat was killed on the 88th day. Sections of the cornea of this rat do not differ in any important respect from those of the normal cornea so that it is unnecessary to append a figure.

C. Rats in which symptoms were apparent in one eye but not in both.

Case 3. Rat No. 7.

*Diet.* Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

This case is one in which symptoms were apparent in one eye whilst the other seemed to be normal.

Histological examination of the apparently normal eye (Fig. 10) shows the presence of a few leucocytes (*l*) in the cornea proper.

Sections of the other eye show a swollen cornea with loosened tissue due to the infiltration of fluid; leucocytes are present in the cornea proper.

Case 4. Rat No. 74.

*Diet.* Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

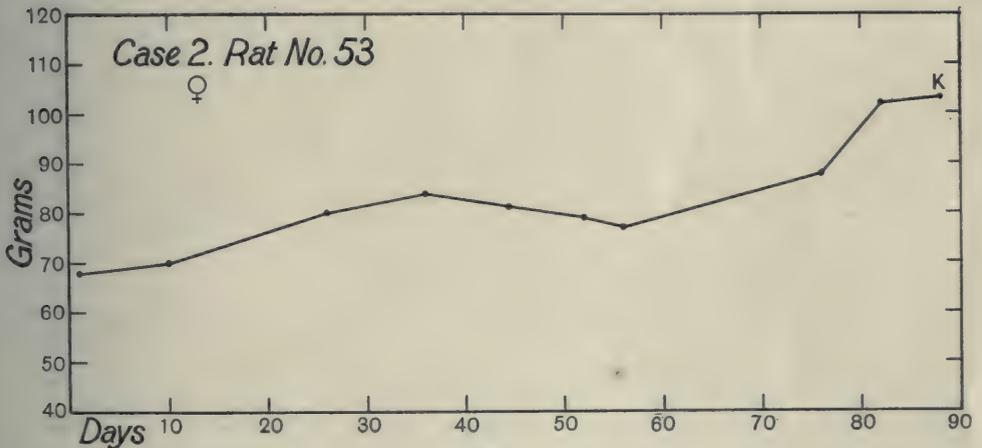


Fig. 3.

As in Case 3 (Rat No. 7) one eye was visibly diseased whilst the other was apparently normal. Histological examination of the normal eye (Fig. 11) shows that, unlike the apparently normal eye in Case 3, no abnormality can be discerned in the cornea.

In the case of the diseased eye of the same rat (Fig. 12) the cornea shows an enormously increased number of corneal corpuscles and a great many well-defined blood-vessels lined with epithelium (*b.v.*). These profound tissue changes are not uniformly distributed throughout the cornea but are more marked in some areas than in others. This observation will be referred to later in the discussion of the significance of the histological changes.

D. Rats in which both eyes are in an advanced stage of the disease.

Case 5. Rat No. 77.

*Diet.* Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, see Fig. 4; symptoms of disease were first noted on the 66th day (A).

Both eyes in this rat were in approximately the same state and show apparently an earlier stage of the disease than does the diseased eye of Case 4.

In Fig. 13 we notice localised increase of corneal corpuscles with well-marked blood vessels (*b.v.*). As in Case 4 these changes are not uniformly distributed throughout the cornea.

Gram-stained preparations of both these eyes show large numbers of Gram-positive cocci of the pneumococcal type, see Fig. 14 (*d*).

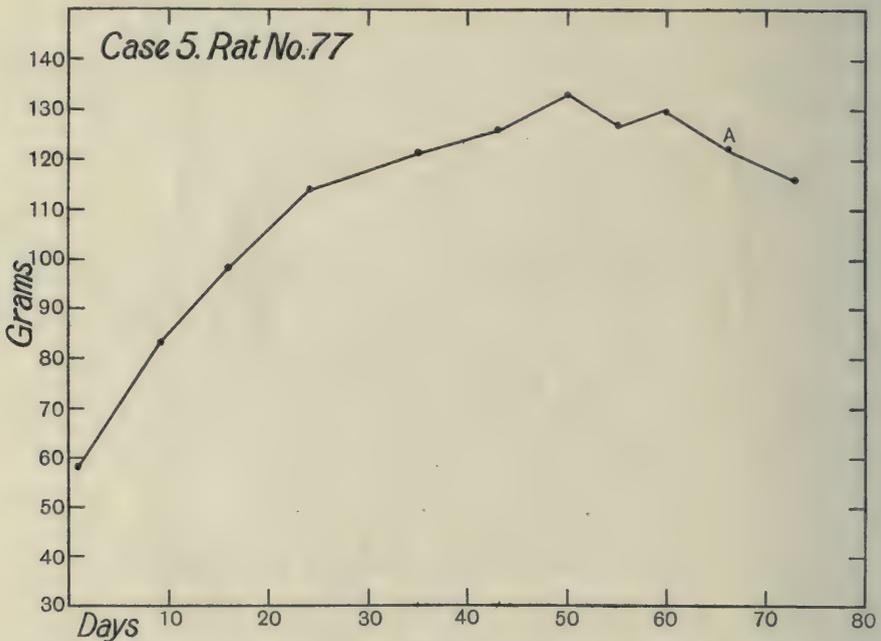


Fig. 4.

*Case 6. Rat No. 60.*

*Diet.* Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

The first symptoms of disease were noted on the 51st day (A); death took place on the 82nd day.

Growth curve, see Fig. 5.

Both eyes of this rat were in an advanced stage of the disease; both corneas were completely disintegrated exposing the front of the lens, which protruded during life.

Sections of the eye showed the lens complete but no anterior structure remaining.

Gram-stained preparations showed the presence of Gram-positive cocci of the pneumococcal type in the sclerotic.

E. Rats in which a cure has been effected and sight restored by the addition to the diet of a fat containing the fat-soluble vitamine in place of the palm kernel oil.

Case 7. Rat No. 113 ♀.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, see Fig. 6.

In order to interpret this growth curve it is necessary to give details of the history of this rat.

The first symptoms of eye disease were noted on the 50th day of experiment, the left eye only being affected (A).

On the 54th day both eyes were affected; the palm kernel oil was then replaced by bone marrow in order to test the vitamine content of this fat (B).

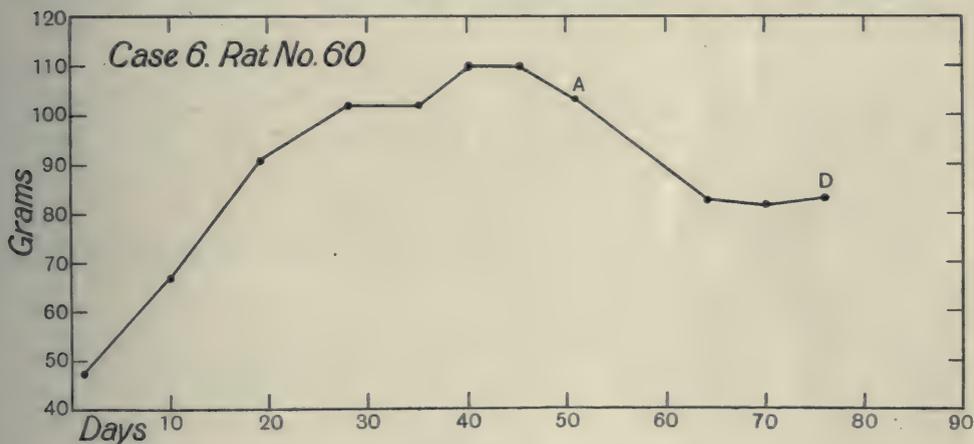


Fig. 5.

On the 57th day the eyes had improved (C), and on the 66th day all purulent symptoms had disappeared and the eyes were apparently normal (D).

Symptoms of eye disease returned however on the 93rd day both eyes becoming purulent (E).

On the 96th day the symptoms were more pronounced (both eyes were then swabbed) (F).

On the 105th day the bone marrow was replaced by butter (G).

On the 119th day the eyes were cured (H).

On the 137th day the rat was killed (K).

It is seen from the above notes that 18 days elapsed between the apparent completion of the cure by butter and the killing of the rat.

Histological examination of the sections showed that the cornea had completely recovered the normal state.

Case 8. Rat No. 114 ♀.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, Fig. 7.

The first symptoms of eye disease were noted on the 72nd day when both eyes were slightly purulent (Z). On the 82nd day both eyes were very purulent and closed (Y). On the 85th day crude light petroleum extract of carrot was added to the palm kernel oil (X); on the 95th day a marked improvement had taken place, a slight discharge only from the right eye being noticeable (W).

On the 98th day the eye was swabbed.

On the 107th day both eyes were clean showing only minute spots of discharge on the lid (V); these had disappeared on the 119th day (U). On the 142nd day the rat was killed (K).

As in Case 7, a considerable interval, in this case 23 days, elapsed between the completion of the cure and the killing of the rat. Histological examination of the sections showed that the cornea had almost completely resumed its normal condition, a slight increase in the number of corneal corpuscles only being still apparent.

F. *Rats in which supply of the fat-soluble factor had cleared up the purulent symptoms and effected the healing and regeneration of the tissue, but in which sight had not been restored.*

Case 9. Rat No. 5.

*Diet.* Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, see Fig. 8.

The earliest symptoms of eye disease in this rat escaped observation. On the 39th day both eyes were opaque and one was bleeding (A). Light petroleum extract of carrot was then added to the palm kernel oil. On the 46th day (B), purulent symptoms had disappeared; the left eye was clean and whole but with an opaque cornea; the right eye was permanently closed. On the 251st day the rat was killed.

Sections of the left eye disclose striking abnormalities. The cornea (Fig. 15) is considerably flattened and large vessels full of blood cells (*r.b.c.*) still remain. Since 90 days elapsed between the completion of the cure and the killing of the rat, this condition of the cornea may be regarded as permanent and not as representing a stage in the cure. In the case of the right eye the cornea degenerated so completely that the lens actually fell out of the eye during life; when the diet was changed the purulent condition of the eye was cured. Histological examination of the sections (Fig. 18) shows that the disappearance of the large lens has caused collapse and shrinkage of the "eye"; in place of the cornea there is a curious regenerated tissue built up from various elements (Fig. 16). This opaque anterior structure contains an enormous number of corneal corpuscles and a mass of fibrous connective tissue that has been built up from behind by the proliferation of corneal corpuscles.

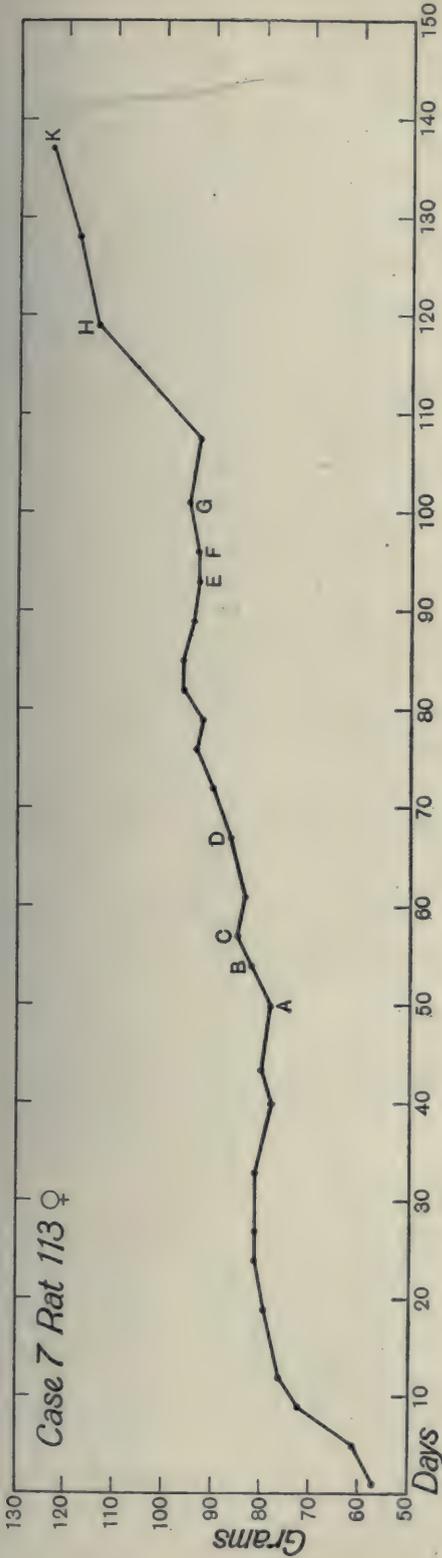


Fig. 6.

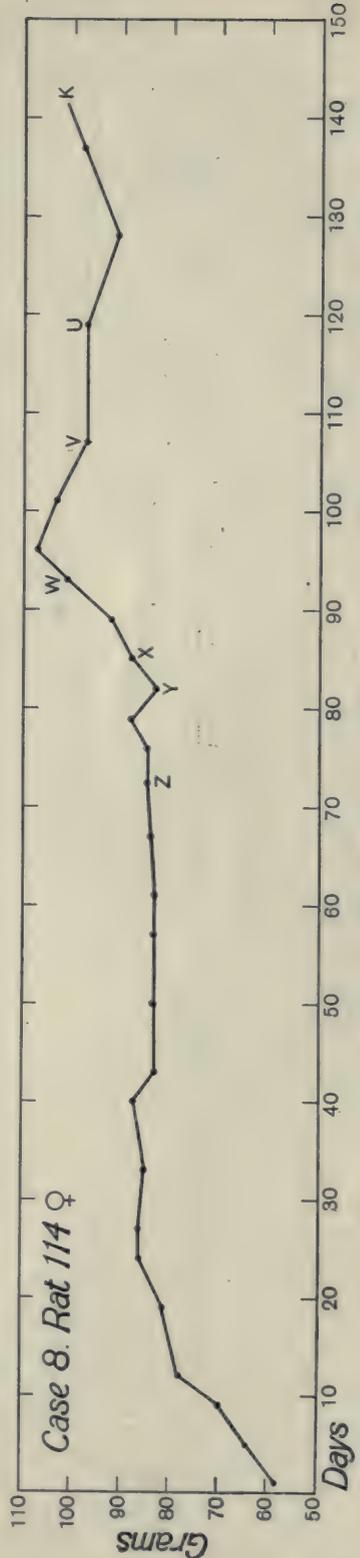


Fig. 7.

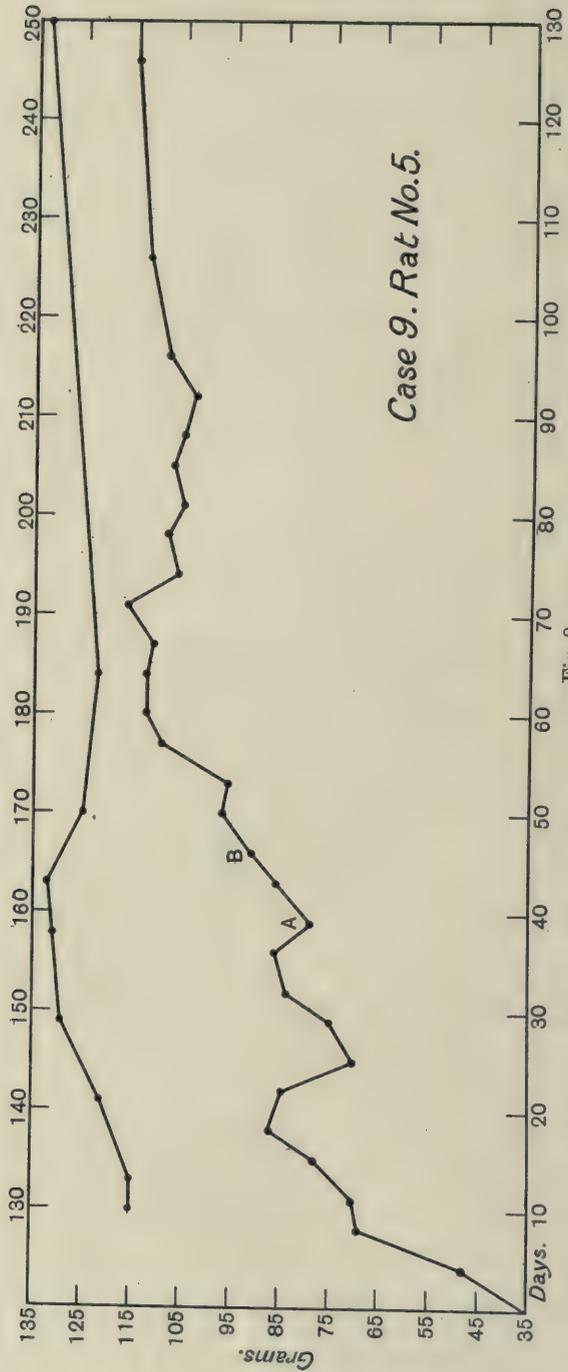


Fig. 8.

*Summary of Histological Results.*

The earliest histological changes noticed in the cornea are those in Case 3 (the apparently normal eye of a rat whose other eye was purulent and degenerated). This apparently normal eye showed the presence of leucocytes in the cornea, a condition which is frequently associated with incipient bacterial infection. We cannot therefore deduce from the condition of such corneas that there is any demonstrable histological change directly attributable to dietary deficiency prior to bacterial invasion. A slightly later stage is shown by the other eye described in Case 3, where purulent symptoms were apparent and the cornea was swollen and oedematous. Again leucocytes are present, but no definite blood vessels are yet discernible.

In Case 5 very definite blood vessels are present, and there is marked increase in the number of corneal corpuscles.

The bad eye of Case 4 shows the same abnormalities in a much greater degree. In both Cases 4 and 5 the tissue changes are more or less localised in definite areas, thus affording strong evidence that they are the direct result of bacterial infection.

In Case 6 the cornea is completely degenerated and here, as in Case 5, organisms of the pneumococcal type were demonstrated.

The histological conditions of "cured" eyes are of different types. In the majority of cases where cure is effected by change of diet, sight is restored and the cornea completely regains its normal condition as in Cases 7 and 8.

Where degeneration has (presumably) proceeded further before cure is commenced, the cornea may remain opaque as in Case 9, left eye. Histological investigation of this cornea shows that it is flattened and contains many large blood vessels. As 105 days had elapsed between the apparent completion of the cure and the killing of the rat, it is justifiable to assume that this condition was permanent and did not represent a stage in the recovery of the cornea.

In some cases the cornea has so far degenerated before cure is begun that the lens is forced through the aperture during life, and "cure" consists in the disappearance of pus and the healing over of the injured tissues. Case 9, right eye, exemplifies this condition.

**B (2). BACTERIOLOGICAL.***Method.*

Pus was collected from rats' eyes with a sterile platinum loop, direct films were examined, and cultures were put up in tryptic broth ( $P_H = 7.4$ ), on tryptic blood agar ( $P_H = 7.4$ ), and on inspissated serum. Anaerobic broth cultures were also incubated, but in no case did they yield the growth of organisms other than those facultative anaerobes that also appeared in the aerobic cultures. The organisms were obtained in pure culture, and fermentation reactions were studied by growth for seven days in litmus milk and

sugars, etc. (1 % of the substance dissolved in diluted tryptic broth, with litmus as an indicator). The organisms present in the conjunctival sac of several normal and "cured" rats were examined in a similar way.

### *Results.*

The following organisms were isolated:

1. Pneumococci; two types (see Table IV).
2. Staphylococci (*S. albus* and *S. aureus*).
3. Gram-positive cocco-bacilli; four types (see Table IV).
4. *Bacillus subtilis*.
5. Gram-positive diphtheroids; two types (see Table IV).
6. Other Gram-positive bacilli; two types (see Table IV).
7. Gram-negative bacilli; two types (see Table IV).

A list of the organisms found in 18 cases is appended:

#### *α. Normal rats on mixed diet; eyes clean and healthy.*

- Case 10. (a) *S. aureus*.  
 (b) Cocco-bacillus, Type III.
- Case 11. (a) Pneumococcus, Type I.  
 (b) Gram-positive bacillus, Type II.
- Case 12. (a) *S. albus*.

#### *β. Rats on diet deficient in fat-soluble factor.*

- Case 13. Rat No. 91; eyes purulent and bleeding.  
 (a) Pneumococcus, Type I.  
 (b) Cocco-bacillus, Type II.
- Case 14. Rat No. 129; eyes closed; purulent.  
 (a) Pneumococcus, Type I.  
 (b) *S. aureus*.
- Case 15. Rat No. 48; eyes purulent.  
 (a) Pneumococcus, Type I.  
 (b) Diphtheroid, Type I.
- Case 16. Rat No. 202; eyes purulent.  
 (a) Pneumococcus, Type II.  
 (b) Cocco-bacillus, Type III.
- Case 17. Rat No. 205; eyes bleeding.  
 (a) Pneumococcus, Type II.  
 (b) Gram-negative bacillus, Type II.

Table IV.

Organism	Glucose	Saccharose	Mannitol	Maltose	Lactose	Raffinose	Inulin	Levulose	Galactose	Dextrin	Dulcitol	Milk	Notes
Pneumococcus, Type I	A	A	A	A	A	A	A	A	A	A	—	A	Long chains formed in broth.
" " II	A	A	A	A	A	—	A	A	A	A	—	A or AC	Long chains formed in broth.
Cocco-bacillus, Type I	A	A	—	A	A	A	—	A	A	A	—	A	Very small colonies on agar.
" " II	A	A	A	A	A	—	A	A	A	A	—	AC	Strong growth on agar.
" " III	A	(A)	A	A	A	—	—	A	A	A	—	(AC)	Spreading greenish growth on agar.
" " IV	A	—	—	—	—	—	—	(A)	A	(A)	—	AC	Strong white viscous growth on agar; pellicle formed on broth.
Diphtheroid, Type I	No growth	—	—	—	—	—	—	—	—	—	—	—	Very small colonies on agar.
" " II	A	—	—	—	—	—	—	A	A	A	—	—	Strong growth on agar.
Gram-positive Bacillus, Type I	A	A	—	A	—	—	—	A	—	A	—	—	White lustrous growth on agar.
Gram-positive Bacillus, Type II	A	A	A	A	A	A	—	A	A	A	—	A	Strong growth on agar; non-motile.
Gram-negative Bacillus, Type I	—	—	—	—	—	—	—	A	A	A	—	A	Liquefies inspissated serum.
Gram-negative Bacillus, Type II	AG	—	—	—	—	—	—	AG	AG	AG	—	A	Strong growth on agar; non-motile.

A=acid.

AG=acid and gas.

AC=acid and clot.

(A)=acid usually but not invariably formed.

Case 18. *Rat No.* 63; eyes purulent.

(a) Gram-positive lancet-shaped diplococci not obtained in pure culture.

(b) *S. albus*.

(c) *B. subtilis*.

Case 19. *Rat No.* 113 (see Case 7); eyes purulent.

(a) *S. aureus*.

(b) *B. subtilis*.

(c) Gram-negative bacillus, Type I.

Case 20. *Rat No.* 201; eyes purulent.

(a) Diphtheroid, Type I.

Case 21. *Rat No.* 203; eyes opaque and bleeding; no pus.

(a) Cocco-bacillus, Type III.

(b) Gram-positive bacillus, Type I.

Case 22. *Rat No.* 204; eyes purulent and bleeding.

(a) Cocco-bacillus, Type III.

(b) Diphtheroid, Type I.

Case 23. *Rat No.* 206; eyes closed; slightly purulent.

(a) Cocco-bacillus, Type IV.

(b) Diphtheroid, Type I.

Case 24. *Rat No.* 207; eyes purulent.

(a) Cocco-bacillus, Type III.

(b) Cocco-bacillus, Type IV.

*γ. Rats which developed eye disease on a diet deficient in the fat-soluble factor, and which were subsequently cured by change of diet.*

Case 25. *Rat No.* 114 (see Case 8).

*Right eye still purulent.*

(a) Pneumococcus, Type I.

(b) *S. aureus*.

(c) *B. subtilis*.

(d) Gram-negative bacillus, Type II.

*Left eye: no pus.*

(a) Diphtheroid, Type II.

Case 26. *Rat No.* 161; eyes still showing symptoms of disease.

(a) *S. albus*.

(b) Diphtheroid, Type II.

Case 27. *Rat No.* 63 (see Case 1 before treatment).

The diet was changed on the 73rd day, and on the 78th day the eyes were quite clean. Two days later the eyes were swabbed.

(a) Cocco-bacillus, Type I.

An attempt was made to determine whether the eyes of rats which had subsisted for a prolonged period on a diet deficient in the fat-soluble factor were any more susceptible to bacterial infection than the eyes of control rats on butter diet. For this purpose four rats were selected, two normal rats and two which had not developed eye disease although they had been eight weeks on a diet deficient in the fat-soluble factor. The right eye of each of these four rats was smeared with pus just removed from a purulent eye. The experiment was repeated four times and in each case no symptoms of eye disease developed in any of the four rats.

#### *Interpretation of Bacteriological Results.*

Cases 10, 11 and 12 indicate that the normal conjunctiva of the rat has a varying flora. When interpreting the results from diseased eyes, it must be remembered that pus from the eye is open to contamination from the air, and that therefore an organism isolated from the pus is not necessarily concerned with the production of lesions in the eye.

Pus from the diseased eyes in many cases (13, 14, 15, 16, 17, 25) showed the presence of a Pneumococcus, but this organism was not invariably present, in fact no organism that we obtained was common to all the purulent cases, so that we have not been able to show that the specificity of the disease is related to bacterial species.

From the results given above, it appears that when the rat has been on a deficiency diet for such a length of time that the resistance of the cornea is affected, those pathogenic bacteria which happen to be present in the conjunctival sac invade the corneal epithelium and induce the destruction of tissue.

#### SUMMARY AND DISCUSSION.

The histological and bacteriological evidence shows that keratomalacia among rats consists in a breakdown of the corneal tissue, caused by bacterial invasion.

The dietetic and statistical evidence shows that bacterial invasion of the cornea occurs only after a prolonged course of diet in which the fat-soluble factor is absent. The condition therefore directly attributable to dietetic deficiency is a predisposition to bacterial infection of the cornea leading to purulent symptoms and the destruction of tissue.

The question then to be decided is whether this predisposition of the cornea to infection is to be regarded as a true deficiency disease. The answer to this question is complicated by the following consideration.

We have hitherto failed to demonstrate with certainty any histological change in the cornea preceding bacterial invasion. We cannot therefore state the precise moment at which the predisposition to infection begins. The only criterion we possess for determining the preliminary change caused by the deficient diet is the appearance of the secondary symptoms caused by bacterial

invasion. Supposing a variable time to elapse between the preliminary change in the cornea and the actual invasion by bacteria—inflammation, pus, etc.—then the statistics collected in the earlier part of the paper are invalidated, although the evidence on the question of the cure is unaffected. If however we may assume that the organisms normally present in the conjunctival sac ensure the infection of the cornea as soon as the absence of the fat-soluble factor has rendered it liable to infection, then we are justified in regarding, for statistical purposes, the appearance of the purulent symptoms as an immediate notification of the preliminary change in the cornea due to dietary deficiency, and the evidence adduced on the incidence of the disease remains valid.

Assuming—as seems probable—the latter hypothesis to be the true one, the statistical evidence described in the earlier part of our paper can be applied to decide whether predisposition of the cornea to infection is a true deficiency disease. The conclusions then arrived at may be briefly recapitulated.

1. Corneal disease is not coincident with failure of growth culminating in death; it forms only 28 % of the cases examined.

2. This disease is further differentiated from the cessation of growth-death symptoms by occurring at a later stage in the experimental period and rising more rapidly to a maximum.

3. At no period in the experiment are all the deaths preceded by eye disease or all the survivors afflicted with it.

4. Cure of the corneal disease was effected by the replacement of the fat-soluble factor in 100 % of the cases attempted.

Before this evidence, however, serves to place preliminary deterioration of the cornea among deficiency diseases, it requires the acceptance of one of the three supplementary hypotheses A, B or C below:

*either*

A. The symptoms caused by experimental fat-soluble deficiency disease among rats vary in such a way that in some cases the cessation of growth-death symptoms predominate to such a degree that death ensues before the deterioration of the cornea commences, whereas in other cases the nutritive integrity of the cornea is disturbed before the cessation of growth symptoms reach an acute stage;

*or*

B. The concentration of the fat-soluble factor in the tissues of the rat necessary to protect the cornea was less than that required for life and growth in 72 % of the cases examined (*i.e.* in those dying without eye disease); whilst the reverse was true in 28 % of the cases (*i.e.* in those developing disease before death);

*or*

C. Two factors are involved; one responsible for the continuance of growth and the maintenance of life, and another for the protection of the cornea.

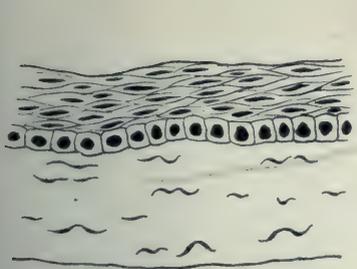


Fig. 9

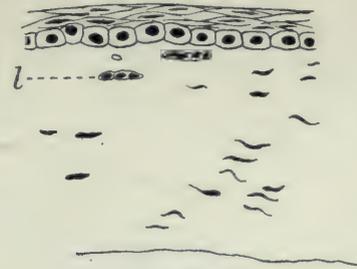


Fig. 10

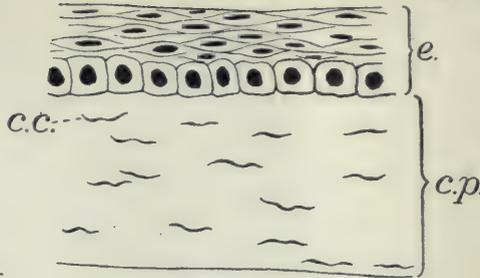


Fig. 11

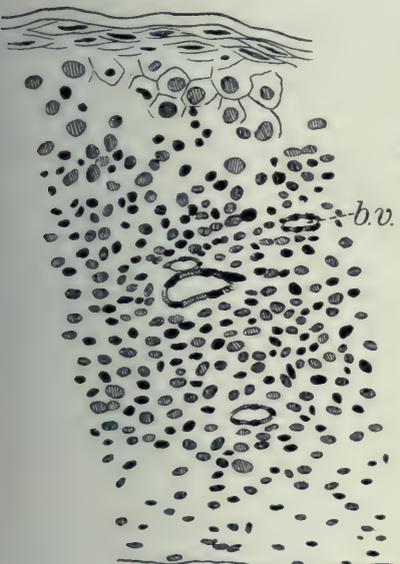


Fig. 12

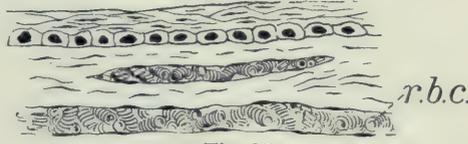


Fig. 15



Fig. 14

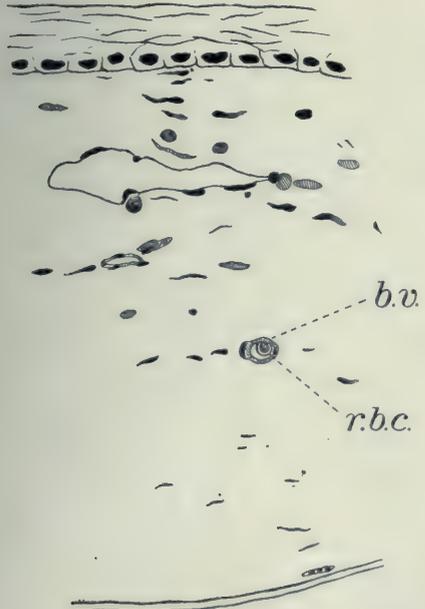


Fig. 13

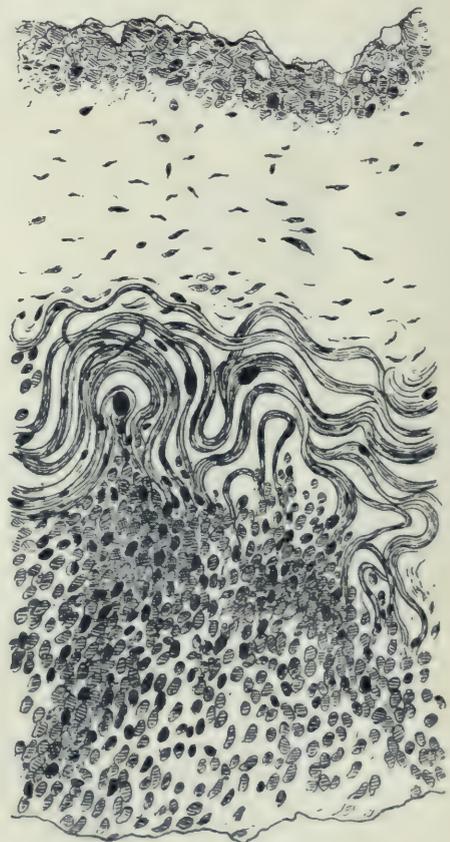


Fig. 16

0 10 20 30 40 50μ

Figs. 9, 10, 11, 12, 13, 15, 16.

0 10 20 30

Fig. 14



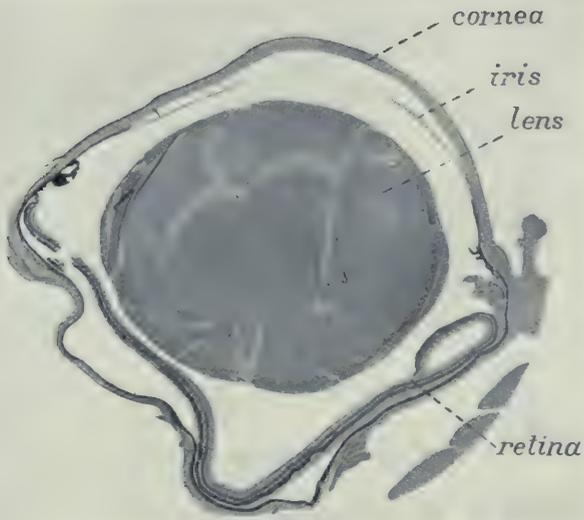


Fig. 17



Fig. 18



The authors gladly avail themselves of this opportunity for thanking Professor Hopkins for his stimulating help and advice in the course of this investigation. They are also deeply indebted to Dr H. K. Anderson, Master of Gonville and Caius College, Cambridge, for his invaluable help in interpreting histological evidence.

The expenses of this research were defrayed from funds contributed by the Planters Margarine Co., and the Maypole Co.

The authors are also indebted to the above firms for their courtesy in permitting the results to be published.

#### EXPLANATION OF PLATES IX AND X.

Fig. 9. Normal cornea.

Fig. 10. Case 3, Rat 7. Cornea of apparently normal eye.

Fig. 11. Case 4, Rat 74. Cornea of normal eye.

Fig. 12. Case 4, Rat 74. Cornea of diseased eye.

Fig. 13. Case 5, Rat 77. Cornea.

Fig. 14. Case 5, Rat 77. Portion of degenerated corneal epithelium stained by Gram's method.

Fig. 15. Case 9, Rat 5. Cornea of left eye.

Fig. 16. Case 9, Rat 5. Cornea of right "eye."

Fig. 17. Microphotograph of normal eye. ( $\times 25$ .)

Fig. 18. Microphotograph of Case 9, Rat 5, right "eye." ( $\times 25$ .)

*b.v.* = blood vessel. *c.c.* = corneal corpuscle. *c.p.* = cornea proper. *d.* = Gram-positive diplococci. *e.* = epithelium. *l.* = leucocytes. *r.b.c.* = red blood corpuscles.

Microphotographs by A. C. P. Lunn, King's College, Cambridge.

#### REFERENCES.

- Bloch, C. E. (1917). *Ugeskrift for Laeger*, **79**, 349.  
 Bulley, E. C. (1919). *J. Biol. Chem.*, **13**, 103.  
 McCollum and Simmonds (1917, 1). *J. Biol. Chem.*, **32**, 181.  
 — (1917, 2). *J. Biol. Chem.*, **32**, 347.  
 Mori, M. (1904). *Jahrb. Kinderheilk*, **59**, 175.

# XLIII. BARGER'S MICROSCOPICAL METHOD OF DETERMINING MOLECULAR WEIGHTS.

## PART II. ITS APPLICATION TO CASEINOGEN.

BY KUMAO YAMAKAMI.

*From the Lister Institute of Preventive Medicine, London.*

*(Received April 12th, 1920.)*

### A. THE MOLECULAR AND IONIC CONCENTRATION OF ALKALI CASEINOGENATE SOLUTIONS OF NEUTRAL AND ALKALINE REACTION.

THIS work has been undertaken to study the osmotic concentration of alkali caseinogenate solutions. T. B. Robertson [1909, 1918, p. 336] and T. B. Robertson and T. C. Burnett [1909] have investigated the cryoscopic behaviour of dissolved caseinogenates of alkalies and alkaline earths ( $\text{NH}_4\text{OH}$ ,  $\text{KOH}$ ,  $\text{LiOH}$ ,  $\text{Ca}(\text{OH})_2$  etc.) and found that:

“Keeping the concentration of casein constant and increasing the concentration of base bound by the caseinogen results in a proportionate increase in the observed depression, in other words, the molecular and ionic concentration of caseinate solutions is conditioned by the combined alkali. On the other hand, the increasing of the quantity of dissolved casein does not alter the observed depression in any appreciable degree when casein is dissolved in the solution of base in the proportion of  $80 \cdot 10^{-5}$  gram equivalent or  $50 \cdot 10^{-5}$  gram equivalent base per gram casein.”

From these experimental results they concluded that each equivalent of combined base yields the same number of protein ions derived from the splitting of successive  $\cdot\text{CO}\cdot\text{NH}$  groups, and suggested that “if this relation were maintained for other concentrations, then at zero concentration of combined base, if casein were soluble under such conditions, the freezing point depression due to dissolved casein would be zero. The possibility is therefore indicated that base- and acid-free protein may exert an immeasurably small osmotic pressure, probably owing to the polymerisation of protein as the uncombined protein is set free.”

When I was in the laboratory of Toronto University, it was suggested that I should apply Barger's microscopical method of determining molecular weights to caseinogen. Accordingly I investigated the osmotic concentrations of alkali caseinogenate solutions employing caseinogen which was prepared by a colleague, but I could not demonstrate the parallelism between the

molecular ionic concentrations of caseinogenate solution and the concentration of the base in which the caseinogen was dissolved.

Prof. Robertson suggested that this might be due to the impurity of the caseinogen employed, and this proved to be the case. For, when I repeated the experiments at the Lister Institute, London, with "Hammarsten casein" (Kahlbaum) consistent results were obtained.

The molecular and ionic concentrations of Na-caseinogenate solutions have been observed to be absolutely dependent upon the concentration of NaOH, not only when the proportion of base to caseinogen is between  $80 \cdot 10^{-5}$  and  $50 \cdot 10^{-5}$  g. equivalents base per g. caseinogen, but also for all other ratios between  $180 \cdot 10^{-5}$  and  $50 \cdot 10^{-5}$  g. equivalents.

*Experiment 1.* "Hammarsten casein" (Kahlbaum) was purified according to the directions of Robertson [1918, p. 39] and was dissolved in NaOH solutions of varying concentrations, changing the amount of caseinogen, so that the ratio of caseinogen to base varied between  $180 \cdot 10^{-5}$  and  $50 \cdot 10^{-5}$  g. equivalents base per g. caseinogen and the molecular and ionic concentrations of these caseinogenate solutions were determined by means of Barger's method, employing urea as the standard substance.

Unfortunately there is at present no good test for the purity of caseinogen, but its degree of purity in my experiments is indicated by the following results of the tests which are generally applied.

1. A perfectly white powder which is absolutely dry.
2. Its solution is colourless.
3. The percentage of ash is 0.11.
4. The solution in alkali in the proportion of  $80 \cdot 10^{-5}$  g. equivalent is just neutral to phenolphthalein.
5. The solubility in 5 % NaCl solution is less than 2 %.

If van Slyke and Bosworth's statement [1913] that the usual caseinogen preparations obtained from chemical supply houses are generally contaminated with water-insoluble calcium caseinogenate (monobasic caseinogenate) and calcium phosphate is true, the directions for purification given by Robertson cannot be very effective, but the solubility in NaCl solution as well as the content of ash in the caseinogen preparation employed in my experiments indicate that it is sufficiently pure for the purpose.

The purity of the urea employed as standard had been checked by determining the molecular weights of other pure substances such as cane-sugar, lactose, etc. with its aid.

The first measurement of the drops was made 30-40 minutes after the preparation of the tubes and the second determination after standing for 24 hours in a cold room.

The caseinogenate solutions in these experiments had generally small molecular-ionic concentrations, consequently the change of the drops was very slight even when the tubes were exposed to ordinary room temperature. Nevertheless the tubes were kept cool in the vestibule of a cold room where

the temperature was about  $10^{\circ}$ – $11^{\circ}$  C., because it was feared that the decomposition of caseinogen by micro-organisms which cannot be prevented from entering the solution, might take place more easily at room temperature.

The technique employed is exactly the same as described in Part I [Yamakami, 1920], the directions of Barger being strictly followed. In the first determination the absolute measurements of the drops and the changes in them are given by way of illustration. In all the remaining determinations only the concentrations of the solutions in the limiting tubes are given.

In the first column of the table, the molar concentration of the urea solutions is given, and the drops denoted by odd numbers are those of the caseinogenate solutions.

Table I.

(a) NaOH =  $N/20$ , caseinogen = 10 %; alkali: caseinogen =  $50 \cdot 10^{-5}$  g. eq.: 1 g.

Urea	I	II	III	IV	V
<i>M/12</i>	221 - 7	384 + 7	235 - 7	359 + 11	303 - 10
<i>M/14</i>	251 - 6	279 + 6	270 - 15	331 + 7	209 - 9
<i>M/16</i>	402 - 1	676 + 2	175 - 4	754 + 4	608 - 4
<i>M/20</i>	388 + 2	555 - 1	490 + 1	612 ± 0	224 ± 0
<i>M/25</i>	504 + 12	841 - 1	785 + 5	670 + 4	492 + 2

Limits =  $1/16$ – $1/20$  *M*.

(b) NaOH =  $N/20$ , caseinogen = 8 %; alkali: caseinogen =  $62 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/16$ – $1/20$  *M*.

(c) NaOH =  $N/20$ , caseinogen = 6.2 %; alkali: caseinogen =  $80 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/16$ – $1/20$  *M*.

(d) NaOH =  $N/20$ , caseinogen = 4 %; alkali: caseinogen =  $125 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/16$ – $1/20$  *M*.

(e) NaOH =  $N/20$ , caseinogen = 2.78 %; alkali: caseinogen =  $180 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/16$ – $1/20$  *M*.

(f) NaOH =  $N/20 \cdot 8$ , caseinogen = 6 %; alkali: caseinogen =  $80 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/17 \cdot 5$ – $1/20$  *M*.

(g) Caseinogen solutions containing respectively  $50 \cdot 10^{-5}$  and  $80 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen were compared directly.

I	II	III	IV	V
-2	-2	-3	-3	-2
-2	+1	+1	+1	+1
-3	-2	-2	-2	-1

The drops denoted by even numbers are those of caseinogen solution containing  $50 \cdot 10^{-5}$  g. equivalent alkali.

Table II.

(a) NaOH =  $N/25$ , caseinogen = 8 %; alkali: caseinogen =  $50 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/20$ – $1/25$  *M*.

(b) NaOH =  $N/25$ , caseinogen = 6 %; alkali: caseinogen =  $66 \cdot 7 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/20$ – $1/25$  *M*.

(c) NaOH =  $N/25$ , caseinogen = 5 %; alkali: caseinogen =  $80 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/20$ – $1/25$  *M*.

(d) NaOH =  $N/25$ , caseinogen = 3.75 %; alkali: caseinogen =  $106 \cdot 6 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/20$ – $1/25$  *M*.

(e) NaOH =  $N/25$ , caseinogen = 2.22 %; alkali: caseinogen =  $180 \cdot 10^{-5}$  g. eq.: 1 g.

Limits =  $1/20$ – $1/25$  *M*.

Table III.

- (a) NaOH =  $N/33$ , caseinogen = 6 %; alkali: caseinogen =  $50 \cdot 10^{-5}$  g. eq.: 1 g.  
Limit =  $1/35$  M.
- (b) NaOH =  $N/33$ , caseinogen = 3.75 %; alkali: caseinogen =  $57.6 \cdot 10^{-5}$  g. eq.: 1 g.  
Limits =  $1/35$ – $1/40$  M.
- (c) NaOH =  $N/33$ , caseinogen = 1.7 %; alkali: caseinogen =  $182 \cdot 10^{-5}$  g. eq.: 1 g.  
Limits =  $1/30$ – $1/35$  M.
- (d) NaOH =  $N/50$ , caseinogen = 4 %; alkali: caseinogen =  $50 \cdot 10^{-5}$  g. eq.: 1 g.  
Limits =  $1/40$ – $1/50$  M.
- (e) NaOH =  $N/50$ , caseinogen = 3.7 %; alkali: caseinogen =  $53 \cdot 10^{-5}$  g. eq.: 1 g.  
Limits =  $1/40$ – $1/50$  M.
- (f) NaOH =  $N/50$ , caseinogen = 2.5 %; alkali: caseinogen =  $80 \cdot 10^{-5}$  g. eq.: 1 g.  
Limit =  $1/40$  M.
- (g) NaOH =  $N/50$ , caseinogen = 2.2 %; alkali: caseinogen =  $90 \cdot 10^{-5}$  g. eq.: 1 g.  
Limits =  $1/40$ – $1/50$  M.

The results obtained in the above tables are so regular that detailed explanation and discussion are hardly necessary. The molecular and ionic concentrations of the caseinogenate solutions were always very nearly identical with the concentrations of the alkali solutions in which the caseinogen was dissolved, as long as the proportion of alkali to caseinogen was between  $180 \cdot 10^{-5}$  g. equivalent and  $50 \cdot 10^{-5}$  g. equivalent base per g. caseinogen.

The only thing I feared was that urea might combine chemically with the alkali of the caseinogenate solutions. But this, very probably, would not occur because the urea solution is very slightly acid to phenolphthalein, while the acidity of caseinogen is pretty strong. Thus the urea solution would not be able to attract the alkali which was combined with such a strong acid as caseinogen. This would be particularly true when the reaction of the caseinogenate solution itself was strongly acid to phenolphthalein. Moreover the regularity of the change of the drops suggests that no chemical reaction was taking place between the two solutions.

As to the effect of the impurities in the caseinogen upon the results obtained, the calcium caseinogenate which is alleged to contaminate most preparations of caseinogen would not exert much influence upon the molecular and ionic concentrations.

The possible impurities which must be taken into consideration in these experiments are crystalloid substances which have small molecular weights, because they would change the osmotic concentrations of the caseinogenate solutions to a considerable extent even if present only in small quantities. In order to get rid of this objection, I dialysed my caseinogenate solutions and convinced myself that the caseinogen used was free from crystalloid substances since no lowering of the molecular and ionic concentration of the caseinogenate solution resulted from dialysis. Reference will be made to these experiments later.

On one occasion, the direct comparison of caseinogenate solutions which contained varying amounts of caseinogen and alkali gave the same result,

and this indeed might well be expected, as in the last experiment recorded in Table I, in cases where the reaction of the two caseinogenate solutions compared did not differ very much. But when there existed large differences in the reactions of the two solutions, the change of the drops was quite irregular, so that no conclusion could be drawn from the experiment. This is probably due to the chemical attraction between the two solutions, which interferes with the regularity of movement of the solution from one drop to another because of the physical attraction of solute for solvent.

It is my belief that the observation made by Robertson and Burnett by means of the cryoscopic method is satisfactorily confirmed by my experiments in which Barger's method was employed.

Now, if this relationship between alkali, caseinogen and the molecular ionic concentration of the solution is maintained unchanged up to the highest percentage of caseinogen which is soluble in a given amount of alkali, then the largest molecular ionic weight, we can expect to obtain by Barger's method, must be dependent upon the solubility of caseinogen in the alkaline solution.

If caseinogen is soluble in the alkaline solution in the proportion of  $25 \cdot 10^{-5}$  g. equivalent of alkali per g. caseinogen then we should obtain 4000 as the molecular or ionic weight of caseinogen, if, on the other hand, the solubility of caseinogen is  $12 \cdot 5 \cdot 10^{-5}$  g. equivalent then the figure would become 8000 provided that the osmotic concentration of caseinogenate solution is always conditioned by the concentration of alkaline solution.

The solubility was, therefore, studied before I undertook the investigation of the molecular and ionic concentrations of the alkali caseinogenate solutions of acid reaction in which the proportion of alkali to caseinogen was smaller than  $50 \cdot 10^{-5}$  g. equivalent per g. caseinogen.

#### B. THE SOLUBILITY OF CASEINOGEN IN ALKALI SOLUTION AND THE MOLECULAR WEIGHT OF CASEINOGEN CALCULATED FROM THE SOLUBILITY OR THE COMBINING CAPACITY.

The solubility of caseinogen in alkali solution or "the combining capacity of casein at saturation of the base with casein, that is when the base has dissolved the maximum amount of casein which it will dissolve," as expressed by Robertson, has recently been stated to be  $11 \cdot 4 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen. This is based upon two experimental results, the one, obtained by Robertson, and the other, by van Slyke and Bosworth [1913], and it is supported by deduction from the formula for the lowering of the conductivity of alkali solution which is caused by dissolved caseinogen.

Robertson found that the relation between  $b_1$ , the concentration of alkali solution in which caseinogen is dissolved, and  $\Delta$ , the lowering of its conductivity which is caused by the caseinogen, is expressed by the formula

$$\Delta \times 10^5 = 26 \cdot 880b_1 - \frac{475 \cdot 800}{C} b_1^2 - 2898C.$$

Putting  $\Delta = 0$ , the concentration of alkali solution  $b_1$  becomes  $0.000114C$ . This proportion of alkali to caseinogen occurs at the point where the change in the conductivity of an alkaline solution, which is brought about by dissolving a given percentage of caseinogen, is zero, and coincides exactly with the value for the solubility of caseinogen which Robertson [1918] and van Slyke and Bosworth [1913] claim to have obtained experimentally.

Van Slyke and Bosworth, however, proved the fact that the caseinogenate of an alkaline earth is not soluble when the proportion of base to caseinogen is  $11.4 \cdot 10^{-5}$  g. equivalent: 1 g.; it is soluble in water only when the proportion is larger than  $22.5 \cdot 10^{-5}$ . This fact seems to suggest very strongly that we should not identify the solubility of caseinogen in alkali solution with its combining capacity.

In other words, caseinogen may combine with alkali in the proportion of  $11.4 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen, as deduced from the above-mentioned formula, yet it does not follow that the product of combination must necessarily dissolve in water.

This suspicion with regard to the solubility of van Slyke and Bosworth's monobasic alkali caseinogenate is deepened when we carefully examine the experiments of the authors who claim  $11.4 \cdot 10^{-5}$  g. equivalent as the solubility as well as the combining capacity of caseinogen to alkali.

When they determined the solubility of caseinogen in alkali, they did not dissolve directly as much caseinogen as possible in alkali solution, but in both investigations a measured amount of caseinogen was dissolved in a known quantity of alkali capable of very easily dissolving the caseinogen taken and then the point was determined at which the first permanent precipitation of caseinogen appeared on neutralising the caseinogen solution with HCl, using the refractometric method (Robertson) or centrifuging (van Slyke and Bosworth). The value, which they call the solubility, is therefore the quantity of alkali used to dissolve the caseinogen minus the amount of alkali neutralised by HCl.

But it must be taken into consideration that the resulting solution of caseinogenate in the experiments of these authors is not a pure aqueous solution of alkali caseinogenate, but a caseinogenate solution containing alkali chloride. Thus the resulting solution in van Slyke and Bosworth's experiment contained almost 0.5 % alkali chloride.

The solubility of caseinogen in NaCl solution seems to be undeniable [Robertson, 1918, p. 88], although it is questioned by van Slyke and Bosworth.

Even if the insolubility of uncombined caseinogen is admitted to be true, as van Slyke and Bosworth claim, yet there remains the possibility that the so-called monobasic caseinogenate may be soluble in alkali chloride solution and insoluble in water, just as in the case of the alkaline earth caseinogenates.

No one has succeeded, so far as I know, in dissolving pure caseinogen (uncontaminated by base) in alkali solution in a proportion of  $11.5 \cdot 10^{-5}$  g. equivalent directly. Even when we triturate caseinogen in a mortar with

alkali solution for two or three hours, it is not easy to dissolve caseinogen in a proportion of  $22.5 \cdot 10^{-5}$ . It seems hardly credible therefore that the solubility of caseinogen should be more than twice this value. It seems reasonable to ascribe the results obtained by van Slyke and Bosworth as well as by Robertson to the greater solubility of caseinogen in alkali chloride solution or to the solubility of so-called monobasic alkali caseinogenate in alkali chloride solution, if such a combination indeed exists. And the water-soluble combination of alkali and caseinogen should be accepted as containing  $22.5 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen.

Our experimental results described in the following pages appear to support this view.

*Experiment 2.* In this experiment, the solubility of caseinogen in pure alkali (NaOH) solution was compared with that of caseinogen in 0.5 % saline solution and that of caseinogen in alkali solution containing 0.5 % alkali chloride (NaCl).

The object of this experiment was to investigate whether the solubility of caseinogen in alkali is increased by the addition of alkali chloride to the alkali solution.

0.4 g. or 0.8 g. of caseinogen was accurately weighed and triturated in a glass mortar respectively with 5 cc. distilled water, 5 cc. 0.5 % NaCl solution, 5 cc. *N*/500 NaOH solution, 5 cc. *N*/500 NaOH solution in 5 % NaCl solution, very thoroughly for about 1.5 hours in each case, and then the mixtures were poured into centrifuge tubes. The mortar was washed with 45 cc. of distilled water in each case and the wash water was also added to the solutions in the tubes. They were then stoppered with cotton wool and were allowed to stand at room temperature ( $13^{\circ}$ – $15^{\circ}$ ) for 24 hours, being shaken from time to time, the air over the solution being changed often so that the  $\text{CO}_2$  driven from the solution by the caseinogen was removed.

The solutions were then centrifuged, the supernatant fluid cautiously siphoned off, and the sediment washed carefully with absolute alcohol and ether dried over sodium and then dried and weighed.

By subtracting the obtained weight of sediment from the amount taken (0.4 or 0.8 g.) the amounts of caseinogen dissolved in these four solvents were determined. The results are given in Table IV. The loss of weight of caseinogen triturated with distilled water is apparently manipulative and must be taken into consideration also in the other cases.

Table IV.

Amount of triturated caseinogen. g.	Solvent 50 cc.	Weight of sediment. g.	Amount of caseinogen dissolved. g.
0.4	distilled water	0.3941	0.0059
0.4	0.5 % NaCl	0.3928	0.0072
0.4	<i>N</i> /500 NaOH	0.0246	0.3754
0.4	<i>N</i> /500 NaOH in 0.5 % NaCl	0.0000	0.4000
0.8	distilled water	0.7922	0.0078
0.8	0.5 % NaCl	0.7885	0.0115
0.8	<i>N</i> /500 NaOH	0.4070	0.3930
0.8	<i>N</i> /500 NaOH in 0.5 % NaCl	0.0236	0.7764

As will be seen from the table, 50 cc. of NaOH solution, when alkali chloride is added to it, dissolved 0.7764 g. of caseinogen, while the alkali solution alone dissolved only 0.3754–0.3930 g.; that is, the solubility is almost doubled in the former case. That this is not due to the ability of pure NaCl solution to dissolve caseinogen is evident since the amount of caseinogen dissolved in 0.5 % NaCl solution was only 0.0072–0.0115 g.

This experimental result suggests very strongly that the solubility observed by Robertson and Bosworth and by van Slyke is not the solubility of caseinogen in alkali solution but the solubility of caseinogen in alkali solution containing alkali chloride.

Thus it appears very probable that the alkali caseinogenate which contains  $11.4 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen or the monobasic alkali caseinogenate, as it is called by van Slyke and Bosworth, even if such a substance is admitted to exist, is not soluble in water and soluble only in alkali chloride solution.

As it is impossible to dissolve caseinogen in pure alkali solution in the direct way in larger proportion than 22.5–25.0· $10^{-5}$  g. equivalent alkali per g. caseinogen it seems reasonable to assume that the alkali caseinogenate which is soluble in water must contain this percentage of alkali.

Calculated from this solubility value (22.5–25.0· $10^{-5}$ ), the molecular weight of caseinogen is 4000–4400, one molecule of caseinogen containing one atom of phosphorus and one atom of sulphur.

More evidence must be produced before 8000–8800 can be admitted to be the true molecular weight of caseinogen.

### C. THE MOLECULAR AND IONIC CONCENTRATION AND THE DEGREE OF DISSOCIATION OF ALKALI CASEINOGENATE OF ACID REACTION.

We have seen in Exp. 1 that the osmotic concentration of alkali caseinogenate solution of neutral and alkaline reactions, is dependent upon the concentration of the alkali in which the caseinogen is dissolved, and that the amount of dissolved caseinogen does not affect in any appreciable degree the osmotic concentration between proportions of alkali to caseinogen from  $180 \cdot 10^{-5}$  to  $50 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen.

In Exp. 2, the molecular weight of caseinogen was shown to be 4000–4400, if calculated from the solubility of caseinogen in alkali solution. At any rate, the largest ionic weight of the protein radical in the alkali caseinogenate which is soluble in water must be 4000–4400, even if 8800 is ever proved to be the true molecular weight of uncombined caseinogen.

Now, if the alkali bound by caseinogen does not dissociate at all and no inorganic ions are present in the caseinogenate solution, and if polymerisation takes place in a caseinogenate solution of acid reaction while the caseinogen molecule is split successively into smaller protein ions by adding base, as Robertson [1918] has suggested, then the relationship between the osmotic

concentration of alkali caseinogenate solution and the concentration of the alkali in which the caseinogen was dissolved, must be maintained in caseinogenate solutions of acid reaction. Consequently we should expect to be able to obtain 4400 as the molecular weight of caseinogen by means of Barger's method when caseinogen is dissolved in alkali in the proportion of  $22.5 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen. But it is quite clear that this expectation cannot be realised experimentally.

4400 will not be obtained by Barger's method for the molecular weight of the caseinogenate at the point of the maximum solubility of caseinogen in alkali solution, simply because the reaction of this caseinogenate solution is acid.

The fact that the reaction of alkali caseinogenate solution is acid when the percentage of base in caseinogenate is smaller than  $50 \cdot 10^{-5}$  g. equivalent indicates the presence of free H ions in the solution, that is the molecule of caseinogenate is dissociated into the protein radical and H ions.

The H ion concentration is enhanced from  $P_H$  8.5 to  $P_H$  7.0 when the proportion of alkali to caseinogen is decreased from  $80 \cdot 10^{-5}$  to  $50 \cdot 10^{-5}$  g. equivalent per g. It will be, therefore, very easily realised that many H ions are dissociated when an alkali solution is saturated with caseinogen. Moreover, the hypothesis that the alkali salt of such a strong acid as caseinogen does not dissociate in the solution at all, is very doubtful. Under these circumstances, we believe that the results obtained in the following experiment may not be very erroneous.

*Experiment 3.* In this experiment, the molecular and ionic concentration of alkali (NaOH) caseinogenate solution of acid reaction was determined by Barger's method.

Table V.

- (a) NaOH =  $N/50$ , caseinogen = 5 %; alkali: caseinogen =  $40 \cdot 10^{-5}$  g. eq.: 1 g.  
The molecular and ionic concentration =  $1/35-1/40$  M.  
The mean molecular ionic weight = 1875.
- (b) NaOH =  $N/50$ , caseinogen = 6 %; alkali: caseinogen =  $33.3 \cdot 10^{-5}$  g. eq.: 1 g.  
The molecular and ionic concentration =  $1/30-1/35$  M.  
The mean molecular ionic weight = 1950.
- (c) NaOH =  $N/50$ , caseinogen = 7 %; alkali: caseinogen =  $28.5 \cdot 10^{-5}$  g. eq.: 1 g.  
The molecular and ionic concentration =  $1/30$  M.  
The mean molecular ionic weight = 1750.

Table VI.

- (d) NaOH =  $N/33.3$ , caseinogen = 8.0 %; NaOH: caseinogen =  $37.5 \cdot 10^{-5}$  g. eq.: 1 g.  
The molecular and ionic concentration =  $1/20-1/25$  M.  
The mean molecular ionic weight = 1800.
- (e) NaOH =  $N/33.3$ , caseinogen = 10.0 %; NaOH: caseinogen =  $30 \cdot 10^{-5}$  g. eq.: 1 g.  
The molecular and ionic concentration =  $1/17.5-1/20$  M.  
The mean molecular ionic weight = 1875.
- (f) NaOH =  $N/33.3$ , caseinogen = 10.8 %; NaOH: caseinogen =  $27.8 \cdot 10^{-5}$  g. eq.: 1 g.  
The molecular and ionic concentration =  $1/17.5-1/20$  M.  
The mean molecular ionic weight = 2025.
- (g) NaOH =  $N/33.3$ , caseinogen = 13.5 %; NaOH: caseinogen =  $29.6 \cdot 10^{-5}$  g. eq.: 1 g.  
The molecular and ionic concentration =  $1/15-1/17.5$  M.  
The mean molecular ionic weight = 2194.

As shown in Tables V and VI, the osmotic concentration of alkali caseinogenate solution of acid reaction is not dependent upon the concentration of alkali solution in which the caseinogen was dissolved, thus differing from caseinogenate solutions of neutral or alkaline reaction. The factor which conditioned the molecular and ionic concentration of the solution in this experiment was the amount of caseinogen dissolved. The osmotic concentration was almost parallel with the amount of caseinogen dissolved, and consequently the mean weight of the dissolved molecules and ions in the solution was calculated to be about 2000.

The objection might be raised that the result is due to impurities in the caseinogen. But such cannot be the case, because dialysis of the caseinogenate solution did not lower the osmotic concentration of the solution, as shown in the following experiment.

The impurities which may influence the osmotic concentration of the solution would be crystalloid substances having small molecular weights.

Colloids with large molecular weights would not change the osmotic concentration of the caseinogen solution, even though they might happen to be contained in the caseinogen employed. It would, therefore, be expected that dialysis of the caseinogen solution would remove most of any contaminating substance which could influence the osmotic concentration of the solution.

*Experiment 4.* Caseinogen solution was dialysed in a very thin collodion sack in a cold room for 48 hours, the distilled water outside the collodion tube being changed frequently.

The volume of the solution (0.3 g. caseinogen dissolved in  $N/25$  NaOH solution) increased from 3.0 to 8.8 cc.

Table VII.

The molecular and ionic concentration after dialysis =  $1/60$ – $1/70$   $M$ .

The mean molecular ionic weight = 2206.

(The tubes in this experiment stood 32 hours after their preparation.)

Thus in every case, 2000 appears as the mean molecular and ionic weight of alkali caseinogenate when determined by Barger's method, if the proportion of alkali to caseinogen is smaller than  $50 \cdot 10^{-5}$  g. equivalent per g. caseinogen.

As has been stated previously, the molecular weight of alkali caseinogenate when determined by calculation from the combining capacity, supposing the solubility of caseinogen in alkali to be  $25$ – $22.5 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen, must be 4000–4400. We must therefore admit that one molecule of alkali caseinogenate is dissociated into two ions in solutions of acid reaction when the solutions are weak.

In the case of caseinogenate solutions of neutral or alkaline reaction, there may not exist any inorganic ions in the solution as Robertson suggests, the protein molecule being split into protein ions only, but in the case of alkali caseinogenate solutions of acid reaction, it is beyond doubt that the ions in

the solution are mostly those of the protein radical and hydrogen, since the reaction of the solution is acid.

Whether there are any alkali ions dissociated from the caseinogenate molecule, and whether there are any protein ions derived from the splitting of .CO.NH groups, is a very difficult problem to solve.

After I had finished these experiments, my attention was called to the paper of Plimmer and Bayliss [1906] in which they report that caustic soda of strong concentration (1 %) has the power of splitting off  $P_2O_5$  from dissolved caseinogen. Though they admit from their experimental basis that "dilute alkali sufficient only to dissolve the caseinogen causes no separation of soluble  $P_2O_5$ ," and, as a matter of fact, they themselves employed caustic soda as the solvent of caseinogen in their experiments, and though furthermore the fact that I have obtained quite the same results in my Exp. 1 as those obtained by Robertson and Burnett employing alkaline earths and other alkalies than NaOH, affords clear evidence that the specific decomposing action of caustic soda of strong concentration found by Plimmer and Bayliss was not taking place in my experiments, still I thought it might be of some use to test experimentally whether similar results can be secured when other alkalies than NaOH are used as solvents. Thus I performed a few experiments employing  $NH_4OH$  as solvent. The results are as follows.

- (I)  $NH_4OH = M/50$ . Caseinogen = 4 %. Alkali: caseinogen =  $50 \cdot 10^{-5}$  g. eq. : 1 g.  
Osmotic concentration =  $1/47.5 M$ .
- (II)  $NH_4OH = M/50$ . Caseinogen = 6.0 %. Alkali: caseinogen =  $33.3 \cdot 10^{-5}$  g. eq. : 1 g.  
Osmotic concentration =  $1/33.3 M$ .
- (III)  $NH_4OH = M/33.3$ . Caseinogen = 10.8 %. Alkali: caseinogen =  $27.8 \cdot 10^{-5}$  g. eq. : 1 g.  
Osmotic concentration =  $1/15 M$ .

These results are quite the same as observed when NaOH was used as solvent of caseinogen.

#### SUMMARY.

In the present paper, the solubility of caseinogen and the osmotic concentration of alkali caseinogenate solutions have been investigated, employing Barger's method of determining molecular weights, and it has been found that:

1. The solubility of caseinogen in alkali solution containing a certain amount of alkali chloride, is almost twice that of caseinogen in pure alkali solution.

2. The molecular and ionic concentration of alkali caseinogenate solutions of neutral and alkaline reaction is conditioned by the concentration of the alkali solution in which the caseinogen is dissolved.

3. The osmotic concentration of alkali caseinogenate solutions of acid reaction is dependent upon the amount of dissolved caseinogen, and the mean weight of the ions in the solution is about 2000.

As a result of these experiments, it is suggested that the solubility of caseinogen in alkali solution obtained by Robertson, as well as by van Slyke

and Bosworth, seems very probably to have been that of caseinogen in alkali solution containing alkali chloride. The true solubility of caseinogen in pure alkali, therefore, must be  $22.5 \cdot 10^{-5}$  g. equivalent alkali per g. caseinogen.

The molecular weight of alkali caseinogenate which is soluble in distilled water must be 4000–4400, and one molecule of caseinogenate seems to be dissociated in such dilute solutions as were investigated ( $1/15$ – $1/70$  *M*) mainly into two ions, consisting principally of H-ions and protein ions, beside the ions of the alkali and split protein.

In conclusion, I wish to express my thanks to Prof. C. J. Martin who supplied me with material and apparatus.

## REFERENCES.

- Plimmer and Bayliss (1906). *J. Physiol.*, **33**, 453.  
Robertson (1909). *Univ. of California, Publ. Physiol.*, **3**.  
— (1918). *The Physical Chemistry of Proteins*.  
Robertson and Burnett (1909). *J. Biol. Chem.*, **6**, 105.  
van Slyke and Bosworth (1913). *J. Biol. Chem.*, **14**, 203, 207, 211.  
Yamakami (1920). *Biochem. J.*, **14**, 103.



5-3

## XLIV. OXIDISING ENZYMES. II. THE NATURE OF THE ENZYMES ASSOCIATED WITH CERTAIN DIRECT OXIDISING SYSTEMS IN PLANTS.

BY MURIEL WHELDALE ONSLOW.

*From the Biochemical Laboratory, Cambridge.*

(Received May 6th, 1920.)

THE present and the following communications are a continuation of work undertaken upon the oxidising enzymes of various fruits for the Food Investigation Board of the Department of Scientific and Industrial Research.

In a previous paper [Onslow, 1919] it has been stated that the tissues and tissue extracts of those plants which give a blue colour with guaiacum tincture contain some substance with the catechol grouping in addition to a peroxidase. In the case of other plants the blueing of guaiacum is only brought about after addition of hydrogen peroxide, and from these plants substances with the catechol grouping are absent. It was shown, moreover, that in the first type of plants, the tissues could be freed from the "catechol" substance by thoroughly pounding and extracting with cold alcohol. A water extract of the tissue residue will then only blue guaiacum on addition of hydrogen peroxide, that is, it apparently only contains a peroxidase. Also when this extract is added to solutions of substances containing the catechol grouping, e.g. catechol, protocatechuic acid or the compound from the plant itself, oxidation of these takes place with a brown coloration and the production of a peroxide (probably hydrogen peroxide from decomposition in aqueous solution of a peroxide of the catechol compound). The resultant combination, peroxide and peroxidase, will then blue guaiacum. It was suggested that the enzyme present, which could only be spoken of as a peroxidase since it did not blue guaiacum without hydrogen peroxide, catalysed the oxidation of the catechol compound with the production of peroxide. Enzyme extracts prepared in a similar way from plants without catechol substances were without effect on catechol, protocatechuic acid, etc. After heating, the enzyme extract loses its power to oxidise catechol as well as the power to blue guaiacum in the presence of hydrogen peroxide. Two reactions are thus catalysed by the enzyme extract in the first of the above-mentioned classes of plants. One is the oxidation of the catechol substance by means of molecular oxygen with the formation of a peroxide: the other, the decomposition of the peroxide

with the setting free of "active" oxygen. The catalysis of the second type of reaction has always been defined as the sole function of peroxidases. If a peroxidase only is present in the enzyme extract of "catechol" plants, it must be allowed that the peroxidases in the two classes of plants are very different, in that the enzyme of one class appears to catalyse rapidly the oxidation of the catechol substance in addition to the usual function, whereas that of the other class is without effect. Since autoxidation is undoubtedly a complex process, it is not impossible that one enzyme may in some way catalyse both reactions, though the hypothesis is not satisfactory. As an alternative, it is now suggested that a second enzyme, in addition to the peroxidase, is present in the first class of plants. The function of this additional enzyme is to catalyse the oxidation of the catechol substance with the formation of a peroxide, which, when formed, is acted on by the peroxidase in the usual way. It is proposed to call this second enzyme an oxygenase<sup>1</sup>, a term originally used by Chodat and Bach for the portion of an oxidase which can be replaced by hydrogen peroxide. Chodat [1910] speaks of oxygenases thus: "...es sind fermentartige Körper, die sich mit dem Sauerstoff der Luft zu einem Peroxyd verbinden können." Thus these authors make no clear differentiation between the organic compound from which the peroxide is formed and the enzyme which forms the peroxide, as a result, in all probability, of not having effected in their experiments a separation of the catechol substance, or similar compound, from the enzymes involved. In recent years the term oxygenase has fallen into disuse in favour of the simpler explanation of oxidase action, *i.e.* as being due to a peroxide formed by autoxidation only, and a peroxidase.

In the present communication, the following additional evidence is offered in connection with these problems.

*Spontaneous autoxidation of solutions of various phenols and aromatic acids.*

Solutions of the following substances were exposed to air for 6-7 days: adrenaline, caffeic acid (crude extract only), carvacrol, catechol, cresol (ortho- and para-), gallic acid, guaiacol, orcinol, phenol, phloroglucinol, protocatechuic acid, pyrogallol, quinol, resorcinol, tannic acid and vanillin. One per cent. solutions were used, except in the cases of adrenaline and caffeic acid, of which the strength of the solutions was unknown. In the cases of caffeic acid, gallic acid, phenol, protocatechuic acid, pyrogallol and tannic acid, before exposure to air, dilute caustic potash was added to the solutions until they just failed to change the yellow colour of bromocresol purple, *i.e.* the  $P_H$  ranged between 5.2-6.8. After the long exposure to air, a few cc. were then tested for peroxide with a mixture of 5 cc. of 2 % potassium iodide, 2 cc. of 1 % acetic acid and

<sup>1</sup> The term oxygenase is not very satisfactory, since the function of the enzyme is not oxygen-splitting. It has been adopted for the present, however, to avoid further confusion of nomenclature. Should the enzyme be definitely isolated at some future date, the desirability of giving it a more suitable name might be considered.

1 cc. of 1 % starch solution. A blue colour appeared within half an hour in the cases of adrenaline, caffeic acid and catechol. No colour appeared in the other solutions on standing an hour. If 1 % sulphuric acid is substituted for 1 % acetic acid, in addition to the above three, the following also gave the peroxide test more or less rapidly: gallic acid, phenol, protocatechuic acid, pyrogallol, quinol and tannic acid. The remaining solutions gave no colour within the hour allowed. Controls of starch, iodide and acid also gave no colour on standing for the same length of time. It is evident, therefore, that a number of the above substances tend to form peroxides (or hydrogen peroxide by decomposition) on autoxidation, and those with the catechol grouping, on the whole, most readily. In no case did a freshly-made solution of any of the above substances give a reaction with iodide, starch and either acetic or sulphuric acid.

*Behaviour, with peroxidase, of the above autoxidised solutions of various phenols and aromatic acids as peroxides.*

The behaviour of the autoxidised solutions with a peroxidase (from horse-radish) and guaiacum was next investigated. The peroxidase solution was prepared by well pounding and extracting horse-radish root with 96 % alcohol, and then extracting the residue with water. On addition of the enzyme extract to the above solutions, followed by guaiacum tincture, a blue colour was obtained in the case of adrenaline, caffeic acid, catechol, protocatechuic acid and to some extent tannic acid. Hence such peroxides formed by autoxidation can function in the peroxide-peroxidase system, and, on the whole, those formed by compounds with the catechol grouping are most efficient. In no case was any result obtained when freshly prepared solutions were used.

*Action on freshly-prepared solutions of the above phenols and aromatic acids of the crude enzyme extracts (oxygenase and peroxidase) from plants containing a "catechol" compound.*

The potato tuber and pear fruit were used as sources of enzymes. The enzyme extracts were prepared in the usual way by rapidly pounding the tissues with cold alcohol, filtering on a pump, and repeating the process several times. A water extract of the colourless residue is used for the test. The results with extracts from both plants were as follows. A rapid browning of solutions of catechol and of caffeic and protocatechuic acids on neutralisation. On subsequent addition of guaiacum, a blue colour appeared in all three cases. A solution of adrenaline is rapidly turned red, but no blueing follows on addition of guaiacum (in contrast to the result in the last section). A solution of tyrosine is reddened (rapidly in the case of the potato, slowly with the pear) finally becoming black, but no blue colour appeared on addition of guaiacum at any stage. A solution of *p*-cresol is turned orange (rapidly with potato, slowly with pear), but unlike the case of tyrosine, a blue colour is

given on subsequent addition of guaiacum. No action was obtained with the remainder of the substances.

The reactions with tyrosine and *p*-cresol are presumably due to tyrosinase which has long been known to be present in the potato, though no previous reference can be discovered to its occurrence in the pear fruit. The reaction with adrenaline may be due, either to the presence of tyrosinase, which, according to some authors, acts on this substance, or to the presence of the oxidising enzyme which reacts with the catechol grouping, or to both. Points of interest are the non-blueing of guaiacum in the case of adrenaline and tyrosine, whereas it occurs in the case of *p*-cresol. Also the fact that when an autoxidised solution of adrenaline is used with a peroxidase, as in the last section, blueing occurs on subsequent addition of guaiacum.

*Separation of two enzymes, oxygenase and peroxidase, from the enzyme extract of the potato.*

In view of the suggestion that two enzymes, an oxygenase and a peroxidase are present in the pear and potato, an attempt was made to separate them by fractional precipitation. Such a method was originally employed by Chodat and Bach [1903: Bach, 1910] for separating their oxygenase component from the peroxidase in the case of an extract from the fungus, *Lactarius* (Basidiomycetes). Their extract was precipitated with 40 % alcohol which produced a precipitate rich in oxygenase, but poor in peroxidase. After continued fractional precipitation with alcohol, a final precipitate was obtained containing peroxidase only.

The method adopted for the potato was as follows. The tuber was pounded and extracted with cold alcohol as previously described. The residue was then extracted for an hour with about three times its weight of water, and the extract squeezed out through muslin. The extract was then centrifuged, the opalescent solution decanted and absolute alcohol added until the concentration reached 40 %. The precipitate taken up with water gave in several experiments a stronger oxygenase reaction (blueing with catechol solution and guaiacum in the presence of peroxidase) than peroxidase reaction (blueing with hydrogen peroxide and guaiacum). Reprecipitation of the first fraction failed to remove the peroxidase, nor was the result more successful by making the concentration of alcohol 20 or 30 % for the first precipitation. By further precipitation of the first filtrate four or five times with additional volumes of alcohol equal to that first added, a precipitate was eventually obtained which ceased to give the oxygenase reaction though it still gave a good peroxidase reaction. By adding saturated ammonium sulphate (20 cc. to 80 cc. of extract) instead of alcohol for the first precipitation, in one trial a first fraction was obtained which gave only a slight peroxidase, but a strong oxygenase reaction. The experiment, however, could not be repeated with any certainty.

It therefore appears that the method of fractional precipitation is not wholly satisfactory for the separation of the two enzymes. More recent experiments of Bach [1916] would seem to confirm this result since he has tried to separate Chodat and Bach's two components of *Lactarius* oxidase by ultrafiltration. Of the two, he found the peroxidase to be much the more rapidly adsorbed by ultrafiltration. The oxygenase remains on the ultrafilter, and its oxidising power can be regenerated by addition of horse-radish peroxidase.

The above results furnish evidence in favour of the existence of two enzymes in plants which contain a catechol substance, turn brown on injury and blue guaiacum without addition of hydrogen peroxide. In plant tissues, in cases so far investigated, we conclude then that three factors are necessary for the blueing of guaiacum: an aromatic substance with a dihydroxy-grouping in the ortho-position; an enzyme, oxygenase, which catalyses the oxidation of such substances with the production of an organic peroxide (or hydrogen peroxide by decomposition); and, finally, a peroxidase which transfers "active" oxygen to readily oxidisable compounds, *e.g.* guaiacum. Artificially, the place of the oxygenase and aromatic compound can be taken either by hydrogen peroxide or by peroxides formed by the autoxidation of certain substances. If the oxidase (synonymous with laccase, phenoloxidase and phenolase) of the pear and potato is to be defined as an enzyme blueing guaiacum, then all three components are essential to its constitution, for the mixture of enzymes alone, oxygenase and peroxidase, is unable to blue guaiacum unless contaminated with the catechol substance.

It is probable that the oxidases (laccases, phenoloxidases, phenolases) obtained by previous workers by precipitation of plant juices with alcohol, are in many cases mixtures of oxygenase and peroxidase with adsorbed catechol compound. Such mixtures would blue guaiacum, and would transfer oxygen to many other substances, such as phenols and aromatic acids, to a greater or less degree. The enzyme mixture, oxygenase and peroxidase (which needs care to be prepared free from catechol compound), has, by virtue of the oxygenase, so far been found only to act on the dihydroxy-grouping in the ortho-position. It can be readily seen that the combination, catechol compound and oxygenase, behaves in every way like Chodat and Bach's organic substance of an enzyme-like nature endowed with the power of forming peroxides, which they call oxygenase.

#### CONCLUSIONS.

1. Solutions of catechol, caffeic acid, protocatechuic acid and adrenaline, compounds which have the dihydroxy-grouping in the ortho-position; tend to autoxidise slowly when left in air with the formation of peroxides.
2. When a solution of a peroxidase is added to any of the above peroxides, the combination constitutes an oxidase system which will blue guaiacum.

3. The autoxidation, with production of peroxides, of catechol, proto-catechuic acid and caffeic acid is accelerated by enzyme extracts of plants which turn brown on injury, and of which the juices blue guaiacum without addition of hydrogen peroxide.

4. Such plants have been found to contain a compound giving the "catechol" reaction. It is suggested that they also contain, in addition to a peroxidase, a second enzyme, which will be termed an oxygenase. The function of the latter is to catalyse the autoxidation of the "catechol" compound with the formation of a peroxide.

5. Three components therefore, in the cases investigated, are present in what has been termed an oxidase (laccase, phenoloxidase or phenolase): a "catechol" compound from which a peroxide can be formed, and two enzymes, an oxygenase which catalyses the production of peroxide, and a peroxidase which decomposes the peroxide with the formation of "active" oxygen. All three components are essential for the blueing of guaiacum.

6. The peroxidase can be separated from the oxygenase by fractional precipitation with alcohol, though the converse has not been accomplished.

#### REFERENCES.

- Bach (1910). *Ber.* **43**, 362.  
— (1916). Genève. *Arch. sci. phys. et nat. C.R. soc. chim.* **41**, 424.  
Chodat (1910). *Handbuch der biochemischen Arbeitsmethoden*. E. Abderhalden, Berlin, **3**, 42.  
Chodat and Bach (1903). *Ber.* **36**, 606.  
Onslow (1919). *Biochem. J.* **13**, 1.

# XLV. OXIDISING ENZYMES. III. THE OXIDISING ENZYMES OF SOME COMMON FRUITS.

BY MURIEL WHELDALE ONSLOW.

*From the Biochemical Laboratory, Cambridge.*

*(Received May 6th, 1920.)*

IN carrying out a general survey of the oxidising enzymes of fruits, certain additional observations have been recorded.

The first point to be emphasised is the fact that divergent results may be obtained in testing for oxidising enzymes, according to whether tests are made on the fresh tissue, or on extracts prepared in various ways from the tissues. Some workers have obtained their results by placing the tissues in the testing reagents. Others have used tissue extracts only. It appears desirable to examine the materials in both ways.

The maximum "oxidising capacity" of the tissue is usually shown when the tissue is placed directly in the reagent. In the case of extracts, the reaction may be modified by the following conditions. First, the enzyme itself may not be extracted; secondly, the relative concentration of enzyme, sugars, organic acids and tannins occurring in the extracts are different from those in the cells, and the "oxidising capacity" of the tissue is not always shown until special steps have been taken to remove some of the products present in solution. In order to understand, however, the nature of the reactions *in situ*, extraction of the various components of the system, its inhibitors, etc., is essential.

Further, it may happen that certain substances, such as organic acids, tannins, etc., prevent even the tissues from giving the reactions, and these must be removed by solvents, e.g. alcohol, leaving the enzymes in the tissue residue. Moreover, sometimes the enzymes may be adsorbed by the tissue residue and not readily extracted. In this case, the reactions will only occur when the fragments of the residue are placed in the reagents, the enzyme and reacting substances having been adsorbed on to these surfaces.

It has been stated by various observers that tannins have an inhibiting effect on oxidising enzymes, and the present results confirm this observation. It is further shown that malic acid to the extent in which it occurs in many fruits, even in the form of neutral salts, may also have an inhibiting effect.

Observations have been made on the fruits according to the following procedures. (a) *Reactions of tissues.* Pieces of tissue are placed in the reagents,

*viz.* alcoholic solution of guaiacum (with or without hydrogen peroxide): 1 % solution of benzidine in 50 % alcohol with hydrogen peroxide; 1 % solution of  $\alpha$ -naphthol in 50 % alcohol with hydrogen peroxide. (b) *Reactions of water extract.* A water extract of the pounded tissues is tested with the above reagents. If acid, it is neutralised to litmus. (c) *Reactions with extract of enzymes.* The tissues are pounded rapidly with cold 96 % alcohol, sucked dry on a filter funnel, and the process repeated several times. The residue ("tissue residue") is extracted with water, and in this way a crude extract ("enzyme extract") of the enzymes practically free from sugars, tannins and organic acids is obtained. The enzyme extract is tested for peroxidase with guaiacum and hydrogen peroxide, and for oxygenase with catechol and guaiacum<sup>1</sup>. (d) *Extraction of aromatic compounds and their reaction with the enzyme extract.* The tissues are extracted with hot alcohol and the alcohol removed by distilling *in vacuo*. The residual extract is diluted with a little water and precipitated with powdered lead acetate. The precipitate so formed chiefly consists of salts of aromatic substances (including tannins) and organic acids. It is decomposed with the minimal amount of sulphuric acid and filtered from lead sulphate. The filtrate is neutralised to litmus, and tested for the catechol reaction with a few drops of ferric chloride followed by a few drops of a 1 % solution of sodium carbonate. A green colour becoming blue, purple or red on addition of the carbonate denotes the presence of a substance with the catechol grouping. To another portion some enzyme extract [see (c) above] is then added. If a catechol reaction has been given and an oxygenase has been detected, a brown colour may be produced in the mixture followed by a blue colour on adding guaiacum; this denotes that the whole oxidase system is present. In a few cases such a result is only obtained by using the tissue residue itself. If both oxygenase and catechol substance have been detected and yet no oxidase system can be synthesised in the above way, the absence of the reaction is usually due to the presence of organic acids or tannins.

If considerable quantities of malic acid are present, this is removed by adding calcium chloride solution, and then excess of alcohol, to the neutralised filtrate obtained by treating the lead salts with dilute sulphuric acid. The calcium malate is insoluble in alcohol and can be filtered off. The alcohol is removed by distilling *in vacuo*, and the residue tested with the enzyme extract.

If tannin is present, this is removed by adding a little concentrated gelatin solution. After filtering, the filtrate is tested with the enzyme extract.

Employing the above methods, the following fruits have been investigated. The results may vary in detail, according to the variety or ripeness of the fruit used.

I. The Apple (*Pyrus malus*). The fruit turns brown on injury. (a) *Reactions of tissues.* In guaiacum tincture the tissue usually turns blue, though in some varieties the reaction may be slow. It is accelerated by bruising the tissue.

<sup>1</sup> Since peroxidase has always been present with oxygenase, it has not been necessary to supply a peroxidase from another source.

On addition of hydrogen peroxide, the reaction is obtained at once. The benzidine and  $\alpha$ -naphthol tests are positive. (b) *Reactions of water extract.* All the tests are positive, though the reaction with guaiacum alone may be delayed or slight. (c) *Enzyme extract.* This gives both the peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract.* When the solution, obtained by decomposition with sulphuric acid of the lead salts of the aromatic compounds and malic acid, is neutralised, a yellowish precipitate is obtained. On taking up this precipitate in very dilute sulphuric acid, the solution gives a strong catechol reaction and is precipitated by gelatin; the solution also does not react with the enzyme extract. The substance precipitated appears to be a catechol tannin which is soluble in alcohol and the dilute acid of the fruit itself, but is insoluble in water. The filtrate from the tannin precipitate also gives the catechol reaction, though less strongly. When tested with enzyme extract and guaiacum no blueing is obtained. The filtrate is then treated with 2-3 cc. of a concentrated calcium chloride solution and excess of alcohol added. A white flocculent precipitate of calcium malate is formed. This is filtered off and the alcohol removed from the filtrate by distilling *in vacuo*. The residue taken up in water still gives a catechol reaction. With tissue residue and guaiacum it gives a blue colour. With the enzyme extract and guaiacum it may give a positive reaction if the liquid is slightly acid to litmus.

*Conclusion.* The apple fruit contains an oxidase, that is an oxygenase, a peroxidase, and an aromatic substance with the catechol grouping. The oxygenase will activate the oxidation of the catechol aromatic compounds *in situ* in the cell with the production of a brown colour and of a system which will blue guaiacum: it will also activate the oxidation of catechol supplied artificially with the production, in the presence of the peroxidase, of a similar system. A large part of the aromatic compounds of the fruit appears to be in the form of a catechol tannin, and this cannot be activated by the enzyme *in vitro*. After removing the tannin and malic acid, the residuum of aromatic compounds left is activated by the oxygenase, with difficulty in solution, but more readily when fragments of the tissue residue are suspended in the solution.

II. The Quince (*Pyrus cydonia*). The cut surface of the fruit turns brown less readily than that of the apple, but on pounding in a mortar, considerable browning is produced. (a) *Reactions of tissues.* The tissue gives no blue colour with guaiacum tincture, except on adding hydrogen peroxide. The benzidine and  $\alpha$ -naphthol tests are positive. (b) *Reactions of water extract.* This gives none of the above tests even after neutralisation. (c) *Enzyme extract.* This gives the peroxidase but only a slight oxygenase reaction, unless fragments of tissue residue are added, when they become deep blue. (d) *Extraction of aromatic compounds and their reaction with enzyme extract.* The procedure and reactions are very similar to those for the apple. In the same way, though in greater quantity, the fruit appears to contain a catechol tannin which is

soluble in alcohol and dilute acids. On neutralising the filtrate (from the decomposition of the lead salts) this tannin is precipitated. The filtrate from the tannin precipitate still gives the catechol reaction but contains no perceptible amount of tannin. It, however, gives no blue colour with guaiacum and either the enzyme extract or tissue residue. After removing the malic acid as calcium malate and distilling the filtrate *in vacuo*, the residue gave the catechol reaction less strongly, but would again give no blue colour with guaiacum and either the enzyme extract or tissue residue.

*Conclusion.* The quince fruit contains an oxidase (oxygenase, peroxidase and aromatic substance with the catechol grouping). The oxygenase will activate the oxidation of the catechol aromatic compounds *in situ* in the cell causing browning, but the blueing of guaiacum is largely inhibited owing to the tannin and malic acid present. The oxygenase is not readily extracted from the tissue residue, so that only this residue itself gives a good reaction with catechol solution and guaiacum. No reactions could be obtained between the plant aromatic compounds and enzymes *in vitro* even when the tissue residue was employed. A neutralised water extract of the tissues gives no reactions, probably because of the inhibitory action of the malates.

III. The Pear (*Pyrus communis*). The fruit turns brown readily on injury. (a) *Reactions of tissues.* The tissue turns blue with guaiacum tincture and gives positive reactions with the benzidine and  $\alpha$ -naphthol tests. (b) *Reactions of water extract.* Positive reactions with all three tests. (c) *Enzyme extract.* This gives both the peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract.* The filtrate, after decomposing the lead salts with sulphuric acid, gives no precipitate on neutralisation, and a very slight, if any, reaction for tannin. It gives a strong catechol reaction. On addition of the enzyme extract, browning occurs and a deep blue colour on subsequent addition of guaiacum.

*Conclusion.* The pear fruit contains an oxidase (peroxidase, oxygenase and aromatic substance with the catechol grouping). The oxygenase will activate the oxidation of catechol artificially supplied, with the production, in the presence of the peroxidase, of the oxidase system. When the separate extracts of aromatics and enzymes are reunited, the oxidase system is obtained.

A comparison of the oxidase reactions of the three fruits is rendered more comprehensible if the average percentages [Wehmer, 1911] of tannin and free acids are noted.

	Pear	Apple	Quince
Organic acid (chiefly malic) . . .	0.1-0.2 %	0.7-1.2 %	0.84-3.5 %
Tannin . . . . .	0.05	0.1-0.3	

(No reference to the percentage of tannin in the quince could be found, but large quantities were obtained on extraction.) The tannin and organic acids inhibit the oxidising reactions in both the tissues and extracts, though more so in the latter, the effect of the tannin being to precipitate the enzymes. The presence of tannin also renders the extraction of the enzymes more difficult,

even after the bulk of the tannin is removed by alcohol. This is especially so in the case of the oxygenase, *e.g.* in the quince, which appears to be precipitated and adsorbed by the tissue fragments.

IV. The Plum (*Prunus domestica*). Both red and purple varieties were investigated separately. The fruit turns brown on injury. (a) *Reactions of tissues*. The tissues give a positive reaction with guaiacum alone, and also with both benzidine and  $\alpha$ -naphthol tests. (b) *Reactions of water extract*. The same results as in (a) were obtained. (c) *Enzyme extract*. This gives both peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract*. A heavy whitish precipitate (red plums) or purple precipitate (purple plums) is obtained with lead acetate. After decomposition with sulphuric acid and neutralisation, the extract gives a good catechol reaction, but no reaction with the enzyme extract and guaiacum. After removal of the malic acid, the extract still gives the catechol reaction and, on addition of the enzyme extract and guaiacum, a blue colour. The latter reaction is better if the mixture is slightly acid to litmus.

The same results were obtained with greengage and damson fruits.

The percentages of acid and tannin are as follows: acid, chiefly malic (plum, 0.77–1.01 %; greengage, 0.82 %; damson, 0.92 %); tannin (damson, 0.04 %) [Wehmer, 1911].

*Conclusion*. The plum, greengage and damson all contain an oxidase (peroxidase, oxygenase and aromatic substance with the catechol grouping). Hence the tissues brown on injury and give the oxidase reaction. The oxygenase will activate the oxidation of catechol solution with the production of a peroxide, etc. The extracted aromatic compounds and enzymes from the fruit when recombined will give a blue reaction with guaiacum after the removal of the malic acid.

V. The Banana (*Musa sapientum*). On injury, *e.g.* pounding in a mortar, the skin turns brown but the flesh is less affected. The results recorded below given by the flesh of the fruit varied somewhat in individual fruits and in fruits of different degrees of ripeness. On the whole, however, the reactions were as stated. (a) *Reactions of tissues*. The skin turns blue with guaiacum alone: the flesh only after addition of hydrogen peroxide. Both skin and flesh give positive reactions with the benzidine and  $\alpha$ -naphthol tests. (b) *Reactions of water extract*. The extract of the skin gives a positive reaction with guaiacum alone: that of the flesh no reaction until hydrogen peroxide is added. Extracts of both skin and flesh give positive reactions with the benzidine and  $\alpha$ -naphthol tests. (c) *Enzyme extract*. From both skin and flesh these give the peroxidase and oxygenase reactions. (d) *Extraction of aromatic compounds and their reaction with enzyme extract*. The solution derived from decomposition with sulphuric acid of the lead salt from the skin gives the catechol reaction. On addition of the enzyme extract from the skin followed by guaiacum, a blue colour is obtained. The reaction takes place more readily after precipitation with gelatin owing to the presence of a certain amount of

tannin. The solution from the decomposition of the lead salt from the flesh gives a doubtful catechol reaction, and no blue colour with the enzyme extract from the flesh followed by guaiacum. After removal of malic acid by the usual method, the residue gives no catechol reaction and again no result after addition of enzyme extract and guaiacum.

*Conclusion.* (The results, as mentioned above, given by extracts of enzymes and aromatic compounds of the flesh vary, but on the whole they may be represented as follows.) Both the skin and flesh of the fruit contain peroxidase and oxygenase. The skin also contains substances with the catechol grouping, but these are practically absent from the flesh. The oxygenase of both skin and flesh catalyses the oxidation of catechol, artificially supplied, with great rapidity followed by blueing on addition of guaiacum. The skin oxygenase also activates the oxidation of its own aromatic compound with the production of a blue colour on addition of guaiacum. No reaction could be obtained between the enzyme and aromatic extracts of the flesh.

VI. The Orange (*Citrus aurantium*). The fruit does not brown on injury. (a) *Reactions of tissues.* Rind: positive result with guaiacum and hydrogen peroxide and with the other two tests. Pulp: slight local positive reaction with benzidine and  $\alpha$ -naphthol tests only. (b) *Reactions of water extract.* Rind: positive reaction only with benzidine test. Pulp: after neutralisation to litmus, no reactions with any of the three tests. (c) *Enzyme extract.* Both rind and pulp give some positive reaction with guaiacum and hydrogen peroxide and with the benzidine test. Neither gives the oxygenase reaction. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

VII. The Lemon (*Citrus limonum*). The fruit does not brown on injury. (a) *Reactions of tissues.* Rind: positive reaction with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction with benzidine and  $\alpha$ -naphthol tests only. (b) *Reactions of water extract.* Rind: positive reaction with benzidine and  $\alpha$ -naphthol tests only. Pulp: after neutralisation to litmus, positive reaction with benzidine test only. (c) *Enzyme extract.* Rind: good positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction only with benzidine test. With the other two tests positive reaction only if tissue residue is used. Neither extract gives the oxygenase reaction. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

VIII. The Lime (*Citrus limonum*, var. *acida*). The fruit does not brown on injury. (a) *Reactions of tissues.* Rind: positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction with the benzidine test only. (b) *Reactions of water extract.* Not tried. (c) *Enzyme extract.* Rind: positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: negative reactions with all three tests but positive reaction with the benzidine test if the tissue residue is used. Neither extract gives the oxygenase reaction. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

*Conclusion.* The fruits of the orange, lemon and lime contain a peroxidase in both the rind and the pulp. No substance giving the catechol reaction could be detected in any of the fruits and there is no oxygenase. In the rind the peroxidase reaction is strongest in the lime, less in the lemon and least in the orange. From the pulp, the peroxidase is most readily extracted in the case of the orange, less in the lemon and least in the lime. These fruits afford an example of the possibility of obtaining a greater oxidation reaction with the crudely separated peroxidase than with the tissue itself.

IX. The Raspberry (*Rubus idaeus*). The fruit does not brown on injury. (a) *Reactions of tissues.* Some positive reaction only with benzidine and  $\alpha$ -naphthol tests. (b) *Reactions of water extract.* These cannot be detected because of the anthocyan pigment. (c) *Enzyme extract.* Positive reactions with guaiacum and hydrogen peroxide and with the other two tests. No oxygenase reaction is given. (d) *Extraction of aromatic compounds.* No catechol reaction could be detected.

*Conclusion.* The raspberry fruit contains a peroxidase but no oxygenase and no substance giving the catechol reaction. This fruit affords another example of greater oxidation powers of crudely prepared peroxidase than of the tissue itself.

My thanks are due to Dr F. F. Blackman, F.R.S., for kind criticism and advice in connection with this and the preceding paper.

#### REFERENCE.

Wehmer (1911). *Die Pflanzenstoffe*. Jena

## **XLVI. A STUDY OF SOME BIOCHEMICAL TESTS.**

### **No. 2. THE ADAMKIEWICZ PROTEIN REACTION. THE MECHANISM OF THE HOPKINS-COLE TEST FOR TRYPTOPHAN. A NEW COLOUR TEST FOR GLYOXYLIC ACID.**

By WILLIAM ROBERT FEARON.

*Mackinnon Research Student.*

*From the Biochemical Laboratory, Cambridge, and the Physiological Laboratory, Trinity College, Dublin.*

*(Received May 11th, 1920.)*

IN 1874 Adamkiewicz published [1874] a description of the colour phenomena due to the action of strong sulphuric acid on egg-albumin. In some experiments he used acetic acid as a solvent, and he observed in many such cases that a purple colour was produced on the addition of the sulphuric acid. He considered this colour to be due to "a special influence of the acetic acid upon the colour already produced in the sulphuric acid protein solution."

Udranszky [1888] described the test as a furfural reaction, which explanation was generally accepted till Hopkins and Cole [1901, 1, 2] published the first of their well-known papers, in which they showed that the colour was due to an impurity in the acetic acid. They isolated glyoxylic acid from several samples of acetic acid and showed that in the presence of sulphuric acid it gave a purple colour with certain proteins, the facts thus discovered leading later to the isolation of the amino-acid, tryptophan, which is responsible for the reaction.

Cole [1903] investigated the various protein colour tests with aldehydes, or bodies that gave rise to aldehydes, and proved that the majority of these reactions depended on the presence of tryptophan. He drew attention to the importance of the presence of an oxidising agent in the production of the colours.

Hopkins and Cole's explanation was criticised by Rosenheim [1906], who described a colour reaction between tryptophan and formaldehyde in sulphuric acid containing a trace of an oxidiser, which was similar to that given by glyoxylic acid. Rosenheim considered that the Hopkins-Cole test depended on the presence of formaldehyde in the acetic acid or arising from the reagents used. He remarked upon the ease with which glyoxylic acid is decomposed at high temperature into formaldehyde and carbon dioxide.

Dakin [1907] investigated the tests, and found that glyoxylic acid prepared by different methods gave the test. He thought it "hard to believe that these uniformly positive results with different specimens of glyoxylic acid are due to impurities." Glyoxylic acid preparations in many cases gave the test directly, formaldehyde always required an oxidising agent. Dakin discussed the possibilities of the production of glyoxylic acid from formaldehyde by an aldol type of condensation followed by oxidation. Kondo (1906) described a test for indole similar in many respects to the Rosenheim test. Formaldehyde with indole gives a crimson colour in presence of strong sulphuric acid.

Research was done in the following years on the general chemistry of bodies related to tryptophan, which will be referred to later, as it does not directly concern the Hopkins-Cole reaction [Blumenthal, 1909].

In 1913 Homer published the results of work on aldehyde derivatives of tryptophan [1913]. She believed that the colour bodies were due to an interaction between the aldehyde and the imino-group of the tryptophan. She concluded from the general behaviour of these bodies that the Hopkins-Cole test was due to formaldehyde.

In the same year Mottram [1913] described a failure of the glyoxylic test owing to an excess of oxidising agents in the sulphuric acid used. This was confirmed by Breidahl in 1915, who stated that such acids could be made suitable for the test by the addition of small quantities of reducing agents [1915]. These papers emphasised the fact that a trace of an oxidising agent is necessary in the majority of cases; if the oxidising agent be too strong or too weak the test will not succeed.

Voisenet in 1918, from similarities in appearance and behaviour towards reagents, concluded that the colour produced in the Hopkins-Cole and the Rosenheim tests was the same substance, and that in each test it was due to formaldehyde [1918].

The following research was undertaken originally with the object of finding a satisfactory method for estimating tryptophan colorimetrically. Although such a method has not yet been found, some information has been obtained about the mechanism of the tryptophan colour tests. The first question investigated was the nature of the aldehydes concerned in the Hopkins-Cole and Rosenheim tests with a view to deciding the long-standing glyoxylic acid and formaldehyde controversy.

*The Hopkins-Cole Reaction in the absence of Formaldehyde.* If formaldehyde were liberated from the glyoxylic acid during the process of the test it should be possible to detect its presence, since there are colour tests for formaldehyde in presence of sulphuric acid that are more delicate than the reaction between formaldehyde and tryptophan. Should formaldehyde be detected under the conditions of the Hopkins-Cole reaction, it probably enters into the test. On the other hand, should the test be obtained under conditions that are such that formaldehyde cannot be detected in the reacting solutions, it is strong evidence that the reaction is due to glyoxylic acid.

Glyoxylic acid is more stable in strong sulphuric acid than has been generally supposed. When calcium glyoxylate was added to concentrated sulphuric acid and the mixture warmed gently, decomposition did not begin until the temperature rose above 50°. Carbon dioxide was then evolved, and formaldehyde was recognised in the solution by means of the thiophen test [Fearon, 1918]. The Hopkins-Cole test may be carried out at temperatures below that of the decomposition of the glyoxylate and in the absence of water, by using solid tryptophan and calcium glyoxylate. The characteristic colour develops under conditions where formaldehyde can be shown to be absent. (In passing, it may be noted that if the sulphuric acid be contaminated with nitrous acid an ivy-green colour may be formed on warming the solution, this is due to an entirely different reaction which will be discussed in a later communication.)

Various samples of acetic acid which gave the reaction were tested for formaldehyde, alone and under the conditions of the test, with negative results in nearly every case.

To detect the presence of formaldehyde in excess of sulphuric acid the following tests were employed.

*The Thiophen Test* (1918). This test will show one part of formaldehyde in half a million parts of sulphuric acid. The colour with formaldehyde is distinctive and much more delicate than the colour with glyoxylic acid.

The delicacy of Rosenheim's test for tryptophan is about 1 in 200,000 at most.

*The Pyrogallol-aldehyde Reaction*, described in the present paper.

This is an extremely sensitive test for aldehydes in presence of sulphuric acid; it will show 1 part formaldehyde in over 1,000,000 parts water, and may be used to show the presence of formaldehyde in mixtures containing glyoxylic acid.

As the result of a series of experiments on the Hopkins-Cole test under various conditions and with different preparations of glyoxylic acid, it is concluded that the test can be obtained in the absence of formaldehyde.

*The Rosenheim Test in the absence of Glyoxylic Acid*. Although glyoxylic acid gives many of the general aldehyde reactions, there is no simple colour test for distinguishing it from other aldehydes in presence of sulphuric acid. The test current in the text-books is simply the reverse of the Hopkins-Cole reaction and open to the same criticism of uncertainty as regards the aldehyde. This test was introduced by Eppinger [1905] and modified by Schloss [1906].

#### A NEW COLOUR TEST FOR GLYOXYLIC ACID.

The reagent is 1 % pyrogallol in pure concentrated sulphuric acid, which must be free from any nitrous contamination, otherwise the solution turns brown and is useless. The reagent will not keep for long, after a few days a white condensation product is deposited.

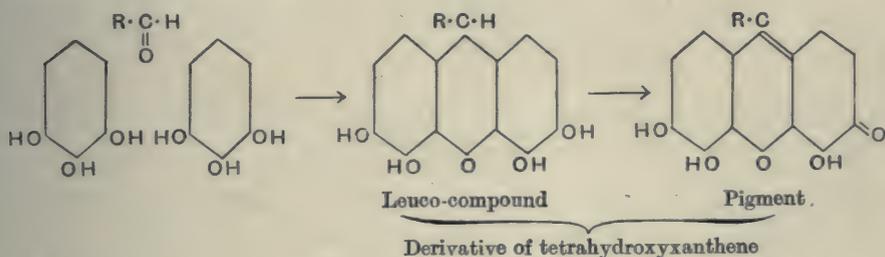
When a trace of calcium glyoxylate, or a couple of drops of reduced oxalic acid solution are added to the reagent, a deep blue colour develops on gentle warming. Dilution with water changes the blue to a deep carmine which becomes blue again on addition of excess of sulphuric acid.

The blue colour is not given by oxalic acid or any of its simple derivatives which do not give rise to glyoxylic acid under the condition of the test.

With the aliphatic aldehydes the reagent gives various shades of red. The presence of glyoxylic acid along with formaldehyde can be shown by the production of a purple due to the formation of two colours. The test is useful for detecting aldehydes in ether, as mentioned later. Syrupy phosphoric acid may be used instead of sulphuric acid as a condensing agent, in which case a red colour is obtained with all the aldehydes on warming.

The delicacy of the test is greatest for formaldehyde; with glyoxylic acid it will show less than one part in a thousand, with weaker solutions the colour takes some time to develop.

The chemistry of the test has only been briefly investigated as yet. The coloured condensation compounds are probably derivatives of xanthene. When separated from the sulphuric acid they show many of the properties of xanthidrol, such as forming typical insoluble compounds with urea in acid solution. The colour is probably due to the formation of the pyrone ring [Palazzo and Onorato, 1905, and references].



On applying the pyrogallol-reagent to solutions of formaldehyde in sulphuric acid in the presence of oxidising agents, under the conditions of the Rosenheim test, no evidence whatever could be obtained of the formation of glyoxylic acid, though on addition to the mixture it could be detected. This supports Rosenheim's view that the test depends on formaldehyde alone.

From a purely chemical aspect, it is not improbable that both formaldehyde and glyoxylic acid should react with tryptophan to give distinct derivatives, though subsequent investigation showed that the glyoxylic derivative readily lost carbon dioxide and changed into the corresponding formaldehyde derivative as might be expected. The fact that the Hopkins-Cole test is considered by many investigators to be more reliable than the Rosenheim test would be explained by the greater tendency, as subsequently observed, of the formaldehyde to react still further with the condensation products. A disadvantage of both tests is the local heating at the junction of the liquids;

this can be greatly reduced by employing glacial acetic acid as a solvent for the glyoxylate or the formaldehyde as the case may be. As will be shown later, an oxidising agent is necessary in both tests, this is usually supplied by the sulphuric acid [Mottram, 1913].

#### THE MECHANISM OF THE HOPKINS-COLE REACTION.

Having concluded from the previous experiments that both glyoxylic acid and formaldehyde gave coloured derivatives with tryptophan, which is confirmed by subsequent research, the nature of the reaction was next investigated. Whilst it is well known that indole and its methyl derivatives yield with different aldehydes a large number of coloured derivatives some of which have been investigated, the aldehyde reactions of tryptophan are known chiefly as qualitative colour tests and the exact nature of the compounds formed never seems to have been fully investigated. This is due partly to the difficulty of obtaining tryptophan in quantities sufficient for extended chemical research and partly to the difficulties of isolating the coloured compounds or their precursors in a stable form.

A number of colour reactions between tryptophan and various aldehydes have been described [Rohde, 1905; Sasaki, 1910], but for the purposes of the present paper it will be sufficient to consider three only: formaldehyde, glyoxylic acid and benzaldehyde. It will also be necessary to consider the reactions of these aldehydes with some substances related to tryptophan, namely: indole, scatole, and carbazole.

#### PREPARATION AND ISOLATION OF THE ALDEHYDE CONDENSATION DERIVATIVES OF INDOLE, SCATOLE, TRYPTOPHAN AND CARBAZOLE.

*Condensation.* Strong sulphuric acid is quite unsuited for use as a condensing agent for these substances. It may contain a large number of active impurities; it acts as an oxidising agent in a variable manner, the heat it is apt to develop may destroy the condensation products. Fischer [1889] employed alcohol and hydrochloric acid gas for his work on the indoles. Homer and others used zinc chloride. Neither of these reagents was found to be quite satisfactory, and most of the present work was done by means of pure glacial acetic acid saturated with hydrochloric acid gas. This gave good results as a condensing agent, and is free from the objections to sulphuric acid. Phosphoric acid was used in a few particular cases. The glacial acetic acid was purified by fractionation until it no longer gave a colour with tryptophan or any of the aldehyde reagents. When pure it will keep for a long time if moisture and light are excluded, and the bottle be kept filled, as the changes leading to the formation of aldehydes appear to occur in the vapour over the liquid.

Acetic acid so prepared will be referred to in the present paper as "pure" acetic acid.

The hydrochloric acid gas for saturation was prepared by the action of pure sulphuric acid on pure dry sodium chloride. If the acid be badly contaminated with nitrous impurities some of them may pass over into the condensation mixture and spoil the preparation.

The aldehydes were used in the following forms:

Glyoxylic acid as the calcium salt.  $(\text{CHO}.\text{COO})_2 \text{Ca}, 2\text{H}_2\text{O}$ .

Formaldehyde as paraformaldehyde. The ordinary solutions of "formol" are unsuitable owing to the presence of methylal and other derivatives [Werner, 1917].

Benzaldehyde as a solution in "pure" acetic acid.

One of the first facts observed during the preparation of the colour condensation compounds by the above method was that an oxidising agent was necessary in every case. The condensation products as formed are colourless or pale yellow; on oxidation the characteristic colour is produced. This fact escapes notice when sulphuric acid is used as a condensing agent since it acts as an oxidiser as well.

For the oxidation a fresh 1 % solution of  $\text{H}_2\text{O}_2$  in "pure" acetic acid was used. In some instances the oxidation was allowed to proceed spontaneously in the air overnight.

*Notes on the Condensation.* It is best to use an excess of the solvent and to pass in the HCl gas slowly, shaking at intervals. After the solution has been saturated, gas is passed in for an additional quarter of an hour. A few cc. of the solution are withdrawn in a test-tube and one drop of the 1 %  $\text{H}_2\text{O}_2$  added, and the tube well shaken, when the colour should develop in a few minutes. The  $\text{H}_2\text{O}_2$  is added carefully up to the point of maximum depth of colour, too much will discharge the colour altogether. The approximate quantity of  $\text{H}_2\text{O}_2$  is noted. Passage of HCl into the mixture is continued until no increase in depth of colour is seen on removing and testing the samples. It is sometimes useful to pass a current of air into the mixture to remove excess of HCl before the final oxidation.

The calculated amount of 1 %  $\text{H}_2\text{O}_2$  is then added to the mixture, which is well shaken till oxidation is complete. It is then poured into a measuring cylinder, three times its volume of purified ether added, and the mixture shaken. The pigment will be precipitated in small flakes. If precipitation is not complete, another volume of ether may be added, but it is advisable not to exceed four volumes if a pure preparation is required, as otherwise the other substances may be precipitated.

*Isolation of the Condensation Products.* This proved to be one of the most difficult parts of the investigation. It was necessary to free the products from unchanged aldehyde, and indole derivatives, and from the condensing agent. Fractional precipitation with *pure* ether was finally adopted and gave satisfactory results. It was found that if three to four volumes of ether were added to one volume of the acetic acid solution of the pigment and the mixture well shaken, the latter came down as a flocculent precipitate and the aldehydes

and unchanged indole derivatives were left in the ethereal solution, as was also the calcium chloride which was formed when calcium glyoxylate was employed.

The purity of the ether is of great importance. It was found that merely washing and drying it was not sufficient. It should also be freed from aldehydes and related substances by distillation from sodium hydroxide, after having been left in contact with it for at least 24 hours. If the ether contains aldehydes it will react with the precipitated pigment on standing, converting it into a greenish paste, which may resinify on drying.

For this reason the coloured compounds should be filtered from the ether as soon as they are precipitated. The pyrogallol test is useful for detecting aldehydes in samples of ether.

*List of Pigments isolated.* The following are the colour derivatives obtained by the condensation of indole, scatole, tryptophan and carbazole with the three aldehydes, formaldehyde, glyoxylic acid and benzaldehyde respectively.

(The figures placed below the colour denote the number of molecules interacting.)

ALDEHYDE	CH <sub>2</sub> O	CHO.COOH	C <sub>6</sub> H <sub>5</sub> .CHO
Indole	Red	Red	Red
	2I + F	2I + G	2I + B
Scatole	Red	Red	Purple
	2S + F	2S + G	2S + B
Tryptophan	Red	Red	Blue
	2T + F	2T + G	2T + B
Tryptophan	Blue	Blue	—
	2T + 3F	2T + 3G	
Carbazole	Blue	Blue	Blue
	2C + F	2C + G	2C + B

No attempt has been made to describe the shade in the above list, anything varying from rose to carmine has been grouped as "red" and similarly for the other colours. The qualitative differences are quite definite in many cases.

Many other aldehyde condensation derivatives were prepared, but the above are the most important in connection with the discussion which follows on the chemical constitution of these bodies.

*General Properties of the Condensation Derivatives.* Both the leuco-compounds and the pigments can be obtained by the ether precipitation method. The leuco-compounds are unstable and soon oxidise to form the pigments.

As precipitated the pigments are all hydrochlorides of nearly colourless bases. The pigments are soluble in strong mineral acids, in the lower aliphatic acids, in esters such as ethyl chloride, in phenol to a less extent. The solubility varies somewhat with the different pigments. The red and blue derivatives of tryptophan are hydrolysed at once when water is added to their solution in the HCl-acetic condensation mixture. The scatole and carbazole pigments are much more stable, the carbazole blues being precipitated on the addition of excess of water. Addition of alkali discharges the colour of the

pigments, as might be expected; in most cases the base is precipitated from the alkaline solution. The pigments were purified by repeated solution in "pure" acetic acid and precipitation with pure ether. They were then rapidly dried in a current of air. When pure they are amorphous deeply coloured powders.

*Melting Points.* These were all high, in the region of  $300^{\circ}$ , depending on the rate of heating. The tryptophan reds and blues tend to lose  $\text{CO}_2$  on heating.

If the pigments are dissolved in absolute alcohol and treated with dry  $\text{NH}_3$ , they are decolorised and gradually precipitated from solution as bases, which in some cases are microcrystalline. Addition of strong acid to the bases restores the original colour of the pigments. The bases were also obtained during an attempt to crystallise the pigments from alcohol. The alcohol apparently combined with the HCl of the pigment and removed it to form ethyl chloride.

*Molecular weights.* The molecular weights of the bases were determined by the freezing point method with comparatively concordant results, using acetic acid or phenol as solvents. It has been shown that compounds of related indigotin type do not tend to form associated molecules in these solvents [Beckmann and Sabel, 1906].

The pigments are the salts of these bases.

*Analyses.* For the present preliminary work, the only element estimated was nitrogen. This was done by a modified Kjeldahl method, using mercury as catalyst instead of copper, and drawing the fumes by means of a Folin absorber into a wash-bottle, so that a control could be kept on any nitrogen which might escape combustion. These bodies can be analysed satisfactorily by the Kjeldahl method provided that the heating is continued for a considerable time after the mixture has cleared [cf. Dakin and Dudley, 1914].

The results of the nitrogen estimations were used to check the formulae of the condensation derivatives deduced from the molecular weights, sufficient tryptophan not being at my disposal for a complete analysis of the various pigments.

#### CHEMICAL CONSTITUTION OF THE CONDENSATION DERIVATIVES.

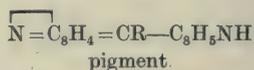
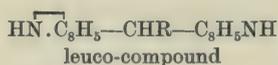
Up to the present time no structural formulae appear to have been suggested for the tryptophan pigments. From the preliminary examination of these bodies it is possible to assign formulae to them which will satisfy the requirements of the Hopkins-Cole and Rosenheim tests without attempting the more ambitious task of giving a complete account of the chemistry of these interesting condensations.

From determinations of the molecular weights of the colour bases, it appears that all the pigments prepared are the salts of compounds formed by the condensation of two molecules of the indole derivative with one of the aldehyde, except in the case of tryptophan blue, where condensation occurs with three molecules of the aldehyde.

In theory the aldehyde may unite with the indole derivative in three ways:

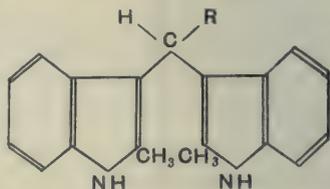
- (1) by replacing hydrogen in the benzene ring,
- (2) by replacing hydrogen in the pyrrole ring,
- (3) by replacing hydrogen in the imino-group.

The fact that all the pigments arise from oxidation of *leuco*-condensation bodies is, on the quinonoid theory of colour and constitution, strong evidence for union in either of the ways (1) or (2). The subsequent removal of hydrogen from the aldehyde residue and from one of the imino-groups occurs on oxidation and leads to the formation of the double bonds<sup>1</sup>.



Fischer [1889] gives the following formula for the aldehyde derivatives of  $\alpha$ -methylindole (methylketole), the union taking place by the aldehyde replacing the hydrogen atoms of the  $\beta$ - or 3-carbon atom of the pyrrole groups.

Freund and Lebach have prepared and investigated several aldehyde derivatives of  $\alpha$ -methylindole (methylketole). They accept the Fischer formula, which, as they point out [1905], accounts for the production of a colour by oxidation.



Although Fischer's formula is satisfactory when applied to the aldehyde condensation derivatives of indole and methylindole, it is not so applicable to the 3-substituted indoles, such as scatole and tryptophan. Here the 3-carbon is already in union with another group and it is unlikely that under the conditions of the condensation the aldehyde displaces these groups, and even if it did, this would not explain the fact that the scatole-benzaldehyde compound is *deep purple* while the corresponding indole-benzaldehyde compound is *red*.

Again, if condensation did occur in the pyrrole ring in scatole-benzaldehyde purple, it is hard to account for the existence of the leuco-precursor. Freund and Lebach accept the formula for the aldehyde derivatives of scatole on the lines of Fischer, though they note its inability to explain the formation of the colour on oxidation.

If the aldehyde condenses with the carbons in the *benzene* ring, instead of the pyrrole ring, all these objections can be overcome. The existence of both leuco-compounds and pigments can be easily explained in the case of all the substances examined, and the general properties of the pigments can be accounted for.

The di- and tri-phenylmethane dyes in common use are quinonoid salts of bases of this type. One of their disadvantages is their sensitiveness to alkalis,

<sup>1</sup> The formulae are printed so as to show that the nitrogen of the imino-group is in union with two different carbon atoms.

which reconvert them into the colourless bases. It will be seen that the pigments described in the present paper have many properties in common with the di-phenylmethane dyes.

The carbazole pigments are similar in many respects to the indole derivatives. Here condensation with the aldehyde has almost certainly taken place in the benzene ring, as the pigments are produced by oxidation of leuco-compounds. The greater stability of the carbazole blues may be explained as being due to the absence of the mobile hydrogen atom of the 2-unsubstituted indoles, which is capable of changing its position from the nitrogen to the neighbouring carbon atom, and *vice versa*. The carbazole ring is stable and the imino-hydrogen is held by the presence of the benzene rings on either side. This tendency on the part of the indoles to undergo tautomeric change has been studied by various investigators [Weissgerber, 1913]. In assigning formulae to the compounds mentioned in the present paper, the hypothesis that aldehyde condensation takes place in the benzene ring has been applied. According to this, when the indole derivative reacts with the aldehyde in the presence of a condensing agent, water is eliminated and the aldehyde links up two molecules of the indole derivatives by uniting with the carbon atoms of their benzene rings to form the leuco-compound, this on oxidation loses one hydrogen atom from the aldehyde residue and one from an imino-group, with the production of the quinonoid configuration in the molecule and the formation of a colour.

These pigments, especially the benzaldehyde derivatives, and their leuco-precursors are comparable on this hypothesis with malachite green.

#### EXPERIMENTAL.

##### *The Scatole Derivatives*<sup>1</sup>.

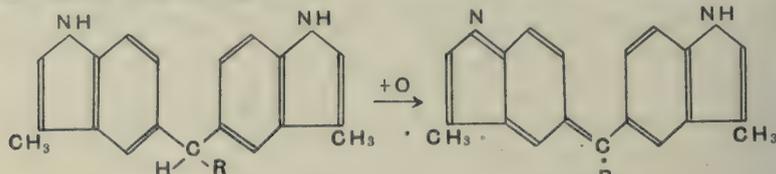
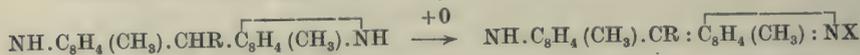
- |   |   |
|---|---|
| (1) <i>Scatole formaldehyde red.</i>    | $\text{NH} \cdot \text{C}_8\text{H}_4(\text{CH}_3) \cdot \text{CH} : \overline{\text{C}_6\text{H}_4(\text{CH}_3)} : \text{NX}$                      |
| M.W. of base, 272.                      |   |
| (2) <i>Scatole glyoxylic red.</i>       | $\text{NH} \cdot \text{C}_8\text{H}_4(\text{CH}_3) \cdot \text{C}(\text{COOH}) : \overline{\text{C}_6\text{H}_4(\text{CH}_3)} : \text{NX}$          |
| M.W. of base, 316.                      |   |
| (3) <i>Scatole benzaldehyde purple.</i> | $\text{NH} \cdot \text{C}_8\text{H}_4(\text{CH}_3) \cdot \text{C}(\text{C}_6\text{H}_5) : \overline{\text{C}_6\text{H}_4(\text{CH}_3)} : \text{NX}$ |
| M.W. of base, 348.                      |   |

Two molecular parts of scatole and one of the aldehyde are dissolved in excess of "pure" acetic acid and condensed by means of dry hydrochloric acid gas as described. The condensation and subsequent oxidation should be done at a temperature not above 20°, otherwise the scatole itself may be oxidised with the formation of a red-brown pigment. The condensation compounds are precipitated by ether and purified as already described. They are amorphous intensely-coloured powders, fairly soluble in dilute HCl and H<sub>2</sub>SO<sub>4</sub>.

<sup>1</sup> The formulae are written so as to show the union of the nitrogen atom with two different carbon atoms. X represents the acid which combines with the pigment base to form the coloured salt. In the present paper X represents HCl.

The benzaldehyde derivative is the one least hydrolysed by dilution with water. They are all decolorised and precipitated by alkalis, boiling with which reconverts them in part to the original scatole. As prepared by the above method the yield is nearly quantitative.

General formula suggested for these compounds:

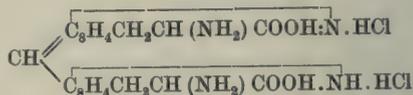


Leuco compound

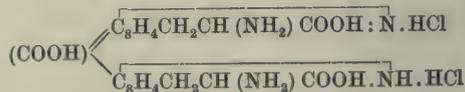
Pigment base

*The Tryptophan Derivatives*

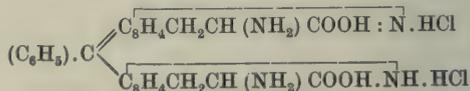
- (4) *Tryptophan formaldehyde red.*  
M.W. of base (average), 418.



- (5) *Tryptophan glyoxylic red.*  
M.W. of base, above 420.



- (6) *Tryptophan benzaldehyde blue.*  
M.W. of base, 494.



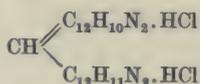
Two molecular parts of tryptophan are condensed with one part of the aldehyde as previously described. The preparation must be carried out in one stage, as the compounds may undergo decomposition if left for long in the condensing reagent. The temperature should not rise above 15°. If the aldehyde be added rapidly at the start in preparing tryptophan formaldehyde red, it may interact with the NH group to form a methylene derivative —N = CH<sub>2</sub>, but this does not affect the general formula type given above. This methylenation of the amino-group is an intermediate stage in the formation of the blue compounds to be described later.

The tryptophan reds are the most unstable of the pigments prepared. They readily lose CO<sub>2</sub> on heating, and for this reason it is difficult to isolate tryptophan glyoxylic red, although there is evidence for its existence in solution. It is very difficult to obtain these derivatives in a pure state, apart from tryptophan benzaldehyde blue. The product from the ether precipitation appears to be a varying mixture of the true tryptophan condensation product with condensation products from decarboxylated tryptophan and tryptophan in which the amino-group has been methylenated. However, there seems to be little doubt that all these reactions are modifications of the condensation along the lines of the formulae given.

Tryptophan benzaldehyde blue is one of the more stable pigments, and seems to be well suited for colorimetric work as well as being a good reagent

for qualitative tests. Cole in 1903 drew attention to the clear colour produced. As obtained by the above method, the pigment is a deep blue powder with a faint lustre. It melts just above  $310^{\circ}$ , and rapidly decomposes according to the rate of heating. It is soluble in the solvents mentioned, but has not been crystallised from any. Like the previous tryptophan derivatives it is largely hydrolysed on diluting the acetic acid solution with water. Readings of the molecular weight of the pure base in acetic acid were on the average below 500 and just above 490, which corresponds to the theoretical value, 494, required by the formula.

- (7) *Tryptophan formaldehyde blue.*  
 M.W., base 1, 354 (average).  
 M.W., base 2, 444 (average).



- (8) *Tryptophan glyoxylic blue.*  
 M.W., variable. Above 400.

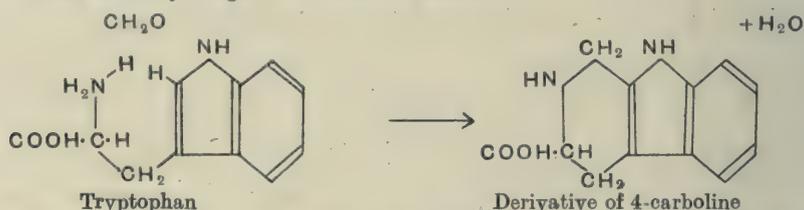
Base 1 is derived from the decarboxylated tryptophan, Base 2 from ordinary tryptophan.

*The Tryptophan Blues.* It was confirmed at an early stage in the present research that the Hopkins-Cole test and the Rosenheim test each gave *two* distinct colours with tryptophan according to the amount of aldehyde used, a fact previously noticed by Mottram [1913] in the former test. With the minimum amount of aldehyde and the test-tube kept cool, the colour in each test is *carmine*. Using more aldehyde and allowing the temperature of the mixture to rise, the colour is *violet to blue*. On studying the reaction using "pure" acetic acid and HCl gas, it was found that tryptophan is able to give two distinct coloured derivatives when condensed with formaldehyde or with glyoxylic acid. One class has been briefly referred to above as the tryptophan reds.

When two parts of tryptophan are condensed with three parts of formaldehyde or of glyoxylic acid at a temperature between  $50^{\circ}$  and  $70^{\circ}$ , the resulting compound on oxidation is deep blue. Also, when tryptophan red was dissolved in "pure" acetic acid saturated with HCl and left for a couple of days, it gradually changed into the blue compound. This change did not occur in acetic acid alone. The change is accompanied by the liberation of some free tryptophan, as was shown by extracting the mixture with ether containing HCl.

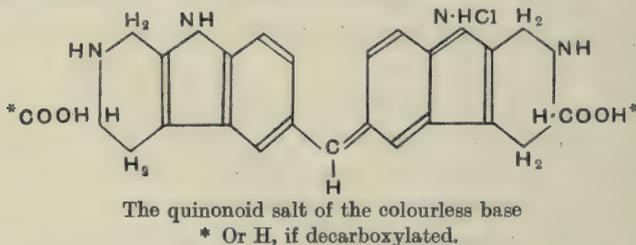
Investigation of the molecular weight of the HCl-free base indicates that the pigment is a di-molecular derivative of tryptophan like the other substances investigated. It was thought probable that the additional molecules of the aldehyde reacted with the *amino*-group. This was shown to be so by the preparation of some indole-hydroxypropionic acid by the action of the calculated amount of  $\text{NaNO}_2$  on tryptophan in HCl solution and removal of any excess of  $\text{HNO}_2$  by means of urea. This substance only gave a red aldehyde derivative under the above conditions. From this it was concluded that for the production of the *blue* pigment interaction was necessary between the aldehyde and the amino-group of the tryptophan. The first effect of such reaction would be to produce a methylene derivative of the amino-group,

—N = CH<sub>2</sub> which would not pass into a methyl group unless the aldehyde were in considerable excess or a reducing agent were present [Werner, 1917]. Neither of these conditions obtains in the preparation as described above. It is, however, hard to understand why the methylenation of the amino-groups should change the red pigment into a blue one, and there is evidence that tryptophan red may contain such substances without alteration of colour. It is probable that there is an additional condensation. Bearing in mind the fact that the corresponding aldehyde derivatives of carbazole are all deep *blue* in colour, and that the H atom attached to the  $\alpha$ - or 2-carbon of indole is labile, it does not seem unreasonable to suppose that a condensation might occur between the carbon of the methylene substituting group in the tryptophan and the carbon atom of the indole group to form a three-ring molecule of the carbazole type, having a pyridine nucleus instead of one of the benzene nuclei. Such a body might be formed as follows:



This ring should resemble carbazole in many of its properties. Perkin and Robinson [1919] have found a similar ring in the harmine series. They have shown that the base C<sub>12</sub>H<sub>10</sub>N<sub>2</sub> prepared by Hopkins and Cole [1903] by the oxidation of tryptophan with ferric chloride is harman [Fischer, 1901]. Consequently there is convincing evidence that closure of the tryptophan side-chain can occur. Perkin and Robinson advance the theory that harmine originates in nature by an acetaldehyde condensation with hydroxytryptophan, a substance already identified by Abderhalden and Baumann [1908]. Additional evidence for the closure of the tryptophan side-chain has been obtained in connection with other reactions which I hope to describe in a future communication.

On this hypothesis, the formula for tryptophan blue is:



There appears to be very little difference between tryptophan blue prepared from formaldehyde or from glyoxylic acid. Loss of CO<sub>2</sub> occurs during the preparation and the final pigment obtained is probably the same in each case. Unless the condensation be carried out thoroughly, the pigment pre-

precipitated by the ether is very impure, containing, as well as the blue derivative, a varying amount of purple material which probably represents an earlier stage in the condensation when the side chain of only one of the tryptophan molecules has closed up. The pigment should not be precipitated until it has stood for some days, by which time most of the substances will have condensed and oxidised spontaneously to the blue.

The blue is precipitated more readily by ether than the red or the purple colouring matters, but, even so, it is almost impossible to separate them completely.

*Tryptophan formaldehyde blue* resembles the benzaldehyde blue in some respects and the carbazole blues in others.

When purified it is moderately stable and has been obtained in solution in dilute HCl. Like the other tryptophan derivatives, it melts in the region of 300°, the exact temperature depending somewhat on the rate of heating.

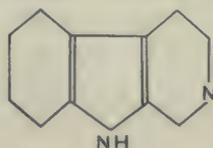
As usually carried out, the Hopkins-Cole test results in a mixture of tryptophan *red* and *blue*, the proportions depending on the conditions. For this reason it is unsatisfactory as the basis of colorimetric work, as two solutions of the same strength may give different shades if the temperature be at all different. Fasal [1912] noticed this difference in tint during his work on the estimation of tryptophan, but considered it entirely due to the amount of colouring matter formed.

For colorimetric work an aldehyde which only reacts in one way with the amino-acid is advisable, if not essential.

The above formula is in agreement with the experimental facts. It accounts for the existence of the leuco-form; for the three molecules of formaldehyde required; and for the colour and general properties of the pigment.

Only aldehydes that react readily with the amino-group, and are also capable of condensing with the indole nucleus, can give the carbolinine blue compound. Tryptophan benzaldehyde and salicyl aldehyde blues are simple condensation products and do not show any evidence of a closure of the tryptophan side-chain.

A study of the freezing-points of the bases obtained by removal of the HCl from the pigments indicated that the molecular weights of the formaldehyde and glyoxylic blues were probably the same, 352. Some preparations had higher molecular weights of the order of 400, which would be explained by the preparations not being fully decarboxylated. The pigment ultimately obtained in each case is one in which CO<sub>2</sub> has been lost by all the COOH groups. It will be seen that the condensation product formed by the closure of the tryptophan side-chain is derived from the ring nucleus:



Perkin and Robinson, who have described a similar ring, suggest the name "carboline" for this structure, indicating an analogy both to carbazole and quinoline. According to this nomenclature, tryptophan blue would be a carboline blue in which the C atoms of the pyridine nucleus are fully saturated, such a ring might be termed "carbolinine" by analogy to the relation between pyrrole and pyrroline. Tryptophan blue then would be "di-4-isocarbolininemethane," and the leuco-precursor would be, "di-4-carbolininemethane."

#### THE CARBAZOLE CONDENSATION DERIVATIVES.

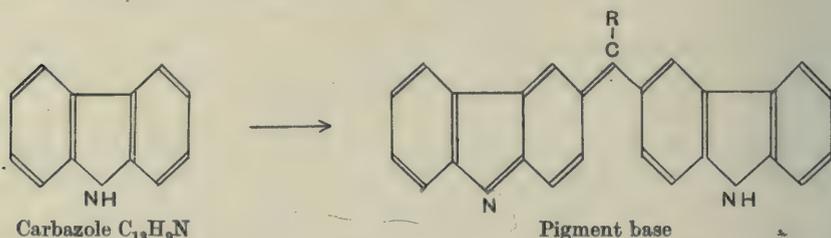
Several of these bodies were prepared: the following are of most interest in connection with the present paper.

- (9) Carbazole formaldehyde blue.
- (10) Carbazole glyoxylic blue.
- (11) Carbazole benzaldehyde blue.

Only a brief reference will be made to these bodies.

Their preparation and principal properties resemble those of the pigments previously described. They are of different shades depending on the aldehyde and the solvent. They are more stable than the other pigments and will keep for a long time in aqueous solution. They are precipitated by alkalies. The melting points are high, the benzaldehyde blue beginning to decompose at 280°.

The general formula is probably:



This reaction is a good general test for the presence of carbazole; benzaldehyde is perhaps the most convenient aldehyde to work with, but the colours are good with all three.

#### A SIMPLE QUALITATIVE REAGENT FOR TRYPTOPHAN AND THE INDOLES.

The reagent is 10 % salicyl aldehyde in alcohol, which must be free from acetone. A small quantity of the liquid under examination is mixed with excess of strong hydrochloric acid. Four or five drops of the reagent are added and the mixture gently warmed for a few minutes, a drop of 10 % hydrogen peroxide is then added and the warming continued. An intense blue denotes tryptophan, a deep purple denotes scatole, while indole gives a bright carmine. If too much peroxide be added the colours are destroyed, otherwise, they are very stable and are not discharged on dilution with water. The reagent will

give a deep blue with less than 0.1% tryptophan; with weak solutions care must be taken in the oxidation. By modifying the strength of the reagents the test can be made very delicate. In properties the pigments resemble very closely the benzaldehyde derivatives, their greater stability towards water is comparable with that of the malachite greens derived from substituted benzaldehyde. The fastness of these latter dyestuffs depends on the presence of a sulpho-group in the ortho-position to the methylene carbon, and one is justified in assuming the hydroxyl of the salicyl aldehyde to operate in a similar manner.

Compare the behaviour of salicyl aldehyde and benzaldehyde when condensed with acetone in presence of hydrochloric acid.

#### SUMMARY.

(1) A new colour test for glyoxylic acid is described, depending on its interaction with pyrogallol in presence of sulphuric acid.

(2) The Hopkins-Cole test is due to glyoxylic acid, the Rosenheim test is due to formaldehyde. In each test at least two distinct colour derivatives of tryptophan are formed, a red and a blue.

(3) A method is described for the preparation of the aldehyde derivatives of indole, scatole, tryptophan and carbazole, and a number of these have been isolated.

(4) The possible formulae of these substances is discussed, and a general type of structure is suggested.

(5) The closure of the tryptophan side-chain is put forward as an explanation of the formation of tryptophan formaldehyde blue.

(6) Salicyl aldehyde is recommended as a useful qualitative reagent for the indole derivatives.

I should like to express my obligation to Professor F. G. Hopkins for his kind advice and interest in the research, also to Professor E. A. Werner for having read the proofs of this paper.

I received much help in the earlier stages of the work from Mr Robin Hill, of Emmanuel College, Cambridge.

The expenses of the research were defrayed by the Mackinnon Research Studentship of the Royal Society.

#### REFERENCES.

- Aberhalden and Baumann (1908). *Zeitsch. physiol. Chem.* **55**, 412.  
Adamkiewicz (1874). *Pflüger's Arch.* **9**, 156.  
Beckmann and Sabel (1906). *Ber.* **39**, 3140.  
Blumenthal (1909). *Biochem. Zeitsch.* **19**, 521.  
Breidahl (1915). *Biochem. J.* **9**, 36.  
Cole (1903). *J. Physiol.* **30**, 311.  
Dakin (1907). *J. Biol. Chem.* **2**, 289.  
Dakin and Dudley (1914). *J. Biol. Chem.* **17**, 275.

- Eppinger (1905). *Beiträge*, **7**, 459; 466.  
Fasal (1912). *Biochem. Zeitsch.* **44**, 392.  
Fearon (1918). *Biochem. J.* **12**, 179.  
Fischer (1889). *Ber.* **19**, 2988.  
Fischer, O. (1901). *J. Chem. Soc. Abstr.* i, 405.  
Freund and Lebach (1905) *Ber.* **38**, 2640.  
Homer (1913). *Biochem. J.* **7**, 116  
Hopkins and Cole (1901, 1). *Proc. Roy. Soc.* **68**, 21.  
— (1901, 2). *J. Physiol.* **27**, 418.  
— (1903). *J. Physiol.* **29**, 451  
Kondo (1906). *Zeitsch. physiol. Chem.* **48**, 185.  
Mottram (1913). *Biochem. J.* **7**, 249.  
Palazzo and Onorato (1905). *Gazzetta*, **35**, 465, 476.  
Perkin and Robinson (1919). *J. Chem. Soc.* **115**, 933, 967.  
Rohde (1905). *Zeitsch. physiol. Chem.* **44**, 161.  
Rosenheim (1906). *Biochem. J.* **1**, 233.  
Sasaki (1910). *Biochem. Zeitsch.* **23**, 402.  
Schloss (1906). *Beiträge*, **8**, 455.  
Udranszky (1888) *Zeitsch. physiol. Chem.* **12**, 395.  
Voisenet (1918). *Compt. Rend.* **166**, 789.  
Weissgerber (1913). *Ber.* **46**, 651.  
Werner (1917). *J. Chem. Soc.* **111**, 844.

## XLVII. THE HEAT INACTIVATION OF DIPHTHERIA ANTITOXIN.

BY ANNIE HOMER.

(Received May 18th, 1920.)

AMONGST serum workers there is an impression that the addition of from 1.5 to 2 % of sodium chloride to antitoxic plasma, prior to its being heated, guards against the undue inactivation of the antitoxin, and it is generally accepted that the protective influence of the sodium chloride in this respect is still more marked during the heating of cresylised plasma or serum.

In order to furnish definite evidence on this point a study has been made of the percentage inactivation of antitoxin during the heating of:

(A) Oxalated antitoxic plasma,

(B) Plasma (A) to which an addition of 1.5 % of solid sodium chloride was made,

(C) Oxalated antitoxic plasma containing 0.30 % of cresylic acid,

(D) Plasma (C) to which an addition of 1.5 % of solid sodium chloride was made,

for stated periods of time at temperatures ranging from 58° to 72°.

Separate volumes of the respective plasmas (A), (B), (C) and (D), each of 25 cc., contained in 30 cc. sealed ampoules, were heated for definite periods of time in water baths regulated to the specified temperatures by means of thermostats.

Determinations were made of the antitoxic values of the respective liquids before and after heating, and, from the results thereby obtained, was calculated the percentage inactivation induced during the heating period.

The data thus furnished from the study of the plasmas (A) and (B), and of (C) and (D), have been included in Tables I and II respectively.

It will be seen that the rate of inactivation rapidly increases with the rise in temperature. Thus (Table I), while the plasma can be kept at 58° for ten hours without suffering any appreciable diminution of its antitoxic value, there is a 10 % loss shown after three hours' heating at 60.5°, or after two hours' heating at 61.5°, or after one hour's heating at 63°. With the progressive rise in temperature the proportional loss of antitoxic value in a given time rapidly increases until at 72° there is a 60 % inactivation of the antitoxin within ten minutes.

A comparison of the results given in Tables I and II shows that the loss of antitoxin during the heating of cresylised plasma is greater than that resulting from the similar treatment of noncresylised plasma. But, contrary to the ideas current amongst serum workers, the evidence obtained in this investigation indicates that the addition of sodium chloride does not appreciably lessen the heat inactivation of antitoxin either in cresylised or in non-cresylised plasma.

Further information as regards the heat-instability of antitoxin was obtained from a similar study of the inactivation of antitoxin in solutions containing only those protein fractions with which the antitoxin is associated in plasma.

The salt-soluble globulin fraction of an unheated antidiphtheritic plasma (potency 450 units per cc. and protein content 7.48 %) was separated and dialysed in the usual way. The residue from dialysis was diluted with distilled water so as to reduce the protein content of the liquid to 7 %.

This solution of antitoxin and its associated proteins, freed in this way from albumin, euglobulin and the other organic and inorganic constituents of the plasma, was submitted to the same treatment as that described above for the cresylised and noncresylised plasmas, (A), (B), (C) and (D).

The results recorded in Table III indicate that, as was anticipated, antitoxin shows a greater stability to heat when in aqueous solution associated with its own particular proteins than when present in the more complex medium of the plasma.

While studying the effect of heat on the inactivation of antitoxin I also made observations on the accompanying increased precipitability of the serum proteins. From the data, thus obtained, was calculated the percentage denaturation of the serum proteins, the results for the respective liquids being also included in Tables I, II and III.

In all cases it was noticed that, during the heating of those sera or plasmas which contained from 1.5 to 2 % of sodium chloride or cresylic acid and phenol in amounts up to 0.6 %, there was an apparently greater conversion of soluble into insoluble protein than occurred during the heating of non-cresylised plasma. However, by means of the refractometer, it was ascertained that the presence of sodium chloride or of cresylic acid and phenol in the plasma had not affected the extent of the heat-denaturation of the proteins, these substances had merely stimulated the coagulation of the particles of denaturated proteins into aggregates of sufficient size to be more readily discerned by the eye.

From the results recorded both here and in my previous papers it has been found that the extent of the denaturation of the proteins is a function of:

- (a) the temperature at which the heating was conducted;
  - (b) the reaction of the plasma;
- and (c) the duration of the heating of the plasma.

Table I.

Showing the extent of the denaturation of the serum proteins and the inactivation of the antitoxin during the heating of oxalated antidiphtheritic plasma (A) and of the same plasma to which has been added 1.5 % of sodium chloride (B).

Potency of the unheated plasma = 375 units per cc.

Protein-content of the plasma = 8.45 %

P<sub>II</sub> of the unheated plasma, 7.8.

Temperature °C.	Duration of the heating	Plasma (A)		Plasma containing 1.5 % NaCl (B)	
		Percentage denaturation of the serum proteins	Percentage inactivation of the antitoxin	Percentage denaturation of the serum proteins	Percentage inactivation of antitoxin
58	4.5 hours	30.3	nil	30.5	nil
"	10 "	31.0	"	31.2	"
60.5	1 hour	13.5	"	14.0	"
"	2 hours	40.0	"	41.0	"
"	3 "	42.0	10	41.5	less than 10
61.5-62	1.5 "	40.0	—	40.0	—
"	2 "	50.0	8	49.0	less than 8
"	3 "	55.0	slightly greater than 13	55.0	about 20
"	4 "	56.5		57.5	
63	1 hour	52.0	10	53.5	10
"	2 hours	64.0	slightly less than 27	66.0	25
"	3 "	72.0	—	70.0	—
65	10 minutes	40.8	—	42.5	—
"	20 "	57.0	about 27	58.5	about 27
"	30 "	65.0	about 33	66.0	about 33
"	1 hour	practically solid	—	practically solid	—
66	10 minutes	44.5	about 13	45.5	about 10
"	20 "	60.0	slightly less than 33	62.0	slightly less than 33
67.5	10 "	62.5	20	63.5	20
"	20 "	69.0	36	71.0	33
68.5-69	5 "	56.5	—	57.0	—
"	10 "	62.0	33	61.0	33
"	20 "	72.0	slightly less than 46	72.0	slightly less than 46
70	5 " *	65.0	slightly less than 33	65.0	33
"	10 "	75.0	53	75.0	slightly greater than 46
"	15 "	almost solid	—	practically solid	—
72	5 "	80.0	60	80.0	60
"	10 "	practically solid	—	practically solid	—

Table II.

Showing the extent of the denaturation of the serum proteins and the inactivation of antitoxin induced during the heating of antidiphtheritic plasma containing 0.30 % of cresylic acid (C), and of the same cresylised plasma to which 1.5 % of sodium chloride has been added (D).

Potency of the original plasma = 375 units per cc.

$P_H$  of the original plasma, 7.4.

Protein-content of the plasma = 8.45 %.

Temperature °C.	Duration of the heating	Cresylised plasma (C)		Cresylised plasma containing 1.5 % of NaCl (D)	
		Percentage denaturation of the serum proteins	Percentage inactivation of the antitoxin	Percentage denaturation of the serum proteins	Percentage inactivation of the antitoxin
58	4 hours	25.0	nil	25.4	nil
"	8 "	26.0	"	26.0	"
60	1 hour	41.6	negligible	40.6	negligible
"	2 "	45.5	10	45.4	10
"	3 "	48.5	17	48.3	17
62	1 "	45.0	17	47.2	13
"	2 hours	55.0	—	57.5	24
"	3 "	57.0	—	57.5	—
63	1 hour	47.2	17	50.0	17
"	2 hours	64.0	—	65.0	—
"	3 "	70.0	—	practically solid	—
64	30 minutes	53.0	15	52.0	17
"	1 hour	63.5	—	66.0	—
"	2 hours	practically solid	—	practically solid	—
65	20 minutes	53.0	40	52.0	40
"	30 "	65.0	—	64.0	—

The extent of the inactivation of the antitoxin seems to be regulated by:

- (a) the temperature at which the heating is conducted;  
and (b) the time of heating.

The time of heating required to produce the maximum value for the denaturation of the proteins became shorter as the temperature was raised. In this respect the behaviour of antitoxin and of the serum proteins is somewhat analogous. However the heat-inactivation of the antitoxin seems to be mainly a function of the temperature and independent of the extent of the accompanying heat-denaturation of the serum proteins, for, by suitable adjustments, the heat-denaturation of the proteins at a given temperature can be rendered complete without any corresponding change in the coefficient for the heat-inactivation of the antitoxin at that temperature.

These observations lead to considerations as to whether antitoxins should be regarded as separate entities rather than as structural modifications of the serum proteins induced during the process of immunisation. In this connection, it had been my intention to plot curves for the time taken, at each degree of temperature, to reduce the antitoxic value of the liquids to half

Table III.

*Showing the extent of the heat-denaturation of pseudo-globulin and of the inactivation of antitoxin in solutions which have been heated at temperatures between 58° and 72°.*

P<sub>H</sub> of the solution of pseudo-globulin, 7.0.  
Potency = 450 units per cc.; protein-content, 7.48 %.

Temperature °C.	Duration of the heating	Percentage denaturation of the pseudo-globulin	Percentage inactivation of antitoxin
58	4 hours	25.0	negligible
"	10 "	26.0	"
61.5	1 hour	23.7	nil
"	2 hours	26.6	negligible
"	3 "	31.6	"
63	30 minutes	31.0	"
"	1 hour	40.0	"
"	2 hours	51.0	"
"	3 "	55.0	"
66	20 minutes	40.0	"
"	40 "	46.0	"
67.5	10 "	50.7	"
"	20 "	61.7	nearly 13
68.5	10 "	57.0	8
"	20 "	67.0	—
70.0	5 "	50	negligible
"	10 "	70	30
"	15 "	practically solid	—
72	8 "	90	greater than 50

value, and to make therefrom a comparison between the heat inactivation of antitoxin and that of certain enzymes. But, unfortunately, owing to the prolonged scarcity of experimental animals, I have been unable to carry my investigations further in this respect.

The work involved in this investigation was, in part, carried out during my war time appointment with the Lister Institute.

#### SUMMARY.

During the course of the investigation it has been found that:

(1) The addition of 1.5 to 2 % of sodium chloride to plasma, whether noncresylised or cresylised, prior to its being heated, in no way reduces the extent of the heat-inactivation of the antitoxin.

(2) The rate of the heat-inactivation of antitoxin is a function of the temperature at which the heating is conducted.

(3) The extent of the heat-inactivation of the antitoxin in an aqueous solution of the antitoxin bearing proteins is considerably less than that evidenced during the heating of antitoxic plasma.

## XLVIII. NOTE ON "SCURVY" IN PIGS.

By ROBERT HENRY ADERS PLIMMER.

*From the Rowett Research Institute in Animal Nutrition, University of  
Aberdeen and North of Scotland College of Agriculture.*

(Received May 19th, 1920.)

At the beginning of April four young pigs were observed to be ill with symptoms suggesting scurvy, namely:

(1) Stoppage of growth.

(2) On being approached the animals squealed with more than their usual fervour, and on being touched the squealing became frenzied.

(3) Lack of appetite and a condition of great lassitude.

(4) Spasmodic twitching of limb muscles (see description of scurvy in guinea-pigs by Miss Chick and co-workers).

(5) Swollen joints.

(6) Inability to walk or stand properly. On being made to rise or move, the pigs dragged themselves about with the trotters bent underneath the forelimbs. The hind limbs were also feeble and not held straight as in the normal pig, but the trotters were not bent under as in the forelegs.

(7) The gums and teeth could not be examined, as the animals resented being touched and were likely to bite.

On enquiry into the diet it was ascertained that the pigs had been fed entirely on *cooked* food, a mash composed of meal, sharps and turnips. As it seemed obvious that the complaint was due to lack of the antiscorbutic factor, directions were given that the same food should be continued but in a *raw* state. The proportion of turnip (yellow swede) was increased in amount because of the high antiscorbutic value which this vegetable is known to possess. In this case the swedes were obtained direct from the field in which they were growing. Occasionally a little skimmed milk or butter milk was given both with the cooked and the raw food at those times when it was available on the farm.

As was expected the four pigs very soon improved upon the uncooked diet; their appetite showed improvement after a few meals of raw food. The other symptoms gradually lessened until in about 14 days the pig in the best condition roamed about the fields of his own free will, and on handling squealed no more than is customary with pigs. The other three pigs, whose symptoms had been more acute, took five weeks to reach the same state of recovery. The best pig walked properly after a week. The others walked on

straight limbs after four weeks, and their limbs, though still weak, are gradually gaining strength.

The animals had the following weights in kilograms which show their improved condition:

	April 9th	April 20th	April 27th	May 17th
(1)	25	32	34.5	41.6
(2)	25	29.4	35	44.5
(3)	38	37.4	41.5	50.5
(4)	20.7	22.8	25.5	31.5

The change to uncooked food has thus not only improved their condition, but has caused renewed growth. The animals were about four months old at the time they first came under observation. No. 3 took longer than the others to recover its appetite.

This case exemplifies the application of recent scientific results to agricultural problems; with the absence of suitable appliances for scientific work it was not possible to make closer study such as weighing of the food and more details of the symptoms of the animals. This disease is said to be fairly common amongst pigs reared during the winter and fed on cooked food as well as being much confined to styes. It is most frequently referred to as "rheumatism," sometimes as "rickets" or "pig gout"; raw potatoes are in some places recommended for its cure. The term rickets as here applied is evidently incorrect, since the antirachitic factor is comparatively stable to heat. Delf [1920] has shown that cooking rapidly destroys the antiscorbutic factor.

*Addendum, August 11th, 1920.*

Since the above note was written, the pigs have been sold on the market and realised the current prices. Fortunately it was possible to see the ribs and bones of pig (4). The ribs on one side appeared normal: on the other side one rib showed a healed fracture (of old standing) and five other ribs showed haemorrhage; two of these ribs were curved and not straight. The leg bones of one side looked normal: those of the other side showed (a) thickening of the radius and ulna, (b) fusion and thickening of the tibia and fibula.

REFERENCE.

Delf (1920). *Biochem. J.* 14, 211.

# XLIX. NOTE ON THE OXIDATION OF QUININE WITH HYDROGEN PEROXIDE.

BY MAXIMILIAN NIERENSTEIN.

*From the Biochemical Laboratory, Chemical Department,  
University of Bristol.*

*(Received May 19th, 1920.)*

THE observations recorded in this note refer to experiments which were carried out in the hope of oxidising quinine to haemoquinic acid, a disintegration product of quinine found in the urine, especially in blackwater fever [Nierenstein, 1918, 1919]. The results were disappointing, since it was only possible to identify quitenine, a well-known oxidation product of quinine, though a very careful search was made for haemoquinic acid. In this connection it is of interest to note that quitenine is also present in the urine after the administration of quinine [Nierenstein, 1918, 1919] and that it is produced from quinine by emulsions from guinea-pig and ox liver [Lipkin, 1919].

Ten grams of quinine hydrochloride dissolved in 100 cc. of water were heated on a water-bath for 6 hours with 50 cc. hydrogen peroxide (40 volumes commercial) and the oxidation repeated with a fresh amount of hydrogen peroxide (50 cc.) for a further 6 hours. To the solution, which had acquired a deep, dark-brown colour, the theoretical amount of  $N/10$  sodium hydroxide was added, so as to neutralise the hydrochloric acid present. The solution, which contained a fair amount of a precipitate, was extracted several times with ether, and the ethereal extract shaken with a 10% solution of sodium bicarbonate saturated with carbon dioxide. The aqueous layer was acidified and extracted with ether. The ethereal extract, carefully dried over anhydrous sodium sulphate, left on evaporation a residue which crystallised from dilute alcohol in slender needles, melting at  $280-282^{\circ}$  when quickly heated, carbon dioxide being evolved. This melting point was not depressed when mixed with quitenine prepared according to Skraup's method [1893]. The melting point for quitenine is given by Skraup as  $286^{\circ}$  and by Nierenstein [1919] as  $283-284^{\circ}$ . Three different preparations, dried at  $150^{\circ}$ , were used for analysis.

0.1384 g.: 10.0 cc.  $N_2$  at  $11^{\circ}$  and 753 mm.

0.1562 g.: 11.2 cc.  $N_2$  at  $15^{\circ}$  and 757 mm.

0.1614 g.: 11.5 cc.  $N_2$  at  $15^{\circ}$  and 747 mm.

Found: N = 8.6; 8.3; 8.2.

Calculated for  $C_{19}H_{22}O_4N_2$ : N = 8.2%.

The quitenene prepared both by the hydrogen peroxide method and according to Skraup's method gave the thalleioquin reactions described by Lipkin [1919], which confirms his very exact observations.

## REFERENCES.

- Lipkin (1919). *Ann. trop. med. parasit.* **13**, 149.  
Nierenstein (1918). *J. Roy. Army Med. Corps*, **32**, 218.  
— (1919). *Report on the Excretion of Quinine in Urine*. War Office Observations on Malaria, edited by Sir Ronald Ross. His Majesty's Stationery Office.  
Skraup (1893). *Monatsh.* **14**, 431.

## L. GLYCINE AND ITS NEUTRAL SALT ADDITION COMPOUNDS.

BY HAROLD KING AND ALBERT DONALD PALMER.

*From the Department of Biochemistry and Pharmacology, Medical Research  
Council.*

*(Received May 26th, 1920.)*

THE work of Bugarsky and Liebermann [1898] showed that proteins could combine with acids and alkalis but not with sodium chloride. It is not however universally accepted that no combination takes place between proteins and neutral salts in general. The behaviour of amino-acids, as forming the fundamental units of the protein molecule, towards neutral salts is therefore of some interest.

Scattered throughout the early literature, there are several references to the addition compounds of glycine and inorganic salts. It is, however, to Pfeiffer and his co-workers that we are indebted for a fuller and more systematic account of the addition products of neutral salts, particularly the halides of the alkali and alkaline earth metals with glycine, its simpler derivatives, and alanine.

By mixing glycine and the chlorides or bromides of the alkaline earth metals or of lithium in suitable proportions in aqueous solution Pfeiffer and Modelski [1912, 1913] obtained on evaporating the solution, or on adding alcohol, crystalline addition complexes, containing the components in some simple molecular proportion. Thus calcium chloride was found to furnish three compounds,  $\text{CaCl}_2, \text{C}_2\text{H}_5\text{O}_2\text{N}, 3\text{H}_2\text{O}$ ;  $\text{CaCl}_2, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, 4\text{H}_2\text{O}$ , and  $\text{CaCl}_2, (\text{C}_2\text{H}_5\text{O}_2\text{N})_3$  and lithium bromide two compounds,  $\text{LiBr}, \text{C}_2\text{H}_5\text{O}_2\text{N}, \text{H}_2\text{O}$ , and  $\text{LiBr}, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, \text{H}_2\text{O}$ . The formation and isolation of these pure substances were materially assisted by the addition to the clear solution of a little acetic acid. Bayliss [1915, 1919, 2] has on several occasions taken exception to the description of these addition products as compounds, his own preliminary experiments having indicated that the problem was more complex and involved the formation of mixed crystals.

One of the present authors, having had experience of addition compounds of the alkaline earth halides and also of the alkali metal halides with an amphoteric substance of quite a different type, it was decided to attempt to throw further light on these addition compounds of glycine under such conditions as would lead to results of significance. The addition of acetic acid

and precipitation by alcohol were therefore avoided throughout. We have, moreover, restricted the experiments to solutions containing one molecular proportion of glycine in the case of lithium, sodium and potassium chlorides, bromides and iodides, and two molecular proportions in the case of calcium strontium and barium chlorides and iodides, except where the behaviour of such a solution rendered it necessary to start with different proportions of the components.

In the main we confirm the results of Pfeiffer and Modelski with regard to the definite nature of the compounds of glycine with calcium, barium and strontium chlorides, of the type  $MCl_2, (C_2H_5O_2N)_2, H_2O$ , and with lithium chloride and bromide of the types,  $LiCl$  (or  $Br$ ),  $C_2H_5O_2N, H_2O$  and  $LiCl$  (or  $Br$ ),  $(C_2H_5O_2N)_2, H_2O$ . We have been unable to prepare any addition compounds of the potassium halides, but have succeeded in preparing from sodium bromide and iodide, addition compounds of the same type as the second lithium type figured above. We are also able to support Bayliss's contention of the formation of solid solutions, which in our opinion are formed by the mutual solubility of the addition compounds in one another.

The different degrees of complexity of behaviour over the somewhat restricted range of composition of the solutions as studied by us are shown by the following examples.

Barium chloride and glycine form a compound,  $BaCl_2, (C_2H_5O_2N)_2, H_2O$ , which can be obtained pure from a solution containing the components in widely differing molecular proportions.

Ratio $BaCl_2/C_2H_5O_2N$	1 : 2	3 : 4	1 : 4
% yield of $BaCl_2, (C_2H_5O_2N)_2, H_2O$	60	64	48
Analysis	Cl=18.8 N=7.6	Cl=18.8 N=7.5	Cl=18.7 N=7.7
Calculated for $BaCl_2, (C_2H_5O_2N)_2, H_2O$ ; Cl=18.8; N=7.4 %.			

This table furnishes a proof of the true molecular compound nature of these addition products. Moreover, the water of crystallisation present in all of these addition compounds is in some simple molecular proportion and is not the same as is present in the pure inorganic constituent, when in equilibrium with its saturated solution at the ordinary room temperature.

The behaviour of lithium bromide and glycine is of interest as it affords evidence that perfect homogeneity of crystalline appearance is not in itself a sufficient criterion of a definite chemical compound. A solution of these components in molecular proportions deposits on concentration first well-formed transparent needles, which are soon followed by a voluminous deposit of smaller white needles. If the transparent needles be collected before the second type has appeared, their Br/N ratio is approximately 0.87/1. This ratio is intermediate between the two compounds,  $LiBr, C_2H_5O_2N, H_2O$  and  $LiBr, (C_2H_5O_2N)_2, H_2O$ , which were obtained by Pfeiffer and Modelski and have also been obtained by us, and the crystals represent a solid solution of the one compound in the other.

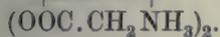
The iodides of calcium, strontium and barium when crystallised from a solution containing two molecular proportions of glycine do not give pure compounds. The crystals are generally poor in appearance when compared with the corresponding chlorides, and probably represent solid solutions of more or less complete miscibility. From the mother liquor of such a calcium iodide—glycine solution, which, having already deposited crystals whose I/N ratio was 0.57/1, was relatively richer in calcium iodide, the most markedly crystalline compound yet encountered in these experiments was obtained. It crystallised in large tablets of the composition,  $\text{CaI}_2, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, 3\text{H}_2\text{O}$ . These were shown to be stable only in contact with solutions containing excess of calcium iodide. This behaviour is strictly analogous to the well-known instability of carnallite,  $\text{KCl}, \text{MgCl}_2, 6\text{H}_2\text{O}$  except in contact with solutions containing excess of magnesium chloride.

Lithium iodide and glycine in molecular proportions did not furnish a pure homogeneous compound.

From aqueous glycine solutions or from solutions of glycine containing neutral salts, we have repeatedly isolated an unstable form of glycine crystallising in long stout needles or columns. On keeping in contact with the solution these gradually disappear with simultaneous deposition of the oft-described and pictured, squat monoclinic crystals [Schmelcher, 1892]. Curtius [1882] seems to have observed the formation of these two kinds of crystal from aqueous solution, but did not recognise their relative stability. The remarkable experiments of Ostromisslenski [1908] on the inoculation of *dl*-asparagine solutions with glycine, to explain which he postulates the existence of two enantiomorphous forms of glycine and moreover states that glycine crystallises in the rhombic hemihedral system and not in the monoclinic, may be intimately connected with the existence of the two forms observed by us. Fischer [1905] obtained a form of glycine which was reactive towards phosphorus pentachloride by precipitating its aqueous solution with alcohol. This form we consider to be probably identical with our unstable form from aqueous solutions. Falk and Sugiura [1918], repeating Fischer's preparation, showed that this reactive form, precipitated by alcohol in the form of fine needles, differed from the ordinary form in its greater absorption of bromine in the dry state and in its slightly slower evolution of gas in the determination of the van Slyke values. The difference in behaviour in the region of the melting point as described by these authors we have shown to be independent of any difference of structure. To account for these two glycines they suggest as a possibility a difference of structure,  $\text{NH}_2.\text{CH}_2.\text{COOH}$  and  $\overline{\text{NH}_3.\text{CH}_2.\text{COO}}$ .

It is of interest that in reviewing the nature of the linking of affinities in these addition compounds, Pfeiffer and Wittka [1915] reject the view expressed in the earlier paper of Pfeiffer and Modelski that the addition compounds were amphi-salts of the type  $\text{LiOOC}.\text{CH}_2.\text{NH}_2.\text{HCl}$  and now suppose that

the metal portion of the inorganic salt is attached to the closed ring form of glycine by means of the residual affinity of the carbonyl group, e.g. ClM...



#### EXPERIMENTAL DETAILS.

##### *Glycine Addition Compounds of Calcium, Strontium and Barium Chlorides.*

*Barium Chloride.* 5 grams of glycine ester hydrochloride were boiled for about 45 minutes with 20 cc. of water and 5 g. of barium carbonate. The solution was then filtered and on concentration yielded well-formed transparent plates. The first and second crops possessed a perfectly homogeneous appearance and amounted respectively to 2.3 and 1.2 g. The air-dried substance was analysed.

0.2033; 0.1557 g. AgCl. Cl=18.9 %.

0.2982; NH<sub>3</sub> equivalent to 16.08 cc. N/10 acid. N=7.6 %

0.1981 dried at 120° lost 0.0103 g. H<sub>2</sub>O=5.2 %.

Calculated for BaCl<sub>2</sub> (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, H<sub>2</sub>O; Cl=18.8; N=7.4; H<sub>2</sub>O=4.8 %.

The same compound was obtained from a mixture of one molecule of barium chloride and two molecules of glycine. Thus 1 g. of glycine and 13.9 cc. of 10 % barium chloride solution gave on concentration 1.5 g. (60 % yield) of addition compound crystallising in stout needles.

0.2092 g. air-dry; 0.1586 g. AgCl. Cl=18.8 %.

0.2089 g. air-dry; NH<sub>3</sub> equivalent to 11.28 cc. N/10 acid. N=7.6 %.

When the proportion of barium chloride was increased to three molecules to four of glycine, the same compound was isolated pure. One gram of glycine gave 1.6 g. (64 % yield) of addition compound.

0.2045 g. air-dry; 0.1557 g. AgCl. Cl=18.8 %.

0.2045 g. air-dry; NH<sub>3</sub> equivalent to 11.01 cc. N/10 acid. N=7.5 %.

When, however, the proportion of barium chloride was decreased to one molecule to four molecules of glycine, crystals of the addition compound separated followed by free glycine. By filtration before the glycine crystals began to appear, 1.2 g. (48 % yield) of pure addition compound were obtained from 2 g. of glycine.

0.2017 g. air-dry; 0.1522 g. AgCl. Cl=18.7 %.

0.2021 g.; NH<sub>3</sub> equivalent to 11.06 cc. N/10 acid. N=7.7 %.

*Calcium Chloride.* When the preparation of the calcium and strontium addition compounds of glycine was attempted starting with glycine ester hydrochloride and excess of calcium or strontium carbonates it was unexpectedly found that after prolonged boiling there was only slight decomposition of the ester hydrochloride, for on concentrating, the first crop of crystals consisted in each case of pure glycine ester hydrochloride, crystallising in the characteristic long silky needles, melting about 140° and analysing correctly. (Found Cl = 25.3; N = 10.0; 9.9; C<sub>4</sub>H<sub>9</sub>O<sub>2</sub>N, HCl requires Cl = 25.4; N = 10.0 %.)

The required addition compound was readily obtained by boiling the ester hydrochloride of glycine with excess of halogen-free slaked lime, filtering, saturating with carbon dioxide and further boiling to decompose the bicarbonate and carbamate formed. The calcium chloride addition compound crystallised in prismatic needles.

0.1995 g. air-dry; 0.1700 g. AgCl. Cl=21.1 %.

0.2023 g. air-dry; NH<sub>3</sub> equivalent to 12.06 cc. N/10 acid. N=8.3 %.

Calculated for CaCl<sub>2</sub>, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, 4H<sub>2</sub>O; Cl=21.3; N=8.4 %.

*Strontium Chloride.* The strontium chloride addition compound crystallised similarly in prismatic needles.

0.2044 g. air-dry; 0.1573 g. AgCl. Cl=19.0 %.

0.2030 g. air-dry; NH<sub>3</sub> equivalent to 11.12 cc. N/10 acid. N=7.7 %.

Calculated for SrCl<sub>2</sub>, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, 3H<sub>2</sub>O; Cl=19.5; N=7.7 %.

The somewhat divergent chlorine value was accidental, for, on recrystallisation, the compound gave the following figures on analysis.

0.2053 g. air-dry; 0.1613 g. AgCl. Cl=19.4 %.

0.2033 g. air-dry; NH<sub>3</sub> equivalent to 11.10 cc. N/10 acid. N=7.6 %.

0.2381 g. dried at 140° lost 0.0356 g. H<sub>2</sub>O=15.0 %. (Theory requires 14.9 % H<sub>2</sub>O.)

*Glycine Addition Compounds of Calcium, Strontium and Barium Iodides.*

*Barium Iodide.* An aqueous solution of two molecular proportions of glycine to one of barium iodide was concentrated under reduced pressure to a small volume. It deposited, when cold, a felted mass of woolly needles. On analysis the I/N ratio showed it to be a mixture.

0.2049 g. air-dry; 0.1508 g. AgI. I=39.8 %.

0.2054 g. air-dry; NH<sub>3</sub> equivalent to 9.8 cc. N/10 acid. N=6.7 %.

Whence I/N=0.65/1.

*Strontium Iodide.* Glycine (two molecular proportions) and strontium iodide gave a felted mass of crystals. Analysis indicated a mixture.

0.2031 g. air-dry; 0.1496 AgI. I=39.8 %.

0.2073 g. air-dry; NH<sub>3</sub> equivalent to 12.46 cc. N/10 acid. N=8.4 %.

Whence I/N=0.52/1.

*Calcium Iodide.* Glycine and calcium iodide were mixed in aqueous solution in the proportion of two molecules of glycine to one of calcium iodide. On concentration under reduced pressure and keeping at room temperature clusters of small needles were deposited. These were collected, air-dried and analysed.

0.2029 g.; 0.1674 g. AgI. I=44.6 %.

0.2013 g.; NH<sub>3</sub> equivalent to 12.4 cc. N/10 acid. N=8.6 %.

Whence I/N=0.57/1.

This sample was recrystallised from water and separated in small needles. The air-dry substance was analysed.

0.2021 g.; 0.1603 g. AgI. I=42.9 %.

0.2013 g.; NH<sub>3</sub> equivalent to 13.96 cc. N/10 acid. N=9.7 %.

Whence I/N=0.49/1.

In spite of the apparently satisfactory atomic ratio the crystals do not, probably, represent a pure individual compound.

The first mother liquors, after further concentration, deposited a felted mass of needles, but after standing for three or four weeks these had almost completely dissolved with deposition of large clear tablets. These were carefully picked out and analysed.

0.2040 g. air-dry; 0.1922 g. AgI. I=50.9 %.

0.2063 g. air-dry; NH<sub>3</sub> equivalent to 8.55 cc. N/10 acid. N=5.8 %.

Calculated for CaI<sub>2</sub>, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, 3H<sub>2</sub>O; I=51.0; N=5.6 %.

On keeping overnight the filtrate gave a further deposit of well-formed smaller tablets. These on analysis proved to be the same compound.

0.2010 g. air-dry; 0.1890 AgI. I=50.8 %.

0.2084 g. air-dry; NH<sub>3</sub> equivalent to 8.55 cc. N/10 acid. N=5.7 %.

With these crystals in hand a fresh calcium iodide solution containing two molecular proportions of glycine was prepared and concentrated. In spite of numerous attempts, inoculation with this well-formed crystalline trihydrate failed to cause any further separation of this form, the solution invariably yielding small needles. Nor did these change on prolonged keeping.

When, however, a solution of molecular proportions of glycine and calcium iodide was concentrated, inoculation with the trihydrate caused immediate separation of this compound.

0.2058 g. air-dry; 0.1919 AgI. I=50.4 %.

0.2046 g. air-dry; NH<sub>3</sub> equivalent to 8.44 cc. N/10 acid. N=5.8 %.

This compound is therefore only stable at room temperature in contact with solutions containing excess of calcium iodide.

#### *Glycine Addition Compounds of the Lithium Halides.*

*Lithium Chloride.* Glycine ester hydrochloride was boiled with an aqueous solution of one molecular proportion of lithium carbonate. On concentration the solution deposited, when cold, well-formed prismatic needles. When crystallisation of this form was almost complete a few crystals, smaller and opaque white in appearance, separated. The proportion of this latter was, however, quite small, and its effect is shown by the analysis of the total solid separation.

0.2059 g. air-dry; 0.2094 g. AgCl. Cl=25.2 %.

0.2034 g. air-dry; 0.2073 g. AgCl. Cl=25.2 %.

0.2015 g. air-dry; NH<sub>3</sub> equivalent to 15.61 cc. N/10 acid. N=10.8 %.

Calculated for LiCl, C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N, H<sub>2</sub>O; Cl=26.2; N=10.3 %.

It was recrystallised and separated in transparent prismatic needles of perfectly uniform appearance.

0.2013 g.; 0.2114 g. AgCl. Cl=26.0 %.

0.2073 g.; NH<sub>3</sub> equivalent to 15.53 cc. N/10 acid. N=10.5 %.

*Lithium Bromide.* A concentrated solution of molecular proportions of glycine and lithium bromide deposited first a crop of moderately long transparent needles, but before crystallisation was complete, a voluminous mass

of fine white needles separated as well. The total crop was collected and analysed when air-dry.

0.2021 g.; 0.1477 g. AgBr. Br=31.1 %.

0.2010 g.; NH<sub>3</sub> equivalent to 14.38 cc. N/10 acid. N=10.2 %.

Calculated for LiBr, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, H<sub>2</sub>O. Br=31.3; N=11.0 %.

A repetition of this experiment reproduced an identical behaviour.

Found on analysis, Br=32.9; N=10.6 %.

The experiment was now repeated, but the first crop of transparent needles, possessing all the apparent attributes of homogeneity, was collected before the second kind of crystal had begun to separate.

0.1767 g. air-dry; 0.1721 g. AgBr. Br=41.4 %.

0.1571 g. air-dry; NH<sub>3</sub> equivalent to 9.37 cc. N/10 acid. N=8.4 %.

Whence Br/N=0.87/1.

Two repetitions of this preparation in which the first, apparently homogeneous, separation of crystals was collected, gave similar figures on analysis.

Found Br=41.4; N=8.5. Whence Br/N=0.85/1.

Found Br=42.2; N=8.4. Whence Br/N=0.88/1.

In the presence of one-half a molecular proportion of lithium bromide in excess, a uniform crop of transparent prismatic needles was obtained. These were collected and analysed.

0.1943 g. air-dry; 0.2015 g. AgBr. Br=44.1 %.

0.2023 g. air-dry; NH<sub>3</sub> equivalent to 10.92 cc. N/10 acid. N=7.6 %.

Calculated for LiBr, C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N, H<sub>2</sub>O. Br=44.4; N=7.8 %.

When the proportion of lithium bromide was reduced to three-fourths of a molecule, a crop of crystals was obtained possessing a homogeneous appearance and crystallising in transparent needles. This result was not obtained without some difficulty as the solution had to be heated up and allowed to crystallise several times before what were undoubtedly uniform crystals were obtained.

0.2001 g. air-dry; 0.1489 g. AgBr. Br=31.7 %.

0.2005 g. air-dry; NH<sub>3</sub> equivalent to 15.71 cc. N/10 acid. N=11.0 %.

Calculated for LiBr, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, H<sub>2</sub>O. Br=31.3; N=11.0 %.

When the proportion of lithium bromide was lowered still further, to one-half a molecule, it was not found possible to obtain a crop of crystals of homogeneous appearance. Pfeiffer and Modelski, using these proportions, admittedly after some difficulty, obtained tablets of the compound LiBr, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, H<sub>2</sub>O, which we obtained as described above, from a solution somewhat richer in glycine, but crystallising in needles.

*Lithium Iodide.* From a solution of molecular proportions of glycine and lithium iodide, clusters of fine white silky needles separated, not unlike the mixed crystals obtained from glycine and the iodides of the alkaline earth metals.

0.2017 g. air-dry; 0.1980 g. AgI. I=53.1 %.

0.2028 g. air-dry; NH<sub>3</sub> equivalent to 11.01 cc. N/10 acid. N=7.6 %.

Whence I/N=0.77/1.

A second experimental solution of molecular proportions was prepared and several attempts made to obtain uniform crystals of a different type, but without success.

*Glycine Addition Compounds of the Sodium Halides.*

*Sodium Bromide.* Glycine and sodium bromide in molecular proportions gave, on concentrating the solution, a homogeneous crop of well-defined transparent needles. These were collected and analysed.

0.2016 g.; 0.1442 g. AgBr. Br=30.4 %.

0.2023 g.; NH<sub>3</sub> equivalent to 15.09 cc. N/10 acid. N=10.4 %.

Calculated for NaBr, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, H<sub>2</sub>O. Br=29.5; N=10.3 %.

A second solution was prepared with the same proportions, but considerable difficulty was experienced in obtaining a crop of crystals of homogeneous appearance. On inoculation, however, with the compound obtained in the first experiment, the same compound, crystallising in well-defined transparent needles, was obtained as before.

0.1562 g. air-dry; 0.1079 AgBr. Br=29.4 %.

0.1695 g. air-dry; NH<sub>3</sub> equivalent to 12.65 cc. N/10 acid. N=10.4 %.

*Sodium Iodide.* Glycine and sodium iodide in solution in molecular proportions gave, without difficulty, a crop of well-defined needles grouped in clusters.

0.2025 g. air-dry; 0.1501 g. AgI. I=40.0 %.

0.2007 g. air-dry; NH<sub>3</sub> equivalent to 12.63 cc. N/10 acid. N=8.8 %.

Calculated for NaI, (C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N)<sub>2</sub>, H<sub>2</sub>O. I=39.9; N=8.8 %.

A second solution of the same initial composition was prepared and on concentration readily gave the same compound.

0.1983 g.; 0.1463 g. AgI. I=39.9 %.

0.2028 g.; NH<sub>3</sub> equivalent to 12.59 cc. N/10 acid. N=8.7 %.

*Sodium Chloride.* Glycine ester hydrochloride was boiled in aqueous solution with one molecular proportion of anhydrous sodium carbonate. On concentration and allowing the solution to cool slowly or rapidly a mixture of crystals was invariably obtained, the glycine and sodium chloride separating side by side.

*Glycine and Potassium Halides.*

Using molecular proportions of glycine and one of the halides of potassium, namely chloride, bromide or iodide, no evidence was obtained of the formation of a crystalline addition compound. In one experiment with potassium bromide, a homogeneous crop of crystals was obtained. These contained a trace of halide, melted from 227–240°, and when analysed for nitrogen by the Kjeldahl process, proved to be practically pure glycine:

0.2026 g.; NH<sub>3</sub> equivalent to 26.58 cc. N/10 acid. N=18.4 %.

Calculated for C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>N. N=18.7 %.

*Other Crystalline Forms of Glycine.*

Glycine usually crystallises in compact crystals in the monoclinic system. When recrystallised from water, we have repeatedly observed the formation of another crystalline form of glycine, separating in long bold needles or columns and devoid of water of crystallisation. In one instance these were about two inches long. On keeping in contact with the solution, they invariably pass into the monoclinic variety, the unstable form gradually dissolving. The purest stable glycine that we have prepared decomposes in the neighbourhood of  $256^{\circ}$  (uncorr.). This temperature depends to a large extent on the rate of heating. There is also progressive darkening from about  $240^{\circ}$  onwards. The comparative behaviour of successively crystallised glycine is shown by the following table.

Crude glycine, becomes pale brown, about $220^{\circ}$ , decomposes $240^{\circ}$ .					
Once crystallised	"	"	"	"	$225^{\circ}$ , " $242^{\circ}$ .
Twice	"	"	"	"	$228^{\circ}$ , " $247^{\circ}$ .
Thrice	"	"	"	"	$235^{\circ}$ , " $254^{\circ}$ .
Four times	"	"	"	"	$240^{\circ}$ , " $256^{\circ}$ .

Fischer's reactive glycine, precipitated by alcohol, prepared from the four times crystallised glycine, when compared side by side in the same bath with the stable form showed an identical behaviour. The observations of Falk and Sugiura ascribing a difference of range of progressive deepening of colour of the two forms on heating, the glycine precipitated by alcohol darkening over a shorter range by 10 to  $20^{\circ}$ , must therefore be due to the presence of extraneous impurity such as is present in the fewer times crystallised glycine shown above. Moreover, we had available a sample of glycine precipitated by alcohol, from some of which glycine acid chloride had been prepared by Fischer's method. When compared with our purest four times crystallised glycine it became progressively coloured several degrees in advance of the stable form and decomposed at  $252^{\circ}$ , whereas the stable form, heated in the same bath simultaneously, decomposed at  $253^{\circ}$ . This again is exactly the contrary of the behaviour described by Falk and Sugiura.

The unstable form from aqueous solution, which was prepared from a sample of pure glycine, when compared with the four times crystallised ordinary glycine, exhibited an identical behaviour.

## SUMMARY.

1. The existence of the following addition compounds of glycine prepared by Pfeiffer and his co-workers is confirmed under severer experimental conditions.  $\text{BaCl}_2, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, \text{H}_2\text{O}$ ;  $\text{CaCl}_2, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, 4\text{H}_2\text{O}$ ;  $\text{SrCl}_2, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, \text{H}_2\text{O}$ ;  $\text{LiCl}, \text{C}_2\text{H}_5\text{O}_2\text{N}, \text{H}_2\text{O}$ ;  $\text{LiBr}, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, \text{H}_2\text{O}$ ;  $\text{LiBr}, \text{C}_2\text{H}_5\text{O}_2\text{N}, \text{H}_2\text{O}$ .
2. The following new addition compounds are described.  $\text{CaI}_2, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, 3\text{H}_2\text{O}$ ;  $\text{NaBr}, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, \text{H}_2\text{O}$ ; and  $\text{NaI}, (\text{C}_2\text{H}_5\text{O}_2\text{N})_2, \text{H}_2\text{O}$ .

3. In agreement with Pfeiffer no compounds were obtained with the potassium halides or with sodium chloride.

4. An unstable form of glycine from aqueous solutions is described and its relationships discussed.

## REFERENCES.

- Bayliss (1915). *Principles of General Physiol.* 105, 220, 281.  
— (1919, 1). *Brit. Assoc. Colloid Rep.* 138.  
— (1919, 2). *J. Physiol.* 53, 162.  
Bugarsky and Liebermann (1898). *Pflüger's Archiv*, 72, 51  
Curtius (1882). *J. pr. Chem.* 26, 155.  
Falk and Sugiura (1918). *J. Biol. Chem.* 34, 29.  
Fischer (1905). *Ber.* 38, 2914.  
Ostromislenski (1908). *Ber.* 41, 3040.  
Pfeiffer and Modelski (1912, 1913). *Zeitsch. physiol. Chem.* 81, 329; 85, 1.  
— and Wittka (1915). *Ber.* 48, 1289.  
Schmelcher (1892). *Zeitsch. Kryst.* 20, 113.

# LI. NOTE ON THE PRODUCTION OF A CONTRACTING CLOT IN A GEL OF GELATIN AT THE ISO-ELECTRIC POINT.

BY DOROTHY JORDAN LLOYD.

*Report to the Medical Research Committee.*

*From the Biochemical Laboratory, Cambridge.*

(Received June 2nd, 1920.)

With Plate XI.

IN a recent paper published in this *Journal* [Jordan Lloyd, 1920] the theory was developed that gelatin gels consist of a solid framework of pure uncombined gelatin, with an interstitial fluid which consists of a solution of gelatin salts, either acidic or basic. Equilibrium in the system was held to be the resultant of two opposing sets of forces, the elastic forces of the solid framework which tend to make the gel contract, and the osmotic forces due to the dissolved salts in the interstitial fluid which tend to keep it expanded. The special case of a hot solution of pure gelatin cooling at a reaction of  $P_H = 4.6$  was considered, and the following deduction drawn from the theory:

“The iso-electric gelatin will be precipitated at numerous crystallisation centres, the solid drops will run together to form a framework, but since there can be no osmotic forces in the system... the framework will contract under the action of its own surface forces and the internal phase will be squeezed out.” This condition of the formation of a contracting clot in a gelatin gel has now been realised in the laboratory and is shown in Plate XI, fig. 1. The gel was prepared by the following method: sheets of Coignet's Gold Label gelatin were dialysed against two changes of  $N/1000$  hydrochloric acid, then against three changes of distilled water, and finally against dilute hydrochloric acid at  $P_H = 4.6$ . The dialysing acid was tested after twenty-four hours for change of reaction and was replaced daily by fresh acid at  $P_H = 4.6$  until no change of reaction after twenty-four hours' dialysis could be detected by the colorimetric method. The indicator used was methyl red, and the colour was standardised by comparison with that obtained by adding an equivalent amount of indicator to a solution consisting of 50 cc.  $0.2M$  acid potassium phthalate + 13 cc.  $0.2M$  caustic soda in a total volume of 200 cc. [Clark and Lubs, 1917]. The whole course of dialysis lasted about a month.

The soaked sheets of gelatin were then removed from the dialysing fluid, dried, melted in an evaporating basin and filtered hot into a large volume of 97 % alcohol. A litre of alcohol was allowed to every 100 cc. of melted gelatin. The gelatin was precipitated as a white fibrous mass possessing marked elasticity. It was allowed to stand in the alcohol twenty-four hours,

5-842

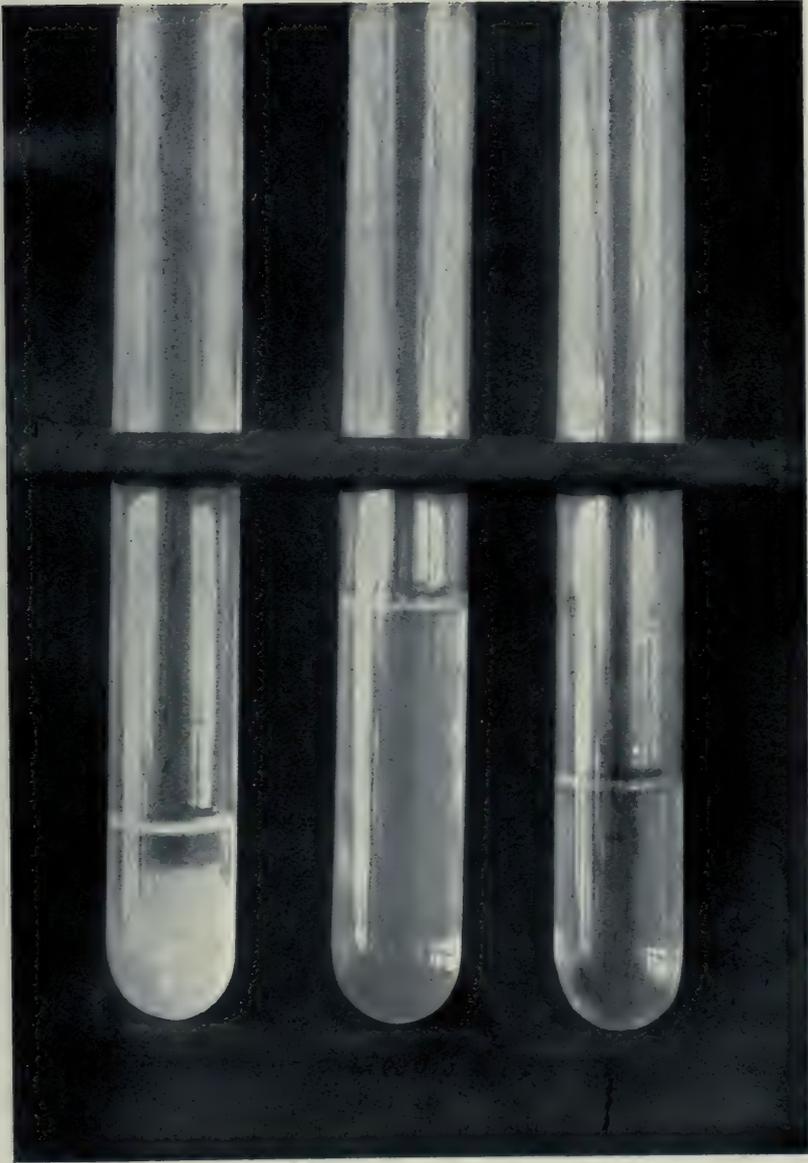


Fig. 1

Fig. 2

Fig. 3



then in three changes of strong alcohol, each for twenty-four hours, and then in three changes of absolute alcohol. Finally the white fibrous mass was washed with ether and dried in a desiccator. This method of preparing pure gelatin differs from that employed by Dheré [1913] and by Dheré and Gorgolewski [1910] in the reaction of the dialysing fluid, but is otherwise similar to that described by these workers. The pure gelatin is snow-white and quite friable. The ash content was 0.1 %. A full account of its properties will be given in a subsequent paper. The three gels made from this gelatin and shown in Plate XI are all of a 1 % concentration. They differ in their content of acid, hydrochloric acid being present in tubes 2 and 3.

*Tube 1* (fig. 1). The gelatin was dissolved in distilled water by heating, and cooled to 18°. The reaction of the solution at this temperature was  $P_H = 6.09$ . The solution set on standing to an opaque white gel. The contraction of the opaque gel was visible in twenty-four hours. The photograph reproduced was taken after standing for fourteen days. The reaction of the expressed clear fluid was taken again and was found to be  $P_H = 4.42$ . The fluid gave a faint positive biuret reaction.

A portion of the clot was stained with methylene blue and examined under a  $\frac{1}{12}$ " oil immersion lens, with compensating eye-piece  $\times 12$ .

The crushed gelatin clot appeared to consist of numerous small spheres similar to the spherites described by Bradford [1920]. They were however very uniform in size, being about  $0.5\mu$  in diameter. No formation of filaments was detected. This, however, may have been due to the crushing of the clot under the cover-glass.

*Tube 2* (fig. 2). The system contained 1 % of gelatin and HCl to a concentration of 0.005 *N*. The reaction of the freshly prepared solution at 18° was  $P_H = 3.43$ . The solution set after two hours to a clear colourless gel, which became faintly opalescent after forty-eight hours' standing. No further change occurred during the following fortnight, *i.e.* till the end of the experiment.

*Tube 3* (fig. 3). The system consisted of 1 % of gelatin in 0.01 *N* HCl. The reaction of the freshly prepared solution after cooling to 18° was  $P_H = 2.53$ . The solution set to a glassy clear gel which showed no changes in transparency on standing.

These observations demonstrate that gelatin gels are unstable at the iso-electric point. Small quantities of caustic soda added to the system have the same stabilising influence as small quantities of hydrochloric acid. The formation of stable gels therefore is only possible in the presence of electrolytes.

#### REFERENCES.

- Bradford (1920). *Biochem. J.* **14**, 91. .  
Clark and Lubs (1917). *J. Bacteriol.* **2**, 1.  
Dheré (1913). *J. Physiol. Path. Gén.* **13**, 158, 167.  
Dheré and Gorgolewski (1910). *Compt. Rend.* **150**, 934; *J. Physiol. Path. Gén.* **12**, 646  
Jordan Lloyd (1920). *Biochem. J.* **14**, 147.

## LII. AN EXPERIMENTAL STUDY OF THE EFFECT OF CERTAIN ORGANIC AND INORGANIC SUBSTANCES ON THE BREAD-MAKING PROPERTIES OF FLOUR AND ON THE FERMENTATION OF YEAST.

BY HELEN MASTERS AND MARGERY MAUGHAN.

*Household and Social Science Department, King's College for Women.*

*(Received July 2nd, 1920.)*

DURING the war, when it was necessary to conserve the wheat supply of the country, the proportion of the flour extracted from the grain was greater than in normal times, and other cereals were being milled and added to the wheat.

The loaf made from such flours was small in size and close in texture, and complaints were also made that it showed a tendency to become sour. Although numerous suggestions for improving the breadmaking properties of the flour were forthcoming, the problem thus raised was an extremely complex one.

The factors on which the breadmaking properties of a flour depend are by no means easy to define and exact data on this subject are difficult to obtain.

It was hoped that these investigations of the effect of certain organic and inorganic substances on the breadmaking properties of flour might yield results which would be of some practical assistance in arriving at a satisfactory solution of the problem. At first the method of investigation adopted was that of making baking experiments. Later, in order to check the behaviour of the various ingredients used in breadmaking, and to obtain some indication of the changes which occur during the different stages of the process, measurements were made of the amount of fermentation produced by the yeast in the absence of flour and also of the expansion of the dough whilst "proving" or "rising" prior to baking.

### BAKING EXPERIMENTS.

Loaves were cooked in batches of four, each loaf containing the same weight of the necessary ingredients, *i.e.* flour, raising agent, salt and water, to which might be added different substances which varied with the nature of the experiment.

In order to minimise experimental errors which are almost inevitable in work of this character, one loaf of each batch was prepared as a control and contained the same ingredients in each experiment. In all cases where it was possible, the experiments were repeated two or three times, and an average of the results taken.

*Ingredients of doughs raised with yeast.*

1. 200 grams of flour. The flours used in these experiments were kindly supplied by Messrs J. and R. Robinson, Deptford Bridge Mills, Greenwich, and their composition was as follows:

I.	Flour containing 80 % of the wheat grain.			
II.	" " 85 % " " "	and barley.		
III.	" " 75 % " " "	which was a weak English wheat.		
IV.	" " 90 % " " "	and rice, maize, barley and rye.		
V.	" " 85 % " " "	and rice, maize, barley and rye.		
VI.	" " 75 % " " "	which was a weak English wheat.		
VII.	" " 83 % " " "	and barley, rice and rye.		
VIII.	" " 85.9 % " " "	and maize, rice and barley.		
IX.	" " 85.9 % " " "	and maize, rice and barley.		
X.	" " 86 % " " "	and barley, rice and rye.		

In addition some white flour similar to the pre-war flour was obtained by a special permit.

2. 5 grams of yeast supplied by a wholesale firm. The age of the samples was known.

3. 2 grams of sugar (or 10 cc. of a 20 % solution).

4. 2 grams of salt.

5. Liquid for mixing. The volume of liquid required for mixing the dough to the necessary consistency varied from 95–120 cc. according to the nature of the flour. The liquid was chiefly tap water, but the total volume included any other liquid, *e.g.* sugar solution, serum, etc., added for the purpose of the experiment.

*Method of making bread raised with yeast.*

The yeast was mixed to a thin cream with the sugar solution, and added to the flour and salt which had previously been mixed and raised to a temperature of 25°. The rest of the liquid (at 25°) was then added to the dough thus formed and kneaded for five minutes. Longer time could not be allowed as the dough cooled rapidly during this process.

*First rise.* The dough was allowed to rise in an incubator kept at 25° until the surface skin cracked. The time required varied with the nature of the flour. A dough made from a "strong flour" (I) required one hour and that from a "weak flour" (II) forty-five minutes. The addition of cereals other than wheat reduced the time necessary for rising.

The dough was then kneaded and put into a greased tin which had previously been weighed.

The tins were cylindrical so that the approximate volume of the loaf could be readily calculated. The dough was weighed in the tins. This weight served as a check on the manipulation of the mixing.

*Second rise.* The dough was put back into the incubator and allowed to remain there until it had about doubled its size. The time necessary for this second rise varied with the flour in the same manner as for the first rise.

*Baking of the dough.* The dough was put into a hot oven, the temperature raised for the first ten minutes and then decreased at intervals. At first a gas oven was used, but later an electric oven was substituted so that the temperature could be more easily controlled. In some of the later work the second rise was omitted, *i.e.* the dough was mixed, put into the tin, allowed to rise for the necessary time and baked. The same range of results was obtained by this latter method as by the longer process, and any possible error introduced by further handling of the dough was eliminated. Some bakings involving the longer process were carried out in all cases in order to check the results obtained by the shorter method.

*Weight and volume of the loaf.* When cold the loaf was weighed, and after keeping twenty-four hours, it was cut through the centre and the greatest and least heights measured.

The volume of the loaf was calculated from the mean height and the area of the base.

The volume of the control was taken as 1000, and the volumes of the other loaves calculated relatively to this. By this means a comparison between experiments carried out at different times could be obtained.

#### *Doughs raised by a chemical raising agent.*

The ingredients of these doughs were as follows:

1. 200 grams of flour.
2. 1 gram of sodium bicarbonate and 2 grams of potassium bitartrate.
3. 2 grams of salt.
4. Sufficient liquid to make a dough.

The raising agent was mixed with the flour and salt, and the mixture passed through a sieve. After adding the liquid the dough was kneaded, placed in a weighed tin, weighed and baked as described above.

#### *The addition of lime-water in breadmaking.*

Bread made from "Government controlled" flour showed a tendency to become sour, which was not observed in the case of pre-war white flour. Jago [1911] states that bread made with lime-water is quite free from sourness.

In order to investigate the power of lime-water to correct the tendency to sourness, baking experiments were carried out with doughs mixed with lime-water, some being raised with yeast and others with a chemical raising agent.

Experiments were made with whole-meal flour, Government controlled flour and white flour.

The loaves made with lime-water were in all cases smaller than their controls. The doughs mixed with lime-water appeared drier than the controls, and whilst rising formed a thick skin.

Some determinations were made of the acidity and also of the moisture content of the lime-water loaves and of their controls, determinations being made both on the freshly baked bread and on bread which had been kept for several days.

The results showed that whilst the lime-water neutralised any acidity in the flour it did not prevent the production of acid.

There was no appreciable difference in the rate of loss of moisture on keeping between the lime-water loaves and the controls. The acidity of the bread decreased as it became drier.

Bywaters [1918] states that the use of lime-water has been largely discontinued as the action of the yeast is thereby delayed. Lapigne and Legendre [1917] and also Le Roy [1917] make reference to this subject.

Observations based on practical experience show that bread which is made on a large scale is more liable to become sour than bread which is made in smaller quantities.

This difference it was thought might be due to the fact that when bread is made on a large scale a longer time is usually allowed for the fermentation or "proving" process than is allowed in a small scale baking.

Some determinations which were made of the acidity of the dough during fermentation showed that the acidity increased slightly as fermentation proceeded. In a typical case the acidity expressed as lactic acid was 0.46 % on the freshly prepared dough and increased to 0.66 % after five hours. This shows that the acidity of the dough is dependent to some extent on the length of time allowed for proving.

It thus appears there is little to be gained by the use of lime-water in breadmaking except, perhaps, in the case of a very acid flour or if the process of fermentation is prolonged.

#### *Experiments on the physical properties of gluten.*

A study of the technology of breadmaking indicates that the size of loaf is partially dependent on the physical properties of the gluten and that to produce a good loaf the gluten must be moderately elastic.

Experiments were made to see if the elasticity of the gluten could be increased and a larger loaf obtained by stretching the dough by some physical process prior to the addition of the yeast. The dough was placed in a vacuum desiccator and the pressure alternately reduced and then increased again to atmospheric pressure three times.

Some expansion of the dough was produced by this process, but after baking the loaf was not appreciably larger than the control.

## THE ADDITION OF OX-SERUM IN BREADMAKING.

At the suggestion of Professor Leonard Hill some experiments were made in which a proportion of ox-serum was added to the liquid used in mixing the dough.

The ox-serum used was separated at the Metropolitan Cattle Market, Islington, and obtained forty-eight hours after the animal had been slaughtered. In cold weather it was possible to keep the serum for three days before it became unfit for use, and experiments were carried out with the serum on the first, second and third day.

Baking experiments were made to ascertain the effect of the serum on the size of the loaf and to find how its action varied with the amount of serum added and the length of time the sample had been kept. The serum was used in proportions varying from 1 to 7 %, expressed by weight in relation to the flour. The results obtained on the addition of fresh serum (first day) in concentrations of 1-3 % with four different flours are shown in Table I.

Table I.

Concentration of serum	Flour	Weight of loaf in g.	Height of loaf in cm.			Volume of loaf	
			Maximum	Minimum	Mean	Calculated	Comparative
First day 1 %	I	281.0	11.25	7.25	9.25	726.8	1156
" " 2 %		282.5	11.25	7.0	9.12	716.9	1124
" " 3 %		279.5	10.25	6.25	8.25	643.2	1023
Control		280.9	9.75	6.25	8.0	628.6	1000
First day 1 %	II	269.9	9.5	7.0	8.25	648.2	1048
" " 3 %		274.9	9.75	6.25	8.0	628.6	1016
Control		274.9	9.5	6.25	7.87	618.7	1000
First day 1 %	IV	290.6	9.0	6.5	7.75	608.9	1051
" " 2 %		287.6	9.0	6.5	7.75	608.9	1051
" " 3 %		292.4	8.75	6.5	7.62	599.1	1034
Control		290.2	8.5	6.25	7.37	579.4	1000
First day 1 %	III	251.0	8.5	6.5	7.75	589.3	1053
Control		246.0	8.25	6.0	7.12	559.8	1000

A marked increase in the volume of the loaf resulted from the addition of 1 % fresh serum.

With concentrations greater than 1 %, the increase on the whole was less marked, although with flour IV the same result was obtained with 2 % as with 1 % of serum. The increase obtained varied with the flour, the maximum increase being 15.6 % with flour I.

It should be noted that the addition of the serum affects only the volume, not the weight of the loaf; the slight differences in weight observed are to be attributed to experimental errors.

Further experiments showed that the increase obtained with 1 % of serum was greater than that obtained with concentrations less than 1 % and also that on the addition of 1 % serum to pre-war white flour a decrease of 1.5 % in the volume of the loaf was obtained.

When the serum was used on the second and third days, the loaf obtained with the addition of 1 % serum was considerably smaller than on the first day. The size of the loaf increased with the proportion of serum used, but even when 7 % of serum was added the increase in volume was not so great as that obtained with the addition of 1 % of serum on the first day.

The maximum increase on the different days obtained with flour I were as follows:

1st day	an increase of 15.6 %	with 1 % of serum.
2nd	„	„ 12.5 % „ 7 % „
3rd	„	„ 12.5 % „ 7 % „

It thus appears that the active principle in serum, which produces an increase in the size of the loaf, is destroyed gradually on keeping, and although the effect of this destruction could to some extent be rectified by increasing the amount of serum used, the maximum effect obtained with the fresh serum could not be produced on subsequent days.

*The effect of heat and preservatives on the properties of serum.*

Some experiments made with serum which had been kept at a temperature of 50–60° for an hour and a half, showed that this treatment had practically no effect on the action of the serum.

Samples of serum, which were heated on arrival and used for baking experiments on the following days, gave a typical first day action on the second day, *i.e.* the maximum increase was obtained with the addition of 1 % of serum, but the increase in the volume of the loaf was not so great as on the first day. Not until the fourth day did the serum heated on the first day give a typical second day reaction, *i.e.* the volume of the loaf increased with the concentration of the serum. Serum heated in this manner did not keep appreciably longer than raw serum. Thus the active principle in the serum is not destroyed by being heated to 50–60°, but it is to some extent protected although not preserved by this treatment.

Serum heated to 100° coagulated and on standing a pale coloured liquid separated out which could be expressed from the solid mass. Baking experiments made with this boiled serum showed that it had very little effect in increasing the volume of the loaf and in some cases the loaf obtained was smaller than the control. The liquid portion was more effective than the solid, but in neither case was a marked increase in volume obtained.

Since the power of the serum to produce an increase in the size of the loaf deteriorates rapidly on keeping, and the maximum effect can only be obtained with fresh serum, some experiments were made to see if it were possible to preserve the serum either by some process of desiccation at low temperatures, or by the addition of chemical preservatives.

An attempt was made to evaporate the serum under reduced pressure at the room temperature, but the process was found to be too slow to be of any practical use.

The protein material in the serum was precipitated by the addition of a saturated solution of ammonium sulphate. The precipitate was filtered and then dried in a vacuum desiccator at the room temperature.

This dried preparation when added to the flour gave similar results to the fresh serum, and did not deteriorate on keeping for a considerable time. Unfortunately the serum obtainable at this stage of the investigations was poor in quality and results yielded were not comparable with those obtained earlier.

The addition of small quantities of formaldehyde (1 part of 40 % formaldehyde to 1000–2000 parts of serum) preserved the serum for several days and in baking on a small scale gave a loaf as large as that obtained with fresh serum; but in one or two experiments which were made on a large scale (see baking on a large scale) a slight decrease was observed.

This may be due to some inhibitory action of the formaldehyde on the yeast owing to the longer time allowed for rising on the large scale, but further experimental work is needed before any definite statement on this point can be made.

Boric acid was found to be ineffective in preserving serum.

#### *Possible substitutes for serum.*

Defibrinated blood was tried as a substitute for serum; its action was found to be similar to that of second day serum but the loaf was dark in colour.

Solutions of the following substances were also used: gelatin, egg and blood albumin, caseinogen in lime-water.

With caseinogen in lime-water the loaf obtained was smaller than the control. (Comp. the addition of lime-water.)

Gelatin gave an increase of 10 % with flour I, but with the other flours the increase was only from 1–2 %.

A slight increase only (1–2 %) was obtained with albumin and it was not considered that any of these substances could be regarded as a satisfactory substitute for serum.

#### *The addition of serum to bread raised with a chemical raising agent.*

Serum appeared to have no action on doughs raised with a chemical raising agent; the loaves obtained were the same size as the controls.

#### *Baking on a large scale.*

In order to decide whether the action of the serum would be the same if the bread were made in larger quantities, one or two experiments were made in which doughs were prepared with 10 lb. of flour, which, after mixing, were divided into eight loaves.

The relative increase in the size of the loaf was less than in the small scale bakings, but the serum was at this time very poor in quality and the experiments were not continued.

Further work is needed to confirm these results and to find whether the optimum concentration of the serum is the same on the large as on the small scale.

The action of serum in the presence of phosphate and of potato is dealt with later.

#### THE ADDITION OF PHOSPHATES IN BREADMAKING.

Jago [1911] states that the addition of mineral phosphates to the flour results in a marked improvement of the loaf, but that the addition of such salts is illegal.

Harden [1914] has shown that the addition of phosphates increases the total fermentation produced by a given volume of yeast juice.

Baking experiments were made using monosodium dihydrogen phosphate,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , and monohydrogen disodium phosphate,  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , and also acid calcium phosphate,  $\text{CaH}_4(\text{PO}_4)_2$ .

The quantities of phosphate used varied from 0.25–3.0 % of the flour.

The average increase in the volume of the loaf obtained on the addition of 1 % of each of these salts to flour was as follows:

Monosodium hydrogen phosphate	+3.5 %
Disodium           "           "	+2.6 %
Acid calcium phosphate	-0.75 %

Although the calcium salt produced a slight decrease in the size of the loaf, it gave a bread which was whiter in colour than the control or that containing the other phosphates. Further experiments showed that the maximum increase in the size of the loaf was obtained on the addition of 0.5 % of monosodium hydrogen phosphate, and that its effect was more pronounced with "weak" than with "strong" flours.

Thus an increase of 7.4 % was obtained with flour III and an increase of only 1.6 % with flour IV.

With disodium hydrogen phosphate the size of the loaf increased with increasing concentrations of the salt up to 3 %, and it was not considered advisable to exceed this amount. The maximum increase obtained with the latter salt was less than that obtained with the monosodium salt.

Judging by the experimental results obtained, there appeared to be no advantage in using a mixture of the salts in the proportion of five molecules of the disodium salt to one of the monosodium salt, although such a mixture was found by Harden [1914] to produce the maximum effect on the fermentation of yeast juice, or in using a mixture of the two salts in the proportions of the concentrations found to produce the maximum effect, *i.e.* 0.5 % of monosodium hydrogen phosphate, and 3 % of disodium hydrogen phosphate.

#### *The addition of phosphates to doughs raised with a chemical raising agent.*

The addition of phosphates to doughs raised with a chemical raising agent did not produce any increase in the size of the loaf.

*The addition of phosphate and serum in breadmaking.*

A comparison of the action of phosphate with that of serum showed that in the case of a weak flour a greater increase in the size of the loaf was obtained by the addition of phosphate than by the addition of serum.

With flour III the addition of 0.5 % of monosodium dihydrogen phosphate produced an increase in the size of the loaf of 7.4 %, and the addition of 1 % of fresh serum an increase of 5.3 %, but with some of the "stronger flours," *e.g.* V and VI, the results were in the reverse order.

If both phosphate and serum were added the increase in the volume of the loaf was greater than that obtained with phosphate alone, but the relative increase in the size of the loaf as compared with serum alone depended on the nature of the flour, *i.e.* the serum and phosphate in most cases produced a larger loaf than the serum alone, but in some cases a smaller one.

## THE ADDITION OF POTATO IN BREADMAKING.

In order to economise wheat flour and other cereals, the addition of potato to the flour used for breadmaking has been extensively advocated.

As the production of potato flour involves special plant for drying the potatoes, it was thought that it would be more convenient, providing that equally good results could be obtained, to use either raw or cooked potato instead of potato flour.

The potato was substituted for a portion of the flour, due allowance being made with raw and cooked potato for the water-content of the material, *e.g.* 70 g. of raw potato were taken as equivalent to 19 g. of flour and a mixture of 181 g. of wheat flour and 70 g. of raw potato as equivalent to the 200 g. of flour used in the other baking experiments.

The average results obtained were as follows:

Raw potato, increase in the volume of the loaf	1.2 %
Boiled " " " " " "	4.2 %
Potato flour " " " " "	1.25 %

Steamed potato had the same effect as boiled potato.

Baking experiments were also made with potato which had been boiled twice as it has been suggested that all enzymes are destroyed by this treatment. This potato had the same effect as ordinary boiled potato.

Experiments made to determine the best proportions of potato to use showed that the size of the loaf decreased with increasing proportions of raw potato, but increased with increasing proportions of boiled potato.

Thus when the potato and flour were in the proportion of 1 : 2 the increase in the volume of the loaf was 3.6 % with raw potato and 10.7 % with boiled potato, and when the potato and flour were in the proportion of 2 : 1 the increase was 1.8 % with raw potato and 14.5 % with boiled potato.

In the latter case, however, the mixture was quite unsuitable for bread-making as the upper part of the cooked dough was hollow and the lower part

had the appearance and consistency of cooked batter. On the average the best results were obtained with a mixture of wheat flour and potato, containing boiled potato equivalent to from 25-33 % of the total weight.

The volume of the loaf decreased with increasing proportions of potato flour, but the flour being dry it was possible by this means to introduce a larger proportion of potato and still obtain a loaf which was satisfactory as regards texture and appearance, although smaller than the control. This bread, however, became stale and dry very rapidly. It was observed that the addition of potato made the dough less spongy. Owing to its high water-content raw potato, which was grated before adding to the dough, tended to make the dough moist and considerably less water was required for mixing than was required by the control. The doughs containing raw potato were greyish in colour owing to changes due to enzyme action which take place when raw potato is exposed to the air and this made the bread a bad colour.

In mixing doughs containing boiled potato the amount of water used was in most cases the same as for the control.

#### *The addition of potato and serum in breadmaking.*

Baking experiments made with the addition of serum to bread containing cooked potato showed that the maximum effect was produced, as in the case of flour alone with the addition of 1 % of fresh serum.

The increase in volume of the loaf obtained with fresh serum in the presence of potato was slightly less than when the potato was omitted, the average difference in the results being about 1 %. Further, when the serum was used on the second day the addition of 2 % of serum gave the maximum increase and the same result was obtained on the third day.

During a spell of cold weather it was possible to bake with one sample of serum on the fourth day. The volume of loaves containing potato and serum then increased with the amount of serum added, *i.e.* the action of the serum was then the same as that observed with second day serum when flour alone was used.

#### *The addition of potato and malt extract in breadmaking.*

The addition of malt extract to dough containing potato did not increase the size of the loaf, and if the proportion of potato to flour was more than 1 : 2 the loaf became smaller on the addition of malt.

It was previously noted that with boiled potato a larger loaf was obtained when the proportion of potato was increased, but on the addition of malt and potato the size of the loaf decreased as the proportion of potato increased.

Further, it was found that the amount of boiled potato which could be introduced into the bread was less in the presence of malt, as the bread showed a tendency to become extremely heavy.

EXPERIMENTS ON THE FERMENTATION OF YEAST AND MEASUREMENTS  
OF THE EXPANSION OF THE DOUGH DURING RISING.

In order to check, as far as possible, the behaviour of the various substances used in making the bread, and to obtain some indication of the changes which occur at the different stages of the process, measurements were made of the amount of fermentation produced by the yeast with the various ingredients in the absence of flour and also of the expansion of the dough prior to baking.

*Experiments on the fermentation of yeast.*

The fermentation experiments were carried out in 100 cc. flasks, each flask being attached to a nitrometer, filled with brine, in which the carbon dioxide evolved could be collected and measured. The flasks were placed in a thermostat kept at a temperature of 25°.

The weights of yeast, sugar, and any additional substance were exactly one-tenth of the weights used in the baking experiments, and, except where otherwise stated, the total volume of liquid was 10 cc.

The experiments were carried out in batches of four. One of each batch contained yeast, sugar, and water only, and was used as a control, and each experiment was done in duplicate.

The mixture to be fermented was placed in a flask and the volume of carbon dioxide evolved read off at regular intervals.

*Experiments on the expansion of dough.*

The proportions of flour, yeast, etc., used in these experiments were exactly half those used in the baking experiments; it was thus possible to get a direct comparison between the baking experiments, the expansion of the dough, and the fermentation experiments.

The dough was introduced into wide-necked glass bottles fitted with rubber bungs through which delivery tubes were passed. The volume of the air displaced by the rising of the dough was measured by the displacement of water. The water was kept alkaline by the addition of sodium hydroxide, so that any carbon dioxide which escaped through the dough should be absorbed.

It was felt that the experiments would be more complete if some arrangement could be devised by which the expansion of the dough during baking as well as during the fermentation process could be measured.

An apparatus for this purpose was constructed of tinned copper, but although several different types of washer have been tried it has so far not been possible to make the screw top absolutely air-tight at the baking temperature, and reliable results have not yet been obtained.

*Results of experiments on the fermentation of the yeast and the expansion of the dough.*

Experiments were first made to compare the amount of carbon dioxide produced by the yeast, sugar, and water only with the expansion of the dough during fermentation.

The carbon dioxide evolved by the fermentation of the yeast was measured at the end of one hour and at the end of four hours.

It was found that the volume of the gas produced varied with the age of the yeast, and the maximum amount of fermentation was produced, not with fresh yeast, but with yeast that was two to three days old.

The amount of fermentation produced varied considerably with the different samples of yeast; the worst sample produced 2.3 cc. during the first hour and a good sample 18.4 cc. in the same time.

These results were confirmed by measurements of the expansion of the dough (see Table II).

Table II.

*Expansion of the dough during fermentation.*

Age of yeast. Days	Increase in volume of the dough.			
	20 mins. cc.	40 mins. cc.	60 mins. cc.	80 mins. cc.
1	10.0	52.0	121	—
2	—	62.0	136.6	230
3	15.0	61.5	124	202
4	12.0	54.0	123	210

Fermentation experiments, measurements of the expansion of the dough and baking experiments were carried out on the same day with the same sample of yeast.

The fermentation produced by the yeast and the expansion of the dough were measured as described above, and the results which would be obtained with the quantities used in the baking experiments were then calculated. A dough was also prepared and baked without the addition of the yeast to act as a control, and it was assumed that the volume of the loaf less the volume of this cooked dough (422.3 cc., see Table III) and less the expansion of dough during fermentation, might be taken as a measure of the expansion on baking.

Thus in Table III the figures in column VI are obtained by subtracting the volume of the baked control (422.3 cc.) and the expansion of the dough (column III) from the volume of the loaf (column V).

As might be expected the amount of fermentation produced in the dough is greater than with the yeast alone, this probably being due to the presence of substances in the flour, *e.g.* gluten, etc., which act as yeast foods.

Further there is a maximum increase on baking which is obtained (under the conditions of the experiment) after the dough has risen for 40 minutes.

Table III.

I	II	III	IV	V	VI	VII
Age of yeast Days	Time of fermentation Minutes	Fermentation of yeast (5 g.) cc.	Expansion of dough during fermentation (5 g. yeast) cc.	Volume of loaf (5 g. yeast) cc.	Expansion on baking cc.	Ratio of fermentation to expansion of dough (III : IV)
—	nil	—	—	422.3	—	—
1	20	6	10	510.7	78.4	1 : 1.7
	40	28	52	579.5	105.2	1 : 1.9
	60	64	121	628.6	85.3	1 : 1.9
2	nil	—	—	422.3	—	—
	40	31	62	599.1	114.8	1 : 2
	60	72	136	628.6	70.3	1 : 1.9
	80	115	230	658.1	5.8	1 : 2
3	20	8	15	500.9	63.6	1 : 1.9
	40	31	61	569.1	85.8	1 : 1.9
	60	71	124	617.1	70.8	1 : 1.8
4	20	6	12	520.5	86.2	1 : 2
	40	31	54	579.5	103.2	1 : 1.7
	60	68	123	628.6	83.3	1 : 1.8
	80	110	210	658.0	25.7	1 : 1.9

These results show that the ratio of the fermentation of the yeast to the expansion of the dough is fairly constant.

This fact may be of practical significance, for if the maximum increase on baking could be ensured the loaf would be increased in volume by a physical process instead of by further fermentation which utilises and wastes some of the ingredients of the dough.

#### *The action of serum and albumin during fermentation.*

At the time when these fermentation experiments were made, satisfactory samples of fresh serum were not obtainable, but some measurements were made of the fermentation produced by yeast and the expansion of the dough in the presence of serum which had been "salted out" of solution and dried in the manner previously described. This serum contained a small amount of ammonium sulphate, but control experiments made with this salt showed that in small quantities it was without action on the yeast.

The serum used had been kept for twelve months. The addition of this serum increased the volume of carbon dioxide produced during fermentation and also the amount of expansion in the dough.

Thus the increase in volume of the loaf observed on the addition of serum must to some extent at least be attributed to the action of the serum as a yeast stimulant.

Some measurements made with albumin showed that it had some stimulating action on the yeast, but that this action was less marked in the dough.

*The action of phosphates during fermentation.*

Fermentation experiments were made with yeast, sugar and phosphates in the proportions found by the baking experiments to give the best results.

In the first series of experiments the phosphate appeared to exert an inhibitory action on the yeast, as the volume of carbon dioxide evolved was less than from the control.

This it was thought might be due to the fact that although the phosphate and yeast were in the same proportions as in the baking experiments, they were used in a more concentrated form.

Harden [1914] found that when phosphate is added to fermenting yeast juice and sugar, the effect varies with the concentration and that with a mixture of 25 cc. of yeast juice and 4.5 g. of sugar, 5–10 cc. of 0.3 molar solution of phosphate in a total volume of 45 cc. produced the maximum effect.

Fermentation experiments were therefore made using 0.5 g. of yeast, 0.125 g. of cane sugar with from 2 to 40 cc. of 0.3 molar solution of monosodium dihydrogen phosphate in a total volume of 45 cc. Table IV shows the results of a typical experiment.

Table IV.

Volume of 0.3 molar solution of phosphate cc.	Percentage increase in fermentation of yeast on the addition of phosphate.			
	1 hour	2 hours	3 hours	4 hours
2	0	3.6	4.3	1.5
5	0	14.3	14.9	11.0
10	8.3	17.9	17.0	13.2
20	33.3	44.9	40.4	29.4
30	41.7	50.0	44.7	35.3
40	41.7	46.4	40.4	29.4

These results show that the maximum increase was obtained with 30 cc. of the 0.3 molar solution (= 0.12 g. of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ), *i.e.* with 0.12 g. of phosphate and 0.5 g. of yeast in 45 cc., which corresponds to 1.2 g. of phosphate and 5 g. of yeast in 450 cc.

The optimum increase on baking was obtained with 1 g. of phosphate and 5 g. of yeast in about 330 g. of dough, so that the results of fermentation experiments confirm those obtained in the baking experiments.

*The action of potato during fermentation.*

In order to study the effect of the addition of potato on the fermentation of yeast, experiments were made with the following preparations of potato:

I. *Raw Potato*: (a) peeled potato, (b) peeled potato washed, (c) juice expressed from potato, (d) potato peel, (e) washed potato peel, (f) juice extracted from potato peel.

The solid matter was finely grated before use.

II. *Cooked Potato*: (a) boiled potato (mashed), (b) steamed potato, (c) boiled potato (washed), (d) potato which had been boiled twice. (These potatoes were boiled for 20 minutes, left over night and boiled again until cooked.)

### III. *Potato Flour*.

In the first experiments 4 g. of potato were used with 0.5 g. of yeast, but later it was found that a more marked effect was obtained when the same weight of yeast was used with 10 g. of potato.

Table V.

Condition of potato	Weight of potato g.	Average percentage increase in the fermentation of yeast with sugar and water produced by addition of potato				
		1 hour	2 hours	3 hours	4 hours	5 hours
Raw potato peeled ... ..	4	68.9	—	—	31.2	—
„ „ „ and washed	4	48.2	—	—	8.9	—
Potato peel ... ..	4	78.1	—	—	29.7	—
„ „ washed ... ..	4	48.8	—	—	12.0	—
Juice of potato ... ..	4	-29.4	—	—	12.2	—
Juice from potato peel ...	4	-59.8	—	—	-25.8	—
Raw potato ... ..	10	92	84	69	64	64
Juice of potato ... ..	10	-28.7	5.5	22	28	—
Boiled potato ... ..	10	82	61	35	21	21
Steamed potato ... ..	10	93	84	60	35	33
Potato flour ... ..	equivalent to 10 g. of potato (i.e. 2 g.)	34	25	20	—	—
Juice of potato filtered and boiled ... ..	10	16	36	54	64	77
Juice of potato filtered, boiled and filtered ... ..	10	9	24	39	48	58

These results show that raw potato produced a marked increase in fermentation, but that if the potato were washed until the liquid filtering through was clear this increase was appreciably diminished.

The juice extracted from the potato inhibited fermentation at first, but later produced a slight increase.

The increase produced by raw potato was greater than the sum of those produced by the washed potato and the juice of the potato.

Potato peel increased the fermentation of the yeast to a greater extent than raw potato. This might be due to the higher water-content of the former, i.e. the total solid matter added was greater in the case of the peel.

The juice extracted from the peel had a more marked inhibitory action than the juice from the potato, and the combined effect of the washed peel and the juice of the peel acting separately was less than that of the peel.

Boiled potato did not increase the fermentation to the same extent as raw potato, but steamed potato had the same effect as raw potato for the first two hours and its stimulating power then became less marked.

This difference in the behaviour of boiled and steamed potato is doubtless due to loss of material in the cooking water during boiling and it is possible

that if the potato were boiled in a small volume of water and the cooking water, as well as the potato, used this difference would be eliminated.

The stimulating action of the potato flour was even less than that of washed potato.

When the juice extracted from the potato was filtered and the filtrate boiled, the filtrate became slightly turbid, this being due no doubt to the cooking of minute particles of starch which had passed through the filter. On filtering this boiled liquid a clear green filtrate was obtained.

A comparison of the results obtained with this boiled juice with those obtained with the raw juice indicates that the boiling to some extent destroyed the principle of the raw juice which exerted an inhibitory action on the yeast. Juice which was only filtered once gave a better result than that which was filtered again after boiling.

Observations made on the colour of this boiled juice showed that when grated potato was exposed to the air it became first pink and then rapidly passed through shades of red until it was almost black. When the juice extracted from this potato was boiled and filtered, the filtrate was always green in colour and the depth of colour increased with the length of time the potato was exposed to the air before the juice was extracted.

It has previously been observed that the yeast showed an optimum activity on the second or third day when fermenting with sugar and water, but in the presence of potato it was found that the yeast was more active on the first day.

The results given in Table IV indicate that the increase in fermentation produced by the addition of potato gradually diminished as fermentation proceeded. This effect, which was also observed in the case of other substances which acted as yeast stimulants, may be due to the fact that the apparatus used was such that an equal but limited supply of oxygen was available in each experiment. The substance which stimulated the yeast may thus have been unable to exert its full action towards the end of the experiment owing to the partial exhaustion of the oxygen.

Measurements made of the expansion of dough containing potato showed considerable variations.

Raw potato produced an average decrease in volume of dough as compared with the control of 10.8 %, whilst boiled potato gave a decrease of 5.7 %. The juice from raw potato inhibited the expansion of the dough and washed potato showed the same effect as raw potato but in a greater degree. Steamed potato decreased the expansion of the dough but to a less extent than boiled potato.

The fermentation experiments as a whole form a valuable check on the baking experiments and also demonstrate the necessity of studying the changes involved during the different stages and not the final stage only if any reliable information as to the processes on which breadmaking is based are to be obtained.

Thus, although the fermentation experiments are in general in agreement with the baking experiments this is not always the case. For example, raw potato stimulated the action of yeast to a greater extent than boiled potato, but the loaf obtained with boiled potato was larger than that obtained with raw potato; also the doughs containing potato were smaller than the controls, the difference being less for cooked than for raw potato, but the loaves containing potato were larger than the controls.

#### SUMMARY.

The amount of fermentation produced by yeast with sugar and water and in the dough varied with the age of the yeast. The maximum effect was obtained on the second or third day after the yeast had been compressed.

The ratio of the fermentation of the yeast with sugar and water to the fermentation in the dough was approximately constant.

The maximum increase in volume on baking was obtained, under the conditions of the experiments, when the dough was allowed to rise for forty minutes, but this factor may vary with the proportions of the different ingredients used. The acidity of the dough increased with the time of fermentation and the acidity of the bread decreased with the moisture content.

The addition of lime-water to bread does not appear to prevent the production of acid, but it neutralises any acid formed; in the presence of lime-water a smaller loaf was obtained.

On the addition of fresh ox-serum the maximum increase in the size of the loaf was produced with 1 part of serum to 100 parts of flour. The power of the serum to increase the size of the loaf diminished rapidly on keeping, but was not destroyed by heating to a temperature of 50–60°.

With phosphates the best results were obtained when monosodium dihydrogen phosphate was added in the proportion of 1 part of phosphate to 200 parts of flour. Neither phosphate nor serum produced any increase in the size of the loaf when the bread was raised with a chemical raising agent.

Raw potato, cooked potato and potato flour, when substituted for an equivalent amount of wheat flour, diminished the expansion of the dough on fermentation, but both raw and cooked potato gave a larger loaf, the increase being greater with cooked than with raw potato.

We wish to express our thanks to Professor C. K. Tinkler, Professor Leonard Hill and Dr Benjamin Moore for suggestions they have made and for the interest they have taken in the progress of the work.

This work has been made possible by the receipt of a research grant by one of us (M. M.) from the Department of Scientific and Industrial Research and for this also we desire to express our thanks.

#### REFERENCES.

- Bywaters (1918). *Report of the Society of Chemical Industry on the progress of Applied Chemistry*, 3.  
Harden (1914). *Alcoholic Fermentation. Monographs on Biochemistry*. Longmans, Green & Co.  
Jago (1911). *The Technology of Breadmaking*. Simpkin Marshall, Hamilton Kent & Co.  
Lapicque and Legendre (1917). *Compt. Rend.* 165, 316.  
Le Roy (1917). *Journal of Society of Chemical Industry*, 1917.

# LIII. AMMONIA EXCRETION, AMINO-ACID EXCRETION AND THE ALKALINE TIDE IN SINGAPORE.

BY JAMES ARGYLL CAMPBELL.

*From the Medical School, Singapore.*

*(Received July 12th, 1920.)*

## INTRODUCTION.

In a paper on nitrogen partition in the urine of the races in Singapore [Campbell, 1919] it was noted that on many occasions the amount of ammonia excreted by apparently normal individuals was high when compared with the European standards. It was suggested that the climatic conditions—hot, moist and still air—in Singapore might be responsible for this acidosis.

The observations recorded in the present paper were made in the first place to determine the hourly variations in the ammonia excretion and their significance, which might explain the acidosis. The excretion of amino-acids and the reaction of the urine were also considered.

## AMMONIA EXCRETION.

The amounts of ammonia excreted hourly by thirty subjects of various races, were estimated by Malfatti's method. In most cases daily amounts, for a week or longer, were also estimated using both Malfatti's and Folin's methods. The former method estimates the ammonia of any amino-acids in the urine in addition. Folin's method was not used in my previous paper [1919], but the results obtained with it corresponded with the results recorded by Malfatti's method, as will be seen from Table I, which shows typical examples of average figures from individuals on their usual diets, mixed or vegetarian, similar to those described in the previous paper. In some cases the average figures for ammonia excreted daily were normal; in others, *e.g.* Chinese II and Tamil IV, the figures were high, due allowance being made for the diets and the total nitrogen excretions. Typical examples of all the figures obtained for ammonia excreted hourly are given in Tables II, III and IV. In Charts I, II and III the results, arranged in the form of curves, cover various periods of time continuous for about four days.

On a total nitrogen excretion of about 8 g. the average daily amount of ammonia nitrogen excreted by a normal person is usually stated to be between 0.5 g. and 0.55 g., if Malfatti's method of estimation be used; this gives

Table I. Ammonia excretion and reaction of urine (average daily figures).

Subject	Height in inches	Weight in kilos	Diet	Number of observations	Total N g.	Ammonia N		Amino-acid N g.	Total N per kilo of body weight	Acidity of Leathes' method %	Volume in cc.	Tetra-NH <sub>3</sub> in table acid in cc. of 0.1 N acid		Total acidity A+N	Ratio A/N			
						Folin's method %	Malfatti's method %					A	N					
European	67.0	54.8	mixed	16	10.54	0.55	5.3	0.68	6.5	0.13	1.2	0.192	—	1210	275	396	671	0.69
Tamil I ...	69.0	54.8	mixed, mainly rice	17	8.64	0.46	5.3	0.58	6.7	0.12	1.4	0.157	—	622	271	328	599	0.82
Chinese I	62.5	54.8	"	9	8.99	0.44	4.9	0.56	6.3	0.12	1.4	0.164	55	857	347	319	666	1.08
Chinese II	65.0	50.0	"	9	7.74	0.55	7.1	0.65	8.5	0.11	1.4	0.155	59	839	330	292	722	0.84
Chinese III	61.5	47.4	"	10	9.83	0.50	5.1	0.63	6.4	0.13	1.3	0.207	51	1164	345	360	705	0.96
Eurasian ...	61.0	51.3	"	10	9.01	0.47	5.2	0.58	6.4	0.11	1.2	0.175	57	1099	343	339	682	1.01
Sikh I ...	65.0	52.0	"	9	7.17	0.39	5.5	0.50	7.0	0.11	1.5	0.137	52	1016	273	279	552	0.97
Sikh II ...	61.5	44.0	"	9	5.02	0.31	6.1	0.39	7.7	0.08	1.6	0.111	52	511	193	224	417	0.86
Tamil II ...	62.0	46.8	vegetarian, mainly rice	7	4.95	0.39	7.9	0.45	9.1	0.06	1.2	0.105	—	807	162	280	442	0.58
Tamil III	65.0	40.0	"	17	4.57	0.29	6.3	0.35	7.7	0.06	1.4	0.114	—	895	94	297	301	0.45
Tamil IV	63.5	51.1	"	10	5.02	0.50	10.0	0.60	12.0	0.10	1.9	0.098	39	649	196	357	553	0.55
Tamil V ...	65.0	52.0	"	10	4.38	0.39	9.0	0.46	10.6	0.07	1.6	0.084	46	699	218	280	498	0.78
Standards for Europe	—	—	mixed	—	16.00	0.53	3.3	—	—	—	—	0.200	—	1231	278	370	649	0.75
	—	—	"	—	8.00	0.40	5.0	0.52	6.5	—	—	—	—	—	—	—	—	—

Cole

Table II.

*Chinese Subjects. 21/11/19.*

(Mixed diet; small meal 6.15 a.m.; large meal 11 a.m. to 12 noon; total nitrogen, 8 g.)

A.—*Urinary Alkalinity per cent. per hour.*

Subjects	a.m.						p.m.				Average
	7	8	9	10	11	12	1	2	3	4	
T.S.W.	36	50	53	34	30	34	44	66	64	34	44
L.E.K.	40	55	98	66	50	56	60	70	65	36	59
H.S.	56	71	60	47	37	34	37	42	56	34	47
T.S.H.	46	86	97	78	23	23	23	71	94	75	61

B.—*Urinary Ammonia mg. per hour.*

	a.m.						p.m.				Average	Remarks
	7	8	9	10	11	12	1	2	3	4		
T.S.W.	41	38	33	44	29	33	33	20	34	25	33	Excess
L.E.K.	36	28	14	27	19	23	25	17	22	20	23	Normal
H.S.	25	37	31	34	22	38	40	23	36	28	31	Excess
T.S.H.	22	16	13	16	13	20	20	17	8	13	16	Normal

(total nitrogen 5 g.)

C.—*Amount of Urine cc. per hour.*

	a.m.						p.m.				Average
	7	8	9	10	11	12	1	2	3	4	
T.S.W.	98	172	54	51	35	36	39	31	48	28	59
L.E.K.	19	29	30	35	23	42	65	42	42	28	35
H.S.	44	92	100	64	26	24	20	15	28	18	43
T.S.H.	46	36	36	34	20	22	22	34	30	25	30

D.—*Amount of Fluid taken cc.*

		Total	Average per hour
T.S.W.	6.20 a.m., 200 cc.; 9, 200 cc.; 11.20, 200 cc.; 3 p.m., 200 cc. ...	800	80
L.E.K.	6.20 a.m., 200 cc.; 8.30, 200 cc.; 11, 400 cc.; 3.30 p.m., 200 cc. ...	1000	100
H.S.	6.15 a.m., 400 cc.; 8.50, 200 cc.; 11, 600 cc. 1 p.m., 200 cc.; 2, 200 cc.; 3, 200 cc. ...	1800	180
T.S.H.	6.30 a.m., 200 cc.; 11.15, 200 cc.; 1.30 p.m., 400 cc.; 3.15, 200 cc. ...	1000	100

the average hourly amount of ammonia nitrogen as 21 or 23 mg. Some of my subjects, whose figures are shown in Tables II, III and IV, excreted quite normal average hourly amounts, whilst others excreted large amounts. The subjects, whose results are given in Charts I, II and III, excreted normal average hourly amounts; and it will be noted that as a rule the ammonia excretion increased as the alkalinity per cent. decreased and *vice versa*; in other words the hourly ammonia varied inversely as the alkalinity or directly as the acidity. This supports the theory that urinary ammonia is there to neutralise acid. The alkalinity per cent. was estimated by Leathes' titration method [1919] in which the reaction of the urine is expressed as so much per cent. alkali or so much per cent. acid.

Attention is now directed to the hourly variations in amount of ammonia excreted when the average was distinctly higher than the normal, that is about 30 mg. As seen in Tables II, III and IV, the hourly variations for large quantities did not differ materially from those for normal amounts, that is the ammonia excreted varied, as a rule, inversely as the alkalinity.

Table III.

*Tamil Subjects. 24/11/19.*

(Mixed diet; small meal 6.15 a.m.; large meal 11 a.m. to 12 noon; total nitrogen, 8 g.)

A.—*Urinary Alkalinity per cent. per hour.*

Subjects	a.m.					p.m.					Average
	7	8	9	10	11	12	1	2	3	4	
A.B.C.	26	34	62	89	75	53	41	73	66	60	57
A.P.P.	45	30	41	40	44	39	36	55	53	52	43
V.D.S.	40	41	42	48	50	46	40	62	72	71	51
C.S.	44	56	73	66	47	52	37	60	65	65	56

B.—*Urinary Ammonia mg. per hour.*

Subjects	a.m.						p.m.				Average	Remarks
	7	8	9	10	11	12	1	2	3	4		
A.B.C.	68	56	27	33	28	30	42	26	32	31	37	Excess
A.P.P.	21	27	29	33	23	25	25	26	23	28	26	Normal
V.D.S.	33	26	27	40	20	27	23	19	15	17	24	"
C.S.	21	27	19	23	17	26	27	27	12	13	21	"

C.—*Amount of Urine cc. per hour.*

Subjects	a.m.						p.m.				Average
	7	8	9	10	11	12	1	2	3	4	
A.B.C.	74	144	45	84	90	80	120	43	37	40	75
A.P.P.	25	26	43	66	52	50	39	27	29	48	40
V.D.S.	100	52	41	60	35	38	24	26	20	25	42
C.S.	32	24	24	26	18	27	31	164	32	53	43

D.—*Amount of Fluid taken cc.*

		Total	Average per hour
A.B.C.	6.30 a.m., 200 cc.; 11.20, 200 cc.; 2 p.m., 200 cc. ...	600	60
A.P.P.	6.30 a.m., 200 cc.; 11.30, 300 cc.; 2 p.m., 200 cc. ...	700	70
V.D.S.	6 a.m., 200 cc.; 8, 200 cc.; 11.30, 200 cc.; 2 p.m., 200 cc. ...	800	80
C.S.	6.30 a.m., 200 cc.; 10.30, 200 cc.; 12.15 p.m., 300 cc. ...	700	70

The main object of the hourly estimations was to endeavour to determine whether the ammonia was excreted in greater quantity during the hours of the day when individuals were most exposed to the effects of the climate. This was not found to be the case. The average hourly ammonia excreted during the period of sleep at night was practically the same as that for the whole day (Charts I, II and III). The total amount for any period of eight consecutive hours of the day was not materially different from that for another period of the same duration. Nevertheless, it must be stated, that

the highest daily amounts of ammonia, in all my cases covering a period of six years, were excreted by those who were, during their work, most exposed to the effects of the climate; they included bakers, rubber estate coolies and rickshaw runners [1918]. In other countries large amounts of ammonia have been detected in the urine of soldiers [Hammarsten and Hedin, 1914]. Koizumi [1919] has found that muscular work at raised temperature causes an increase of urinary ammonia of rabbits.

The subjects described in the present paper attended the medical school, most of them living at the school hostel. Sometimes they showed quite normal amounts of ammonia; at other times there was a distinct increase, the increase lasting sometimes only one day, sometimes several days and sometimes a week, the diet, general routine and total nitrogen excretion remaining almost constant. The average increase for those on a mixed diet

Table IV.

*Eurasian Subjects. 26/11/19.*

(Mixed diet; small meal 6.15 a.m.; large meal 11 a.m. to 12 noon; total nitrogen, 8 g.)

A.—*Urinary Alkalinity per cent. per hour.*

Subjects	a.m.						p.m.				Average
	7	8	9	10	11	12	1	2	3	4	
L.	38	44	56	50	50	34	30	41	55	62	46
G.	36	34	35	35	31	38	30	40	41	42	36
N.	35	35	46	38	38	38	38	57	87	78	42
P.	30	40	42	46	51	40	32	54	75	75	48

B.—*Urinary Ammonia mg. per hour.*

Subjects	a.m.						p.m.				Average	Remarks
	7	8	9	10	11	12	1	2	3	4		
L.	45	39	30	49	20	32	32	40	33	34	35	Excess
G.	62	31	29	45	25	30	34	32	28	32	35	"
N.	25	25	48	36	36	35	35	25	21	21	31	"
P.	33	29	25	37	21	19	19	17	26	19	24	Normal

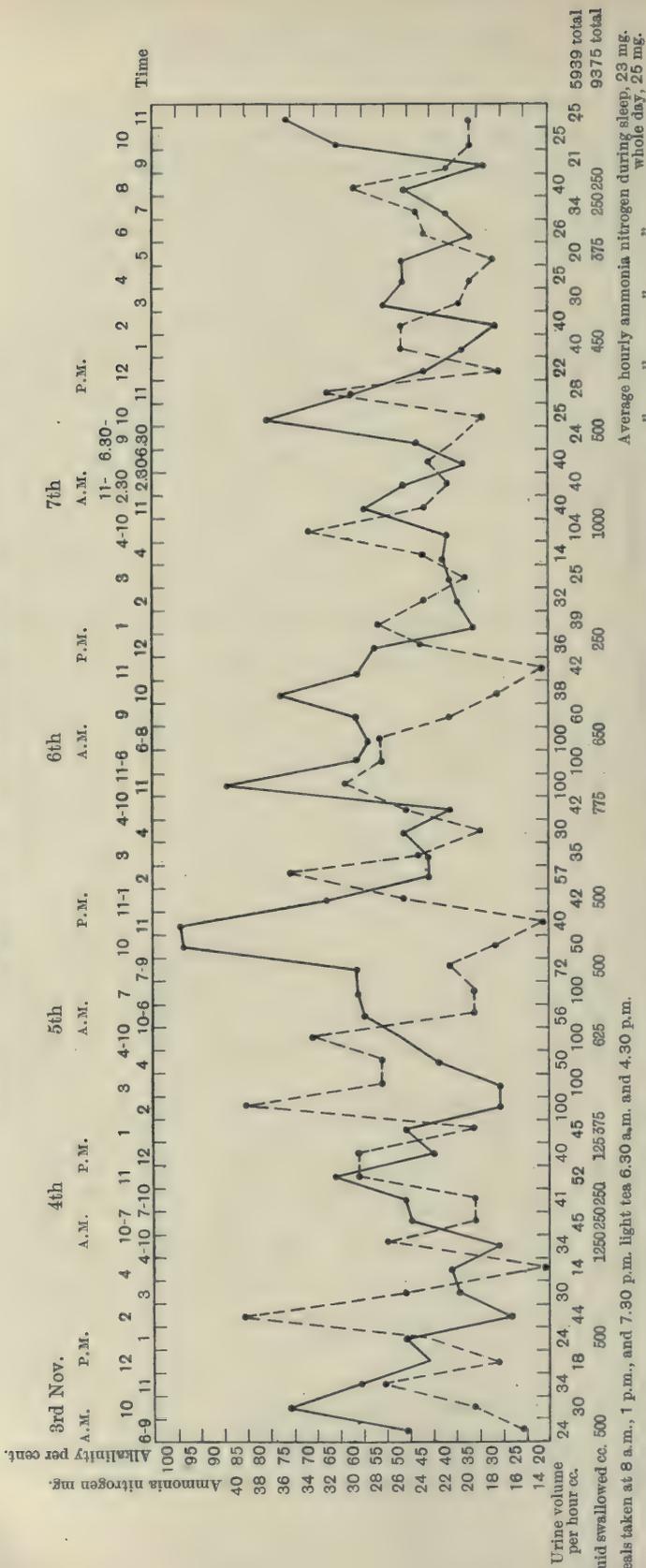
C.—*Amount of Urine cc. per hour.*

Subjects	a.m.						p.m.				Average
	7	8	9	10	11	12	1	2	3	4	
L.	40	38	28	49	20	48	44	49	38	45	39
G.	68	45	38	80	30	45	55	27	27	30	44
N.	11	10	54	26	26	40	40	28	44	58	33
P.	24	22	20	30	18	14	12	20	32	26	21

D.—*Amount of Fluid taken cc.*

		Total	Average per hour
L.	6.15 a.m., 200 cc.; 8.45, 200 cc.; 11.30, 300 cc.; 1 p.m., 200 cc. ...	700	70
G.	6.30 a.m., 200 cc.; 11.30, 300 cc.; 1 p.m., 400 cc. ...	900	90
N.	6.30 a.m., 200 cc.; 8.45, 400 cc.; 12 p.m., 300 cc. ...	900	90
P.	6.30 a.m., 400 cc.; 11.30, 500 cc. ...	900	90

Chart I. *European subject (mixed diet, total nitrogen 10 g.).*



Curves for alkalinity per cent. (continuous line) and for ammonia nitrogen (broken line) of the urine.

Average hourly ammonia nitrogen during sleep, 23 mg. whole day, 25 mg.





and with a total nitrogen excretion of 8 g. was from 23 mg. ammonia nitrogen per hour to 33 mg. per hour. These variations may be normal, but I do not think that such marked variations in the daily ammonia have been noted before in apparently normal individuals, on a constant and customary diet, mainly vegetarian in nature, as taken by most of my subjects. An increase was observed just as frequently in the strict vegetarians as in those using a mixed diet, the absolute quantities of ammonia being of course lower for the vegetarians. It is noteworthy that my subjects appeared to avoid an acid-producing diet.

It was considered that some tropical disease might be responsible, since the high figures resembled those for ammonia excretion in febrile diseases, as described in text books of clinical medicine. Malaria was the only disease of which a history was obtained in a fair number of my subjects; and these did not excrete large amounts more frequently than those without a history of malaria.

In Table I details of the total acidity as arranged by Palmer and Henderson [quoted by Cole, 1919] are given; the figures show that the total acidity was distinctly high in some cases, indicating an acidosis. As in Europe the acidity, generally speaking, varied directly with the total nitrogen. Also the urine was acid on a mixed diet and alkaline on a vegetable diet (see Table I, acidity, Leathes' method). In Europe the volume of the urine is stated to vary inversely as the acidity; but in Singapore the volume more often varied directly as the acidity (see Tables II, III, IV, and Charts I, II and III). Many of the subjects showed that large quantities of water were lost by perspiration, the amount of urine voided being much less than the amount of fluid swallowed. It is evident that a good deal of acid must have been removed by sweat, since perspiration is known to lower the acidity of the urine. Diminution of sweat in a climate like that of Singapore would evidently cause an acidosis.

#### THE ALKALINE TIDE.

Recently Leathes [1919] stated "...the traditional ascription of the alkaline tide to the secretion of hydrochloric acid in the stomach appears to be erroneous. An intense alkaline tide is normal in the morning when no food is taken; alkaline tides after other meals than the first in the day are certainly not the rule, and in the experience of the writer do not occur. It is not even *a priori* probable that they should, since the passage of acid from the stomach is the signal for the secretion of alkaline digestive juices into the small intestine and for absorption into the blood from the intestine to begin." Leathes holds that the matutinal alkaline tide is accounted for by depression of the respiratory centre during sleep and increased activity on waking and some time later, so that in this way acid is removed by the lungs and not by the urine to the usual extent.

My results in Singapore were quite different from those of Leathes, and

certainly confirmed the traditional ascription of the alkaline tide to the secretion of hydrochloric acid. I found that an alkaline tide, although not constant, was the rule about three hours after any meal in Singapore. I employed the method for estimation of alkalinity and acidity described by Leathes in his paper, the reaction of the urine being given as so much per cent. alkali or so much per cent. acid. The subjects were selected from those employed in the first part of this research. Some of them took meals as in Europe about 8 a.m., 1 p.m., and 7.30 p.m., with light tea (tea, bread and butter) about 6.30 a.m. and 4.30 p.m.; others, as was their custom, took large meals about 11 a.m. and 7 p.m. with small meals about 6 a.m. and 4.30 p.m.

My figures for subjects under normal conditions may be summarised as follows. In 100 cases a matutinal tide was possible; in 68 cases it was present (68 %); in 32 cases it was absent. In 78 cases a tide was possible after the midday meal and evening meal; in 57 it was present (73 %); whilst in 34 out of these 57 occasions the tide was better marked than the corresponding matutinal tide. These points are illustrated in Charts I, II and III, and in Tables II, III and IV. It will be observed that the tides appeared also in those subjects who excreted large amounts of ammonia. In these Tables and Charts I have stated the quantities of fluid swallowed and the quantities of urine voided.

In another series of experiments no food was taken in the morning before 11 a.m. Although in some cases the matutinal tide still appeared, it was absent in the majority (80 %) of cases. On the other hand Leathes found that a tide is still present if no food is taken in the morning. It is well known that acid is sometimes present in the stomach when there is no food there. It is likely that acid might appear at the customary time, if food be only occasionally omitted, and thus cause an alkaline tide.

It was noted that in a European subject in Singapore the tide was often absent after lunch if work was commenced soon after the meal; but it was present if two hours' interval was taken. This was the custom for the Asiatics, who rested between 11 a.m. and 1 p.m., taking lunch between 11 a.m. and 12 noon.

I repeated Leathes' experiments with hyperpnoea and found that hyperpnoea readily made the urine alkaline as he points out; but hyperpnoea was not responsible for the tides in Singapore, some of my subjects breathing quietly and slowly all day, in fact being half asleep during the experiment.

My results resembled the conclusions of Hasselbalch whom Leathes quotes: "After each meal the urine was without exception more acid than before, but showed the lowest acidity about three hours later." It seems that the secretion of alkaline saliva renders the urine more acid just after a meal and that the secretion of hydrochloric acid renders the urine more alkaline about three hours after a meal, seeing that it is at this time the acid reaches its maximum in the stomach.

I found that, as a rule, the smallest amounts of ammonia were excreted

three hours after a meal, so that the ammonia may be required in the stomach at that time. It seems likely that this is so from the following theory recorded by Macleod [1918]. The stomach mucosa is particularly rich in ammonia which, it is supposed, carries out the hydrochloric acid into the cavity of the stomach, the ammonia passing back again to the cells of the mucosa. Therefore it seems that most ammonia would be required in the stomach three hours after a meal, when the acid secretion is at its maximum. Schittenhelm and A. Loeb and Gammeltoft [quoted by Hammarsten and Hedin, 1914, p. 767] have also observed a fall in the ammonia elimination a few hours after a meal and *vice versa*.

#### NOTE ON AMINO-ACID EXCRETION.

The amino-acids were estimated by subtracting the result obtained by Folin's method of estimating ammonia, from that obtained by Malfatti's method.

Table I shows that, with the exception of Tamil IV, the amount of amino-acid nitrogen varied directly with the total nitrogen, being about 0.12 g. with a total nitrogen of about 8 g., and about 0.07 g. with a total nitrogen of about 5 g. In Tamil IV the amino-acid nitrogen was 0.10 g., the total nitrogen being 5.02 g. His ammonia nitrogen was also relatively high. When large amounts of ammonia were excreted the amino-acids were sometimes increased; at other times they were not altered.

The average amino-acid nitrogen per cent. of total nitrogen was about 1.2 with a total nitrogen of about 8 g., and about 1.5 with a total nitrogen of about 5 g.

#### SUMMARY.

1. Further observations were made to endeavour to determine the cause of the frequent excretion of relatively large amounts of ammonia in Singapore, as estimated by Folin's and Malfatti's methods. Some results regarding amino-acid excretion and the alkaline tide were also obtained.

2. The hourly excretion of ammonia in subjects excreting a normal amount of this substance, varied, as a rule, directly as the acidity or inversely as the alkalinity. This supports the theory that urinary ammonia is used to neutralise acid.

3. The hourly variations for those excreting large amounts of ammonia resembled those for individuals excreting normal amounts. Any subject was likely to show an increase, which lasted sometimes for a day, sometimes for several days. The increased excretion was spread out over the whole day and was not confined to the hours of greatest exposure to the effects of the climate. When normal amounts were excreted a similar condition existed. Nevertheless, it is considered that the climate was responsible for the acidosis since the largest daily amounts of ammonia, noted in all subjects, were excreted by the individuals who were most exposed to its effects. It is noteworthy that my subjects appeared to avoid an acid-producing diet.

4. Well-marked alkaline tides, although not constant, were the rule three hours after any meal, being just as frequent after the midday and evening meals as after the morning meal. The traditional ascription of the alkaline tide to the secretion of hydrochloric acid in the stomach was therefore supported.

5. The ammonia excretion in the urine varied inversely as the acidity of the stomach. This supports the theory that ammonia is used to carry hydrochloric acid from the stomach mucosa to the stomach cavity.

6. In Singapore the volume of the urine varied directly as the acidity, in this respect differing from Europe. In some cases, the total acidity was distinctly high, indicating an acidosis.

7. The amino-acids in the urine varied directly as the total nitrogen.

#### REFERENCES.

- Campbell (1918). *J. Straits Branch R.A. Soc.* No. 79, 107.  
— (1919). *Biochem. J.* 13, 239.  
Cole (1919). *Practical Physiological Chemistry*, 274.  
Hammarsten and Hedin's text book. *Physiological Chemistry* (1914), Mandel's Translation, 766.  
Koizumi (1919). *Physiol. Abstr.* 4, 437.  
Leathes (1919). *Brit. Med. J.* ii, 165.  
Macleod (1918). *Physiology and Biochemistry in Modern Medicine*, 484.

## LIV. CUORIN.

BY HUGH MACLEAN AND WILLIAM JAMES GRIFFITHS.

*From the Department of Pathological Chemistry, St Thomas's Hospital.*

*(Received July 17th, 1920.)*

THE name cuorin was given by Erlandsen [1907] to a new lipin substance which he isolated from the ether extract of heart muscle. In many of its properties cuorin was very similar to kephalin, but differed from this substance in the relative amounts of nitrogen and phosphorus present in the molecule. It formed one of a constantly increasing group of alleged new lipins isolated chiefly by the German school about a decade ago. At that time it seemed as if the number of different lipins present in the tissues was very great, while their complexity of composition and the difficulties experienced in isolating them made this interesting field of biochemistry appear to be almost hopelessly difficult. Gradually, however, as the result of further research, it became obvious that many of these alleged new bodies had no claim to be regarded as unit substances and were largely mixtures or decomposition products of well-known lipins. Thus MacLean [1918] was able to show that the number of phosphatide lipins present in animal tissues could with certainty be reduced to four—lecithin, kephalin, sphingomyelin and cuorin—but that even this short list was probably too long, since the evidence for the existence of cuorin as a chemical entity was meagre in the extreme. In this connection he remarked that there was no definite proof that the substance was not a mixture, and that though undoubtedly a substance resembling Erlandsen's cuorin could be obtained from tissues, yet the conditions under which this substance was isolated did not exclude the possibility of the body being an artificial product. This opinion was recently substantiated by Levene and Komatsu [1919], who, in a very fundamental investigation into the structure of kephalin, came to the conclusion that cuorin was a mixture composed chiefly of kephalin with certain degradation products.

A review of the literature disclosed the fact that many of the cuorin-like substances isolated differed materially in their nitrogen to phosphorus ratio, and that while some of them had a N : P ratio of 1 : 2, in others this ratio was as high as 1 : 1.5 or even more. All this lent force to the view that cuorin was probably a substance which did not exist in fresh tissue as such, but was formed as the result of the various manipulations utilised in the extraction of the lipins. On this hypothesis an attempt was made to isolate the lipins from

*very fresh tissues as rapidly as possible*, using the purest of solvents, in the hope that decomposition would be largely prevented. If cuorin were a decomposition product it should be possible to prevent its formation, and no substance of this nature should then be found in the tissue. By very careful and rapid manipulation of heart muscle, we found that the substance which corresponded to Erlandsen's cuorin had a N : P ratio of nearly 1 : 1 instead of 1 : 2 and was really slightly impure kephalin. It is, therefore, certain that cuorin is an artificial product and has no further claim to be regarded as a definite lipin. This observation simplifies to a considerable extent the classification of the lipins, for it is now reasonably certain that the tissues contain only three phosphatides—lecithin, kephalin and sphingomyelin—and that the addition to these phosphatides of the two cerebrosides—phrenosin and kersin—completes the list of lipin substances as we know them to-day. Although the constitution of all these bodies has not yet been definitely ascertained, much light has recently been thrown on the question, and soon we may hope to know as much of the structure of the lipins as we do of such a well-defined group as the carbohydrates.

#### EXPERIMENTAL.

Ox hearts, obtained immediately after death, were freed from macroscopic fat and passed through a mincing machine. The resulting material was dried by stirring it up for a short time in a fairly large volume of pure acetone; the mixture was then strained through a piece of muslin, bound up in sailcloth, and freed as much as possible from acetone by the use of a powerful press. The tissue, which was now almost dry, was broken up and spread on a glass plate under a fan for about an hour during which it was constantly broken up with a spatula. It was then passed through a coffee mill and a fine powder obtained. The whole process was carried through as expeditiously as possible, so that the tissue was obtained in the form of a fine dry powder ready for extraction, a few hours after the death of the animals. In our opinion, this is most important, as there is little doubt that decomposition often takes place while the tissue is being dried, especially when this is done more or less slowly at a moderately high temperature, as is often the case.

The next step was to extract the powder with absolute alcohol in the usual way until the extract contained little or no residue on evaporation of the alcohol. After thorough treatment with alcohol the tissue was finally extracted with ether.

The alcohol extracts were mixed, the alcohol removed at ordinary temperature by means of a vacuum pump, and the thick syrupy mass remaining taken up with pure ether. Insoluble substances were removed by the centrifuge and the ether extract treated with excess of acetone. This process was repeated several times. The final precipitate was purified by water and acetone [MacLean, 1912], and the waxy substance obtained dried *in vacuo*. By further treatment with ether followed by centrifuging, some remaining

sphingomyelin was separated, and ultimately a substance was obtained which gave a clear solution in ether. This ethereal solution was again treated with acetone and the precipitate dried *in vacuo* over sulphuric acid. The dry material was then thoroughly shaken up with excess of alcohol, when part of it remained insoluble. The alcohol-soluble fraction consisted of more or less pure "lecithin," and on analysis gave N 1.97 % and P 4.05 %; N : P = 1 : 1.07.

The alcohol-insoluble portion should of course contain kephalin and Erlandsen's cuorin. After dissolving in ether and reprecipitating with acetone it gave on analysis 1.6 % of N and 4.1 % of P. This substance was then treated exactly according to Erlandsen's directions for separating cuorin from kephalin, when a small amount of a body corresponding in solubility and physical properties to Erlandsen's cuorin was obtained. This product which, if it were cuorin, should have a N : P ratio of 1 : 2 gave on analysis 4.06 % P and 1.66 % N, *i.e.* a N : P ratio of 1 : 1.1. The substance was therefore more or less impure kephalin, and no evidence of the presence of a substance of the composition of cuorin could be obtained. It would thus seem that cuorin is only found under conditions where decomposition is liable to take place and is really a mixture containing some decomposition products.

Subsequent treatment of the alcohol-extracted tissue with ether showed that practically all the lipin material present was removed by the alcohol. The ether extract contained only mere traces of phosphatides, and no body of the nature of cuorin was present.

#### CONCLUSIONS.

The cuorin described by Erlandsen is not a chemical unit, but results from decomposition taking place in the tissues during the processes utilised for the extraction of the lipins. This decomposition is probably most active during the drying of the tissue. When precautions are taken to dry the perfectly fresh tissue very quickly by means of acetone, no cuorin is obtained.

The only phosphatides present in animal tissues are lecithin, kephalin and sphingomyelin.

The expenses involved in this work were defrayed from a grant for which we are indebted to the Royal Society.

#### REFERENCES.

- Erlandsen (1907). *Zeitsch. physiol. Chem.* **51**, 71.  
Levene and Komatsu (1919). *J. Biol. Chem.* **39**, 91.  
MacLean (1912). *Biochem. J.* **6**, 355.  
— (1918). *Lecithin and allied substances. The Lipins. Monographs on Biochemistry.* London.

# LV. FACTORS INFLUENCING ALKALOIDAL CONTENT AND YIELD OF LATEX IN THE OPIUM POPPY (*PAPAVER SOMNIFERUM*).

BY HAROLD EDWARD ANNETT.

*From the Agricultural College, Cawnpore, U.P., India.*

*(Received July 21st, 1920.)*

## I. INTRODUCTION.

THE present paper is a summary of a full account of the work of the writer and others which is being published elsewhere<sup>1</sup>. A very large amount of data has been accumulated which it is hoped may be of interest to physiologists but under present conditions it is impossible to reproduce all the figures here. Typical figures only will therefore be given in the present paper.

The work represents an investigation arising out of war conditions. In pre-war days the bulk of the opium required for alkaloidal manufacture and for medicinal purposes was provided by Asia Minor, the Balkans and Persia. The morphine content of Indian opium was too low to enable it to compete in that market. With the entry of Turkey into the war supplies of medical opium were threatened and it became imperative to make use of Indian opium. The writer was placed on special duty in October 1916 to investigate the reason for the low morphine content of Indian opium. The work met with success and there is no reason why India should not put opium on to the market of at least as high morphine content as the product of Asia Minor and the Balkans.

Fortunately for the work the writer has had the advantage of collaboration with Mr H. M. Leake, M.A., Economic Botanist to the Government of the United Provinces. Mr Leake had for several years previously been endeavouring to isolate races of poppy which would be resistant to the Blight disease (*Peronospora arborescens*) which causes very serious havoc annually in India. He was therefore able to supply seed of pure races of poppy for experimental work at once. Much of the work which has been carried out on drug plants appears to suffer from the fact that pure races of plants were not used. In addition to the use of pure races the work has been carried out on a large scale and at several centres, e.g. in the plains and in the Himalayas. Some five or six acres of the same pure race have been under experiment annually.

<sup>1</sup> *Memoirs of the Dept. of Agric. in India.*

## II. DESCRIPTION OF THE METHOD OF EXTRACTION OF LATEX.

It will make the description of the work easier if the method of extraction of opium as practised by the Indian cultivators is first described. The operation is commenced some 10 to 20 days after the fall of the flower petals. The capsules are considered to be ready for lancing as soon as they feel firm to the touch and are yet green. The instruments used are:

(1) A knife which consists of two to four parallel sharp pointed blades bound together with cotton, the binding being so done that the points of each blade are about one-twentieth of an inch apart and all in the same plane.

(2) A small iron scoop.

(3) Unglazed earthen pots in which to store the opium.

The cultivator then usually divides his fields into three portions *A*, *B*, *C*. The lancing is invariably commenced just after midday and is carried out by men, women and children. They begin at the edge of the field and work backwards as otherwise the exuding latex would be brushed off on their clothes. The hand is quickly passed over the capsules. If a capsule is considered to be ready it is grasped in one hand and the knife is drawn vertically upwards over its surface from just above the stalk to just below the stigmatic rays at the top of the capsule. Sometimes the cut is made in the reverse direction, *i.e.* downwards. The second finger of the hand holding the knife is placed near the points of the knife on the surface of the capsules to steady the motion of the knife. Great care is taken not to cut too deeply into the capsule. If the cut be made too deep, then the wall of the capsule may be cut completely through with the consequent secretion of the latex on the inner surface. Good operators can incise 150 to 200 capsules in an hour.

Immediately on incision the latex commences to flow. It varies in colour from milk white and smoky white through pale pink to a very bright pink. It rapidly begins to darken however. Next morning at 6 a.m. or later if there is a heavy dew, the opium is collected. It is scraped off the cut surface with the blunt edged iron scoop. From the scoop it is transferred to the earthen pots. Usually the cultivator lances only one-third of his field (*e.g.* portion *A*) on the first day. After collecting the opium next morning from that portion he then lances portion *B*, that is on the second day. On the third day portion *C* is lanced. On the fourth day he again returns to lance portion *A*. On this occasion many more heads are now ready for a first lancing. In addition a second lancing is given to those capsules which were lanced on the first day. On the fifth day portion *B* receives its second lancing and on the sixth day *C* receives its second lancing. On the seventh day *A* is lanced for the third time and on this occasion more heads are usually ready for a first lancing. Those already lanced once now receive a second lancing and those already lanced twice receive a third lancing. Portions *B* and *C* come in for similar treatment in regular rotation. The lancements are continued as long as the capsules continue to give appreciable yield of latex. Usually each field is lanced

four or five times and occasionally as many as eight times so that some heads would then have received eight lancements. In some poor seasons and on some poor crops the heads cease to yield opium after the second lancing. The whole opium produce of each field is then mixed together and, after three to six weeks' storage, is brought to an official of the Government Opium Department by whom each cultivator's opium is weighed and examined for consistency or dry matter by touch and for certain adulterations (*e.g.* starch by the iodine test). The opium officers reach such a high degree of skill that they can usually tell the moisture content of the opium by touch to within 2 or 3 %. The cultivator at once receives the value of his opium except for a small balance which is paid some two months later when the district officer's examination has been checked by analysis at the Government Opium Factory, to which place all the opium is sent.

### III. THE ALKALOIDAL CONTENT AND YIELD OF LATEX FROM EACH OF A SERIES OF SUCCESSIVE LANCINGS OF THE SAME CAPSULES.

We have established the fact that on the first occasion on which a capsule is incised by the cultivator the opium is far richer in morphine than is the produce of the second lancing and this again than that of the third lancing and so on. Cases have been met with in which the opium obtained from the fifth lancing gave no morphine at all by the method of estimation laid down in the *British Pharmacopoeia*. The following analysis is typical of many hundreds of estimations we have carried out of the morphine content of the opium from a series of successive lancements of the same capsules.

	No. of lancements					
	first	second	third	fourth	fifth	sixth
Morphine percentage on dry matter of opium	13.9	9.6	5.8	3.6	2.2	1.6

We have met with no case in which the opium of a particular lancing was of higher morphine content than the opium of a previous lancing of the same capsules.

As regards the yield of latex at each successive lancing it is difficult to state typical figures. Thus in the hills one usually finds that the capsules will bear a larger number of successive lancements than they will in the plains so that as a rule in the hills the first three or four lancements may each produce about the same amount of opium. In the plains, however, one usually finds the first lancing of a particular capsule produces the highest yield of latex and the yield falls off with each subsequent lancing, provided there are no sudden changes in the weather conditions. The following figures are perhaps typical.

Table showing yield of latex (dried at 100° C.) at each successive lancing.

Locality	Yield of latex in g. dry matter per 1000 capsules					
	No. of lancements					
	first	second	third	fourth	fifth	sixth
Elevation 3500 feet (Himalayas) ...	20.6	33.8	29.5	18.2	5.6	0.5
Cawnpore ...	35.5	12.4	5.4	2.1	0.4	0.1

Varying weather conditions, as will be shown later, exert very great influence on the yield on any particular day. Thus cloudy weather or East winds usually considerably reduce the yield. The author has found similar phenomena in his work on sugar yielding palms [1913].

The rapid fall in morphine content of the latex from each successive lancing, raises the question as to whether any substance is formed in place of the morphine in the later lancements. Indian opiums are particularly rich in codeine and it seemed probable as this alkaloid is so closely related to morphine in its constitution, that one would find an increase in the codeine content of each successive lancing. This however is not the case. Narcotine is the only other alkaloid present in sufficient quantity to be of interest in this connection but here again there is, as a rule, no rise in narcotine content in the later lancements. The following figures show the percentages of morphine, narcotine and codeine in the dried latex of the first four successive lancements of the same capsules.

Number of lancing	Percentage of alkaloids in dry matter of latex			Total of morphine, narcotine and codeine
	Morphine	Narcotine	Codeine	
First ...	11.18	6.85	2.25	20.28
Second ...	9.28	6.34	2.71	18.33
Third ...	6.38	4.18	2.94	13.50
Fourth ...	3.41	3.36	3.64	10.41

As previously stated one always finds the same rapid progressive decrease in morphine content in each successive lancing. As regards narcotine and codeine one does not observe the same regularity in behaviour. Codeine certainly usually shows a slight increase just as in the table. On the other hand narcotine sometimes increases in the later lancements. We are investigating this matter in greater detail. It may be stated here however that the total nitrogen content of the dry matter of the latex from each successive lancing remains practically constant, so that some nitrogenous substance takes the place of morphine in the subsequent lancements. At present our work indicates that the substance is not an alkaloid.

It seemed of great interest to discover if decreasing or increasing the interval of time between the successive lancements would affect the rate at which the morphine content of the later lancements falls off. The following intervals of time between the successive lancements were experimented with:

- |              |             |             |
|--------------|-------------|-------------|
| (1) 4 hours, | (2) 1 day,  | (3) 2 days, |
| (4) 3 days,  | (5) 4 days, | (6) 5 days. |

Our results showed that when the interval between successive lancements was one day or more the morphine content of the opium of each successive lancing fell away in exactly the same manner whether that interval was one, two, three, four or five days. With an interval of only four hours between the successive lancements indications were obtained that the falling off in morphine

content of each successive lancing was not so marked. The bearing of this phenomenon will be discussed in the last section of this paper. Strong indications were obtained that after receiving the first lancing, the capsules require a certain minimum interval of time before the second lancing in order that they may give a free yield of latex at the second lancing.

#### IV. THE VARIATION IN RATE OF FLOW AND MORPHINE CONTENT OF THE LATEX AT DIFFERENT PERIODS OF TIME AFTER INCISION.

Numerous experiments were performed in which latex was collected in fractions at various intervals of time after incision of the capsules. Thus immediately on making the incision latex flows out. This was collected within one minute of making the incision. From the same incised surface latex continues to flow and collections were made at certain set times during the ensuing 16 hours after which time we have only on rare occasions observed any flow of latex from an incised surface. In the latex of each successive fraction there is again a steady fall in the morphine content. Moreover when some two days later a second incision is made on the capsule and the latex again collected in fractions the fall in morphine content again takes place in each successive fraction, the first fraction usually showing about the same morphine content as the final fraction of the previous lancing. Latex collected in fractions from a third successive incision behaves in an exactly similar manner. The table gives a typical result.

No. of lancing	Date of lancing	Percentage of morphine in latex dried at 100° C.				
		Time of collection				
		9 a.m. (immediately on incision)	12 a.m.	3 p.m.	6 p.m.	6 a.m. (next morning)
1	13-4-19	12.3	11.0	10.9	10.1	—
2	16-4-19	9.7	7.8	7.7	5.9	—
3	19-4-19	7.3	5.7	—	—	5.5

The writer considers that these figures indicate that morphine is stored principally in the capsule. When the capsule is lanced the latex first flowing out would therefore be of maximum morphine concentration. As the flow continues the latex would flow in from parts of the plant below the capsule, where the morphine is presumably present in lower concentration. The results also tend to disprove the theory that morphine does not occur as such in the plant [Winterstein and Trier, 1910, True and Stockberger, 1916]. Figures showing the rate of flow of latex at various intervals of time after incision are given in the main publication. It is sufficient to state here that 22.6 to 60.6 of the total yield of latex may be exuded within a minute after incision.

V. THE EFFECT OF DIFFERENT SYSTEMS OF LANCING ON  
THE YIELD AND COMPOSITION OF THE LATEX.

The methods of lancing in use in the various opium producing centres differ greatly. In Asia Minor and the Balkans one spiral cut is made round the capsule and no subsequent lancing is carried out on the same capsule. In Egypt transverse cuts are made. We have already stated that in India one vertical cut with a multiple bladed knife is made at each lancing. It seemed desirable in view of the high morphine content of the latex yielded at the first lancing, to obtain as high a yield as possible at the first lancing. With this end in view experiments were carried out in which two, three, four and five and even six vertical cuts with a four bladed knife were made at the first lancing. A very definite result was obtained from these experiments in that if one obtains a large yield at a first lancing by increasing the number of incisions, then the percentage of morphine in the latex diminishes. Following up this matter, further experiments were made in which only one incision, a very tiny one was made into the capsule so that only a small yield of opium was obtained. The opium obtained in this manner was of distinctly higher morphine content than that obtained by making incisions of the usual length.

Further when only a tiny incision is made at each lancing then the rate of fall in morphine content of the opium of each successive lancing is much less rapid.

Some interesting data have been accumulated both in field and pot experiments on the connection between the yield of latex and the number of incisions made in the capsule. The following table gives the result of a field experiment in which some 5000 capsules were taken for experiment. Some of them were lanced with a single vertical incision, some with three incisions and the remainder received a minimum of five incisions per capsule. In each case a four bladed knife was used. The first two groups received subsequent lancements also but only the results of the first lancing are given in the table.

*Effect of increasing the number of incisions at each lancing on the yield of latex per capsule.*

	A	B	C
	1 vertical incision	3 vertical incisions	5 or more vertical incisions
Gms. latex (dried at 100° C.) per 1000 capsules	30.3	40.9	20.9

Thus by increasing the number of incisions per lancing from one to three a slight increase in yield of latex is obtained. When however the number of incisions per capsule is increased to five or more then the yield of latex per capsule is considerably diminished. These results were fully borne out in an experiment on poppy plants grown in pots. In this case experiments were made on capsules receiving one, two, three and six incisions at each lancing. The table shows the yield of latex dried at 100° C., per capsule for the first lancing only.

*Yield of opium per capsule as affected by using one, two, three and six incisions at each lancing. (Pot experiment.)*

	Number of incisions at each lancing			
	1	2	3	6
Gms. dry opium } per capsule, 1st lancing only }	0.0294	0.0352	0.0374	0.0277

Here again it is seen that increasing the number of incisions does not result in a corresponding increase in the yield of latex, and beyond a certain point may cause a decrease.

The ordinary lancing knife as used in India contains four blades the points of which are set about  $\frac{1}{20}$ th inch apart. We have carried out experiments to determine the comparative effect of using knives with two, four and six blades. Increasing the number of blades does not increase proportionally the yield of latex. The six bladed knife in our experiments gave about a 20 % increase in yield of latex over the yield obtained with the two and four bladed knives which gave practically identical yields.

#### VI. THE YIELD AND ALKALOIDAL CONTENT OF THE LATEX FROM DIFFERENT CAPSULES OF THE SAME PLANT.

One may find as many as 40 capsules in various stages of development on a plant of the opium poppy. Usually, however, a plant has one or two or perhaps three capsules. The oldest is at the end of the main stem and will be referred to as the terminal capsule. The subsequent capsules are borne each at the end of a branch arising from an axil of a leaf on the main stem and the younger the capsule the farther down the main stem does its branch originate. These will be referred to as lateral capsules. We have found as a result of a large number of experiments carried out on 50 pure races of poppy plants that the oldest or terminal capsule produces opium of much higher morphine content than do the younger capsules and it appears that the morphine content of the latex diminishes progressively the later the order of origin of the capsule. It must be clearly understood that this phenomenon has no connection with the unripeness of the later capsules. Our lancements were not carried out until the capsules were at the correct latex yielding stage. Usually also, though not invariably, the terminal capsules yield more latex than the lateral capsules.

The table gives results typical of our experiments. In the experiment to which this table refers 1620 plants each with several capsules in process of development had their terminal capsule lanced. The second, third and fourth capsule on each plant was lanced when ready. The opium obtained from the terminal and from first, second and third lateral capsules was examined separately.

Description of capsule	No. of lancing	Date of lancing	Yield of dried opium per 1000 capsules, g.	Percentage of morphine on dried opium
Terminal ...	1	2-3-18	79.7	14.0
	2	5-3-18	27.0	8.4
	3	9-3-18	8.2	5.3
	4	12-3-18	3.5	3.3
1st lateral ...	1	5-3-18	34.6	12.2
	2	9-3-18	8.0	7.1
	3	12-3-18	2.2	4.9
	4	15-3-18	0.3	—
2nd lateral ...	1	9-3-18	13.7	10.1
	2	12-3-18	6.5	6.1
	3	15-3-18	0.7	—
3rd lateral ...	1	12-3-18	14.3	8.0

In an examination of the latex from the terminal and lateral capsules of 50 pure races of poppy not a single case was met with in which the morphine content of the latex from the lateral capsules was higher than that of the latex of the terminal capsules.

Experiments were carried out to discover if by leaving the terminal capsules unlanced one would obtain latex of higher morphine content from the lateral capsules. It was found that the morphine content of the latex of the lateral capsules was not increased by leaving the terminal capsules unlanced.

Indications were however obtained which showed that by removing all lateral buds a large yield of latex could be obtained from the terminal capsules.

#### VII. THE RELATION BETWEEN THE STAGE OF DEVELOPMENT OF THE CAPSULE AND THE YIELD AND ALKALOIDAL CONTENT OF ITS LATEX.

It has already been shown in Section III that the morphine content diminishes progressively in the latex from each of a series of successive lancements from the same capsules. As will be shown here this phenomenon has nothing whatever to do with alterations in morphine content of the latex with increasing age of the capsule as at first sight might appear possible. We have carried out experiments on this point during three seasons with identical results in each season. Some 20,000 plants have been included in each year's experiments.

Capsules have been lanced at the following intervals of time after flowering: 5, 9, 13, 17, 21, 25 and 29 days, *i.e.* at all stages from soft green to the ripe stage. Controls have been carried out by lancing side by side with the experimental capsules on each day a number of capsules in the same field which were considered by the cultivators to be ready for lancing. The results show that if the capsules are lanced only four or five days after flowering then the morphine concentration of the latex is distinctly low. About nine days from flowering the latex reaches its maximum morphine content and this maximum is maintained at whatever age beyond nine days the capsules are first lanced.

The yield of latex is low when the capsule is very immature but rises to a maximum when the capsules are first lanced at 15 to 17 days from flowering. When the first lancing is carried out later than this the yield of latex again diminishes. The table summarises the results for 1918-19 which are typical of those of the other seasons.

*Table showing connection between stage of development of capsule and the yield and morphine content of the latex.*

No. of days between flowering and first lancing	First lancing		Second lancing (2 days later)	
	Latex dried at 100° C.		Latex dried at 100° C.	
	Percentage of morphine	G. per 1000 capsules	Percentage of morphine	G. per 1000 capsules
5	10.2	27.7	6.5	23.0
9	11.7	28.2	8.4	19.7
13	12.1	30.4	8.3	26.1
17	11.4	36.9	8.7	21.1
21	12.5	26.3	10.7	22.1
25	12.5	22.1	9.1	16.9
29	12.2	25.3	10.4	8.4

#### VIII. THE EFFECT OF CLIMATE AND WEATHER CONDITIONS.

The literature records the results of numerous experiments carried out with the idea of tracing the influence of climate and weather conditions on drug production in various medicinal plants. This work is summarised in our main publication. As regards our own particular problem, the discovery of the reason for the low morphine content of Indian opium, it may be stated at once that climate as such does not control to any important extent the power of the poppy plant to produce morphine. Opium of high morphine content has been produced in every continent, in climates as different as those of the tropics and Sweden.

Our work in this connection has been carried out during four seasons, on a very large scale. We have been fortunate in being able to carry out trials with the same pure race in various places in the plains of northern India and in the same season at altitudes of 4000, 5000 and 6500 feet in the Himalayas. At the latter altitude our poppy was at times under snow. Our remarks apply especially to morphine. As regards other alkaloids our investigations are not so far advanced. Codeine for instance is always high in Indian opium. We have examined the produce of numerous pure races of Indian opium selected by Mr Leake and we have never found less than about 1.8% codeine. Some samples yielded over 4½% and the average for Indian opium we would put at about 3%. Turkish opium only contains about 0.25%.

Narcotine is usually present in opium in quantities amounting to 5 to 8% but French opium has at times been reported to contain none of this alkaloid [van Ittalie and Kerbosch, 1911]. The matter is referred to at a greater length in the complete account of our work.

*Effect of Climate.*

Before dealing with our experimental results it will be advisable to point out that previous investigators on opium have not recognised the large difference which exists in the morphine content of the opium of each successive lancing, nor have they realised the variation in morphine content of the opium produced by the various capsules of the same plant. In our work on climatic conditions we have only used the terminal capsules of each plant as a source of opium and we have kept separate the product of each successive lancing. At each centre we have usually had  $\frac{1}{2}$  an acre of poppy at least, while at our main station at Cawnpore five to six acres of the same pure race have been under experiment. On each area very large numbers of samples were collected amounting to some hundreds at Cawnpore. Much attention has been paid in our work to the determination of the range of variation which occurs over a field which is all under similar treatment. Over a given field provided only terminal capsules are used the various samples of opium of the first lancements usually show quite a small range of variation say from 11 to 13 % morphine for a race averaging 12 % of morphine.

The following table summarises the figures obtained at the various stations in 1916-17, 1917-18, 1918-19 and 1919-20.

*Table showing effect of climate or locality on the morphine content of the opium. Produce of first incision of terminal capsules only.*

No. of samples examined	Locality	Percentage of morphine calculated on opium dried at 100° C.	
		Range of variation	Average
1916-17			
10	Sitoli (Alt. 5000 feet) ... ..	10.9-13.9	12.7
1	Douglas Dale (Alt. 4000 feet) ... ..	—	10.6
1	Chaubattia (Alt. 6500 feet) ... ..	—	10.3
1	Cawnpore ... ..	—	11.1
1917-18			
91	Cawnpore ... ..	11.8-15.8	14.0
1	Fatehgarh (Ankin) ... ..	—	15.1
2	„ (Mianganj) ... ..	12.6-13.1	12.8*
5	Rae Bareli ... ..	11.2-13.4	12.2*
11	Douglas Dale (Alt. 4000 feet) ... ..	11.3-16.7	13.3
1	Sitoli (Alt. 5000 feet) ... ..	—	10.0
2	Chaubattia (Alt. 6500 feet) ... ..	16.1-16.3	16.2
1918-19			
119	Cawnpore ... ..	10.5-15.8	12.6
12	Rae Bareli ... ..	9.2-12.7	11.1
8	Douglas Dale (Alt. 4000 feet) ... ..	9.3-12.4	11.1
1919-20			
71	Cawnpore ... ..	14.0-17.7	15.4
8	Rae Bareli ... ..	11.5-15.0	13.1
1	Fatehgarh ... ..	—	16.6
2	Etawah ... ..	16.1-17.6	16.8
3	Douglas Dale (Alt. 4000 feet) ... ..	13.2-15.3	14.0

\* These samples were mixtures of first and second lancements and would therefore be lower in morphine content than first lancements only.

It should be stated that the pure race grown in 1916-17 was a different one from that used in the subsequent three seasons. In 1916-17 the poppy producing the one sample at 6500 feet altitude was at one stage under snow.

It would not appear that climate is an all important factor in determining the power of the plant to produce morphine. At the same time during the past three seasons in which we have been very carefully studying the behaviour of a pure race of poppy grown at various stations in the plains, indications have been obtained that locality does exert a certain amount of influence on the morphine producing power of a plant. Thus at Cawnpore during the season 1919-20 our main pure race gave an average of 15.4 % of morphine in the opium of the first lancing. At Etawah and Fatehgarh it has given perhaps a slightly better result, whereas in Rae Bareli it has only produced about 13 % of morphine. In the previous season we noticed that the Rae Bareli crop behaved in a similar manner. The climate of all these places shows no marked difference and they are all within 100 miles of Cawnpore. It is hoped to follow up this matter further.

#### *The Influence of Season.*

There is evidence that the morphine content of the opium produced by the same pure race may vary from season to season. We have grown a collection of pure races for three successive seasons and have found a distinct variation in the morphine content in successive seasons. Since only one analysis was carried out in each of these cases in each season it is hardly fair to generalise from these results. We have, however, grown a large area of one pure race at Cawnpore on the same farm during four successive seasons, and numerous analyses were made in each season. The table summarises the results of the four years' experiments.

*The seasonal variation in morphine content of opium produced by the same pure race of poppy. Produce of first lancings only.*

Season	Area under experiment acres	No. of samples analysed	Percentage of morphine in dry matter of opium	
			Range of variation	Average
1916-17	0.5	16	11.7-14.7	12.9
1917-18	5.5	91	11.8-15.8	14.0
1918-19	6.0	119	10.5-15.8	12.6
1919-20	1.5	71	14.0-17.7	15.4

We have accumulated similar data for the same pure race grown during three seasons at Rae Bareli in the plains and at Douglas Dale in the Himalayas.

#### *Weather Conditions.*

Experiments were carried out in 1917-18 and in 1918-19 to trace any connection there might be between temperature and other weather conditions and yield or morphine content of the opium produced. A continuous record

of the temperature was kept by means of a thermograph. Observations were also made of barometric and humidity conditions but produced no results worthy of consideration here. Rainfall, direction of winds and amount of cloud were also recorded. A field of 0.5 acre in extent sown with seed of a pure race was used. The plan of experiment was to select 1000 capsules daily, which were evenly distributed throughout the field, and which the cultivators considered were ready for lancing, and to lance them. In the 1917-18 experiment 1000 capsules were so selected daily throughout the period 28th February to 26th March. The yield of opium and its morphine content were determined in each day's produce. At intervals of three days the capsules received a second and third lancing; after which the plants bearing them were pulled up to prevent confusion. The plants selected each day were labelled with cloth labels of a distinctive colour in order to simplify the finding of them on the field. Only terminal capsules were included in the experiment. The results for the first lancements only of the 1917-18 experiments are set out in the table.

*Table showing yield of opium per 1000 capsules and its morphine content recorded daily throughout duration of opium harvest. First lancements only.*

Date of lancing	Gms. dry opium per 1000 capsules	Percentage of morphine on dry matter of latex
28-2-18	52.1	14.9
1-3-18	62.1	14.5
2-3-18	60.3	14.9
3-3-18	53.1	14.9
4-3-18	60.1	14.6
5-3-18	61.1	14.5
6-3-18	64.5	13.9
7-3-18	53.7	14.2
8-3-18	51.3	13.6
9-3-18	50.4	14.0
10-3-18	43.2	14.2
11-3-18	45.4	14.1
12-3-18	41.5	14.8
13-3-18	33.0	15.3
14-3-18	33.0	15.1
15-3-18	27.5	14.8
16-3-18	24.4	14.6
17-3-18	19.4	15.0
18-3-18	18.1	15.0
19-3-18	12.3	15.7
20-3-18	5.4	13.8
21-3-18	7.7	—

As regards morphine content it will thus be seen that this has kept remarkably constant throughout the period of opium harvest. On comparing the daily yield of latex with the temperature records it appeared that the yield of latex diminished with a rise in temperature and increased with a fall in temperature. After the 9th February, however, the latex yield fell away rapidly probably owing to the drying up of the field and the drying of the capsules and this rapid and continuous fall masked any temperature effect.

It is very interesting to note that in spite of a very rapid fall in the latex yield with advance in season, yet the morphine content of the dried latex is not modified to a detectable extent.

The 1918-19 experiments completely bore out those of 1917-18.

### IX. THE INFLUENCE OF MANURES.

The literature provided numerous references to the effect of manuring on drug production in plants but in the writer's opinion most of the work is very unsatisfactory. Pure cultures of plants appear to have been rarely, if ever, used and it would seem that lack of experience in conducting field experiments has handicapped certain of the investigators. These matters are referred to in our main publication. We have experimented on the same pure race in the seasons 1916-17, 1917-18, 1918-19 and 1919-20.

In 1916-17, on a field of one acre, we tested the effect of  $K_2SO_4$ , superphosphate and nitrate of soda, alone and in all possible combinations. Each manure was applied at the rate of 400 lbs. per acre. These large amounts were put on in order to magnify any possible manurial effect. Each plot was duplicated and the usual precautions taken in field trials were used. In 1917-18, exactly the same experiments were carried out, but castor cake and cattle dung plots were also added to the series. The first two years' experiments having indicated that nitrogen was the dominant manure for poppy, in the next two seasons the trials with superphosphate and  $K_2SO_4$  were discontinued and the effect of increasing amounts of  $NaNO_3$  was determined. Plots receiving castor cake and poppy seed cake were also added to the series in 1919-20. The germination in 1918-19 was so uneven that the experiment was unsatisfactory in that year.

The results obtained in the seasons 1916-17 and 1917-18 were practically identical. Superphosphate and potash alone, or in combination, had no appreciable effect on the yield of latex or on the morphine content. Nitrate of soda and castor cake, however, largely increased the yield of latex but the effect on the percentage of morphine in the dried latex was almost inappreciable.

The results for 1917-18 are summarised in the table.

*Table showing effect of manures on yield of latex and its morphine content.*

Treatment	Percentage of morphine in dry matter of latex from first lancing	Total yield of latex from all lancements as dry matter. Un-manured plot = 100
No manure ... ..	14.1	100
$K_2SO_4$ ... ..	14.9	103
Superphosphate ... ..	15.8	120
$NaNO_3$ ... ..	15.0	188
$K_2SO_4$ + Superphosphate ... ..	13.8	99
$K_2SO_4$ + $NaNO_3$ ... ..	13.8	192
Super. + $NaNO_3$ ... ..	14.9	217
Super. + $K_2SO_4$ + $NaNO_3$ ... ..	14.7	253
Cattle dung ... ..	14.7	121
Castor cake ... ..	14.5	149

In considering the figures one must remember that control plots showed that these variations in morphine content on the different manurial plots are less than the variations to be expected over a field uniformly treated. At the same time the influence of nitrogenous manures on the yield of latex is undoubted.

Careful records have been kept of the weights of the plants, capsules, and of the seed out-turn on each of the plots. It has been shown that these weights are proportionate to the yield of total latex on each plot. The effect of the manure is apparently therefore to produce a bigger capsule which in virtue of its size produces a larger yield of latex. For a particular race of poppy it would seem that the morphine concentration of the latex of the capsule is practically constant.

Our 1919-20 experiments were designed to test the effect of increasing amounts of nitrate of soda on the yield and alkaloid content of the latex. Plots receiving castor cake and poppy seed cake, were also added to the series.

*Table showing effect of increasing amounts of nitrogen on yield of latex and its morphine content.*

Treatment (per acre)	Percentage of morphine in dry matter of latex	Yield of latex at first lancing as dry matter. Unmanured plot = 100	Yield of latex as dry matter, total of all lancings
Unmanured ... ..	14.4	100	100
NaNO <sub>3</sub> 80 lbs. ... ..	15.3	106	127
„ 160 lbs. ... ..	15.5	124	142
„ 320 lbs. ... ..	15.4	113	103
„ 480 lbs. ....	16.2	144	130
„ 640 lbs. ... ..	16.5	158	142
Poppy cake 1600 lbs. containing N = NaNO <sub>3</sub> 530 lbs. ... ..	16.1	153	166
Castor cake 1600 lbs. containing N = NaNO <sub>3</sub> 580 lbs. ... ..	17.0	146	193

The morphine content of the latex shows a small but regular increase with increasing amounts of nitrogen in the form of nitrate of soda. Castor cake produced latex of the highest morphine concentration, though it did not supply as much nitrogen as the maximum amount of NaNO<sub>3</sub> applied. There is however only a difference of 2.6 % in morphine content between the latex produced on the unmanured plot and that produced on the castor cake plot. This difference is no greater than that which it is possible to find in the opium harvested from different portions of the same field uniformly treated. We are convinced that the influence is just significant however, since there were four samples of opium collected corresponding to each treatment and the analyses of these four samples agreed closely in practically every case. The figures given in the above are the averages of these analyses.

As regards yield of latex at the first lancing there has been a uniform increase in latex yield with increasing amounts of NaNO<sub>3</sub> with the exception

of the plots receiving 320 lbs.  $\text{NaNO}_3$  per acre. As regards the total out-turn of opium from all the lancements the plot receiving only 160 lbs.  $\text{NaNO}_3$  per acre has given as big a yield as any of the nitrate plots. The cake plots have given the biggest total yields since they kept up the yields of latex at the subsequent lancements. The important point however is that though the yield of latex has been much increased yet there is little modification in the morphine content of the latex.

#### X. THE INFLUENCE OF STARVATION.

The plants on the unmanured plots in all our manurial experiments were certainly very poor specimens and ill nourished. It seemed of interest deliberately to produce the poorest plants possible and to examine the latex produced by them. For this purpose some hard uncultivated land at Cawnpore was ploughed up and poppy grown on it in the season 1918-19. A most miserable crop resulted and we were able to obtain complete plants only some 5 to 8" high. A portion of this field was dressed with nitrate of soda and this certainly improved to a slight extent the plants growing there but only slightly. The capsules lanced were mostly from  $\frac{1}{3}$  to  $\frac{2}{3}$ " long; though they were slightly larger on the plants receiving nitrate of soda.

The opium from the first lancing only from the unmanured portion yielded 8.5 % morphine. The portion receiving nitrate of soda gave opium with 10.3 % morphine. With the same pure race of poppy grown in the same season on our experimental area the extreme range of variation in the morphine content of opium from first lancements only was from 11.1 to 15.0 %. In the season 1919-20 these results were checked at Cawnpore. Around the edges of fields, owing to the land receiving poor cultivation, there are always a certain number of stunted plants which usually only produce one capsule. The smallest of these plants were selected and their terminal capsules lanced for opium. These plants were not as small as those grown specially in the previous season.

Three separate samples of opium collected yielded 12.4, 12.7 and 10.1 % morphine reckoned in the dry opium. The normal plants in the same field yielded opium containing 15 to 16 % of morphine. Similar results were obtained in experiments in the Himalayas in 1919-20. The stunted plants from the edges of the fields produced opium with 10.3 % and 7.3 % morphine respectively in the two samples examined. The normal plants in the same field produced opium with 13.2-15.3 % morphine.

Undoubtedly therefore stunted plants yield opium very low in morphine content but the stunted plants examined by us were quite abnormal and would never be used by the cultivators for lancing. These results differ from those of Wayne Army [1917] who working on belladonna found that small plants produced the highest percentage of atropine.

## XI. THE INFLUENCE OF HEREDITY.

An account of this will appear elsewhere written in conjunction with Mr Leake. The latex produced by more than 500 pure races isolated by him and of crosses made by him has been examined for morphine content and in many cases for codeine content. The pure races represent practically all types of poppy grown in India including white as well as coloured flowered races. Opium from the first lancements of terminal capsules only varied in its morphine content from 6.6 to 20.1 %, reckoned on the dry material. Most of them gave over 11.5 % morphine. The variation in codeine content is from 1.8 to 4.8 %. Indications have been obtained that a race of poppy producing latex of high morphine concentration maintains that power in subsequent seasons.

## XII. THE BEARING OF THE RESULTS ON THE FUNCTION OF ALKALOIDS IN PLANTS.

For the complete literature on the subject the reader must be referred to our main publications.

Our work has established the following facts:

1. When a capsule is lanced for the first time the concentration of morphine in the latex is at a maximum in the first latex to flow out. As the flow continues the morphine concentration diminishes.

2. At each successive lancing the morphine content of the opium obtained decreases rapidly and if sufficient successive incisions are made latex can eventually be obtained which contains no morphine as measured by the method of the British Pharmacopoeia. The morphine is not replaced in the latex by codeine or narcotine. The interval of time between each successive lancing whether one or five days has no influence on the rate of fall in morphine content of the opium from each successive lancing. If the interval of time between the first, second and third lancements is only a few hours, the fall in morphine content at each successive lancing is not so rapid.

3. If only small incisions are made at each successive lancing, and therefore only a small amount of latex removed each time, the fall in morphine concentration of the latex at each successive lancing is much less rapid than when larger incisions are made and more latex removed.

Similarly when for any reason, *e.g.* bad weather conditions or the making of smaller incisions than usual, the yield of the first lancing is low, then the falling off in morphine content is not so great at the second and third lancements as it would have been with a larger yield at the first lancing.

4. In very young capsules, say six days old, the latex is less concentrated in morphine. After the capsules have reached the stage at which they feel firm, say 16 days old in the writer's experiments, the morphine content of the dry opium is the same however long the first lancing is delayed after that stage and however much the yield of opium may vary from day to day.

5. Manuring with nitrogenous manures largely increases the yield of latex but the percentage of morphine in the latex is not largely modified. The yield of dry opium for a particular race of poppy is roughly proportional to the weight of the capsule and incidentally to that of the plant itself.

6. Morphine exists as such in the latex inside the plant.

7. Even though the capsules are lanced to exhaustion of morphine the seed shows no sign of deterioration, *e.g.* in germinating power. It is the universal practice in India to lance as long as the capsules yield latex and the writer knows of no evidence that the seed suffers in any way. The seed when ripe contains no alkaloids whereas the ripe capsules contain considerable quantities even after a year's storage.

8. Climate and weather conditions do not affect the morphine content of the latex to any appreciable degree. They may, however, have important effects on the yield of latex.

Starting with the above established facts it therefore appears that morphine is stored in the capsule more than in other parts of the plant.

This agrees with Clautriau's remarks [1889], who found that the alkaloids were most concentrated in the epidermis of the capsule and diminished in amount towards the lower part of the plant. That morphine is most concentrated in the latex of the capsule seems the only explanation of facts 1 and 2 above. This would explain why the latex first flowing from the cut surface is richest in morphine. As the flow continues, the latex in parts of the plant below the capsule, where it is poorer in morphine, has to be drawn on. This theory fits in well with fact 3 also, because if one only takes out very small amounts of latex at each lancing it will naturally take longer before the less concentrated latex below the capsule is drawn on. This may be accounted for by supposing that a few hours is not sufficient to exhaust the latex already in the capsule when only one incision is made. Actual observations show that the flow continues for at least 16 hours from one cut. If then the second cut is applied within a few hours of the first the latex exuding therefrom will be partly latex which was already in the capsule and therefore richer in morphine and which with one day's interval between the lancements would have all been exuded at the first lanced surface.

The plant loses its power to produce morphine about the stage that its capsules become firm to the touch (*vide* 4). This explains No. 2, *i.e.* that the morphine content of each successive lancing falls off. The flow of latex appears by No. 6 to be proportionate to the size and the weight of the capsule and incidentally to that of the plants. The effect of nitrogenous manures is to increase the size and weight of the capsules. Yet the morphine concentration in the latex remains nearly the same. It would therefore appear that the amount of morphine produced depends on the amount of plant tissue produced. Different races of poppy however produce latex of different morphine concentration. It may be that plants yielding larger amount of latex may produce

a lower morphine content in that latex and *vice versa*. Data are being accumulated to test this point.

It may be that the amount of morphine produced is proportional to growth whatever the race of poppy. The amount of latex vessels may however be a factor varying with race, and hence a capsule with few latex vessels, *i.e.* one which will give a low yield of latex, may produce opium especially rich in morphine.

It would therefore appear that, during the period of active growth of the plant, morphine is being produced at the same rate as the plant tissue. This fact is against the view of Bayliss [1915], that alkaloids are more or less an accidental product of chemical change. The plant no longer produces morphine when the seed begins to ripen, *i.e.* when its period of active growth has ceased. On the other hand the morphine does not seem to diminish (see No. 4).

The plant deposits morphine chiefly in the capsule, and one of the functions of the lactiferous system would seem to be the removal of alkaloids to the capsule. This theory is supported by the mode of growth of the lactiferous tubes in the case of *Euphorbiae* [Pfeffer, 1900], which send ramifications into each new portion of the tissue in which they occur. No. 7 indicates that the morphine is not used to nourish the seeds. Therefore it would seem that morphine is a useless end product of metabolism. The animal organism takes in complex food materials, and excretes its end products of metabolism which are mainly of a simple structure. The plant on the other hand feeds on simple substances and therefore it is not surprising if some of its end products are complex substances, which it finds difficult to excrete. That the amount of the morphine produced bears a definite relation to the amount of plant growth, would seem to support the theory that it is an excretory product, for the amount of excretory product would naturally depend on the amount of plant growth.

The large number of different alkaloids produced by plants is no objection to the theory that alkaloids are excretory products. Rather it is in favour of it, for certain alkaloids are characteristic of certain families and the metabolism of different families is certain to show differences.

A plant producing much oil in its seed might be expected to form different excretory products from plants producing much starch or protein as reserve material.

#### CONCLUSION.

Morphine in the opium poppy is a useless end product of metabolism. The plant having no mechanism for excreting an end product of such complicated structure stores it in places where it can do no harm to its own metabolism, *i.e.* chiefly in the capsule. The lactiferous system would seem to represent a means of removing waste products of metabolism.

## REFERENCES.

- Annett, Lele and Amin (1913). *Mem. Dept. of Agr. in India*, Chem. Ser. 2.  
Bayliss (1915). *Principles of General Physiology*, p. 727.  
Clautriau (1889). *Ann. Soc. Belge de Mur*, 12, 67, quoted in Tunmann, *Pflanzenmicrochemie* (1913), 297.  
van Itallie and Kerbosch (1911). *Arch. Pharm.*, 248, 613.  
Pfeffer (1900). *Physiology of Plants*, II, 40. Trans. by Ewart.  
True and Stockberger (1916). *Amer. J. Botany*, 3, 1.  
Wayne Army (1917). *J. Heredity*, 3.  
Winterstein and Trier (1910). *Die Alkaloide*, p. 7.

## LVI. DIGESTIBILITY OF GERMINATED BEANS.

BY DOROTHY MARGARET ADKINS.

*From the Royal Holloway College.*

*(Received July 28th, 1920.)*

It is a well known fact that beans contain a comparatively high percentage of protein which is not readily utilised by animals. Recent work by Chick and Hume [1919] has shown that germinated pulses are much richer in vitamins than are the ungerminated seeds. This result suggested the possibility that on germination of a seed changes may occur which affect the digestibility of the protein; experiments were therefore made in order to discover the effect of germination on the digestibility of bean protein.

Haricot, Brown Dutch and Soya Beans were used and they all gave the same results. In the experiments beans were boiled and digested by means of Benger's "Liquor Pancreatin." After regular intervals portions of the digesting mixture were tested by a method adapted from Sørensen's formaldehyde method.

### *Method of Digestion.*

The liquor in which the beans were boiled was poured off and the beans were washed with cold water (1000 cc.) and powdered.

Sodium chloride (0.75 g.) and toluene (10 cc.) were added. Alkali was added until the reaction of the mixture was alkaline to cresol red and acid to phenolphthalein. Benger's pancreatic fluid (25 cc.) was added and the whole made up with water at 37° to a known volume (500 cc. for 50 g. of beans). The flask was shaken well and 50 cc. of the mixture removed for immediate titration. The neck of the flask was plugged with cotton wool and it was placed in a thermostat at 37°. From this digesting mixture portions were removed when required for titration.

### *Method of Titration.*

The portion of the digesting mixture to be tested was filtered. Five drops phenolphthalein were added to 10 cc. of the filtrate, which was then titrated against dilute NaOH till only very faint pink.

To this 5 cc. of a 40 % solution of formaldehyde (containing one drop phenolphthalein and dilute NaOH till faint pink) were added. The resulting mixture was acid and was titrated against  $N/5$  NaOH. This titration was

noted; since the excess of formaldehyde caused any free amino-groups to be neutralised and thus rendered the solution acid, the number of cc. in the last titration gave a measure of the amount of free amino-acids formed by hydrolysis of the protein by the enzyme.

In calculating the amount of nitrogen digested it is assumed that 1 cc. of  $N/5$  NaOH used in the titration in presence of formaldehyde corresponds with  $0.014/5$  g. of N.

#### *Calculation of Percentage Increase in Digestibility.*

Average nitrogen digested at end of 24 hrs.

(a) Ungerminated beans (29 expts) 34.6 mg.

(b) Germinated beans (27 expts) 61.7 mg.

(c) Germinated and dried beans (20 expts) 49.1 mg.

Average increase obtained by germinating = 27.1 mg. N. This is from 100 cc. bean solution. Increase from 1000 cc. bean solution, which includes 100 g. beans = 271 mg. N.

By Kjeldahl experiments it was shown that 100 g. beans contain 3.18 g. N, so that the percentage increase in digestibility due to germination is 8.5.

Similarly the average increase by germination followed by drying is 4.6 %.

Preliminary experiments were made in order to ascertain:

- (1) The correct degree of alkalinity for digestion by pancreatic extract.
- (2) The most suitable length of time for boiling the beans.
- (3) The effect of boiling NaOH and fat with beans on the digestibility of bean protein. (In both cases the increase obtained was negligible.)

#### *Results.*

As a result of 29 experiments the average increase in nitrogen digestibility, as indicated by resistance to tryptic activity, of germinated over ungerminated beans was 8.5 %.

The increase varied from 15.3%, when the beans were germinated rapidly, to 4.3 %, when only slow growth took place.

The increase in nitrogen digestibility of dried germinated beans over ungerminated beans varied from 7.8 % to 1.2 % according to the rapidity with which drying occurred. The best method of drying appeared to be to expose the beans to heat such as strong sunlight for one day and then to keep them at a temperature of 30°.

In all cases when the germinated beans were even partially dried, their protein became more resistant to tryptic digestion.

From these experiments it is clear that when beans are germinated their protein is rendered appreciably more digestible—on drying, the protein becomes again less digestible.

*Tabulated Results.*

No. of Expt.	Beans (50 g.) Series 1	Milligrams of Nitrogen digested after			
		(1) 0 hrs.	(2) 24 hrs.	(3) 48 hrs.	(4) 72 hrs.
(1)	Germinated in saturated air for 24 hrs. at 34°	5.9	80.1	96.6	101.9
(2)	Germinated similarly to (1)	7.0	75.0	95.3	101.2
(3)	Ungerminated	6.4	47.9	66.1	71.9
(4)	Ungerminated	6.4	45.1	64.9	71.1
<i>Series 2</i>					
(1)	Germinated in saturated air for 48 hrs. at 34°	6.7	76.9	110.6	130.0
(2)	Germinated similarly to (1)	7.6	90.0	114.3	127.6
(3)	Ungerminated	6.1	56.0	79.3	98.0
(4)	Ungerminated	5.6	59.8	84.0	98.0
<i>Series 3</i>					
(1)	Germinated: dried in hot sun for 1 day then at 30° for 4 weeks	7.0	70.0	82.0	88.9
(2)	Germinated: dried similarly to (1)	8.1	69.2	84.3	92.0
(3)	Ungerminated	5.8	52.1	56.2	70.3
(4)	Ungerminated	5.8	50.1	62.1	67.4
<i>Series 4</i>					
(1)	Germinated in open air in warm weather for 3 days (radicles 1" long)	3.9	57.1	80.0	95.9
(2)	Ungerminated	2.0	31.9	52.1	61.8
(3)	Germinated similarly to (1)	3.9	63.8	84.3	89.5
(4)	Ungerminated	3.1	36.1	54.1	63.8
<i>Series 5</i>					
(1)	Germinated in greenhouse (30° in daytime) for 3 days	5.0	58.5	74.2	82.9
(2)	Germinated similarly to (1)	5.0	48.2	70.0	80.1
(3)	Ungerminated	3.4	36.1	47.9	52.1
(4)	Ungerminated	3.1	32.2	45.4	47.9
<i>Series 6</i>					
(1)	Germinated in open air for 3 days in warm weather	7.0	45.6	65.0	75.9
(2)	Germinated similarly to (1)	5.9	40.0	58.5	73.9
(3)	Ungerminated	2.0	28.6	47.9	61.9
(4)	Ungerminated	2.0	24.1	31.4	44.0
<i>Series 7</i>					
(1)	Germinated for 2 days: dried in hot sun for 1 day, then at 30° for 6 weeks	3.1	54.0	66.1	72.5
(2)	Germinated: dried similarly to (1)	3.1	47.9	61.3	68.0
(3)	Ungerminated	2.0	28.3	42.0	49.8
(4)	Ungerminated	2.0	30.8	42.6	47.9
<i>Series 8</i>					
(1)	Germinated 2 days: dried for 6 weeks at 30°	3.1	23.0	30.8	33.9
(2)	Germinated 2 days: dried for 4 weeks at 30°	3.4	21.0	29.1	36.1
(3)	Ungerminated	2.0	10.9	17.6	24.9
(4)	Ungerminated	2.0	9.0	16.5	23.0

*Tabulated Results (continued).*

No. of Expt.	Series 9	Milligrams of Nitrogen digested after			
		(1) 0 hrs.	(2) 12 hrs.	(3) 24 hrs.	(4) 48 hrs.
(1)	Germinated in open air in very warm weather for 2 days ... ..	5.9	31.9	45.9	61.9
(2)	Germinated similarly to (1) ... ..	5.0	30.8	42.0	60.4
(3)	Ungerminated ... ..	3.1	12.9	22.1	31.1
(4)	Ungerminated ... ..	2.0	17.1	23.0	29.1
<i>Series 10</i>					
(1)	Germinated in greenhouse for 2 days at 24° in daytime ... ..	5.0	31.1	37.0	42.0
(2)	Germinated similarly to (1) ... ..	5.9	30.8	36.1	43.1
(3)	Germinated similarly to (1): dried in room (15° in daytime) for 4 weeks ... ..	3.0	17.9	26.0	33.0
(4)	Ungerminated ... ..	2.0	15.1	22.1	26.9
<i>Series 11</i>					
(1)	Germinated in greenhouse for 2 days at 26° in daytime ... ..	5.0	40.0	47.0	52.6
(2)	Germinated similarly to (1) ... ..	3.9	35.0	46.5	49.0
(3)	Ungerminated ... ..	2.5	21.0	26.0	28.3
(4)	Ungerminated ... ..	2.0	18.8	23.0	26.6
<i>Series 12</i>					
(1)	Germinated in saturated air at 22° for 2 days ... ..	5.9	52.1	63.9	70.0
(2)	Germinated in saturated air at 20° for 2 days ... ..	5.0	34.2	56.0	73.9
(3)	Germinated similarly to (1): dried at 30° for 4 weeks ... ..	3.9	28.0	42.0	62.2
(4)	Ungerminated ... ..	2.0	22.1	30.0	36.1
<i>Series 13</i>					
(1)	Germinated: dipped into boiling water for 2 seconds, dried at 30° for 4 weeks ... ..	6.4	41.2	50.7	56.8
(2)	Germinated: dipped, dried in laboratory (15° in daytime) for 8 weeks ... ..	7.0	33.0	38.1	40.9
(3)	Germinated: dried in laboratory (15° in daytime) for 8 weeks ... ..	7.3	29.1	33.0	35.7
(4)	Ungerminated ... ..	4.2	26.9	31.9	35.8
<i>Series 14</i>					
(1)	Germinated: dried in hot sun for 1 day then at 30° for 4 weeks ... ..	8.4	50.1	61.3	72.0
(2)	Germinated: dipped into boiling water, dried in laboratory for 4 weeks ... ..	5.9	38.1	48.4	57.7
(3)	Germinated: dried at 30° for 4 weeks ... ..	6.7	47.6	58.5	65.0
(4)	Ungerminated ... ..	3.9	30.5	38.9	44.0
<i>Series 15</i>					
(1)	Germinated in laboratory at 15° in daytime for 3 days ... ..	6.4	31.9	50.1	68.3
(2)	Germinated in saturated air for 24 hrs. at 30° ... ..	9.0	57.7	71.1	80.6
(3)	Germinated in saturated air for 24 hrs. at 34° ... ..	8.4	54.0	75.3	91.6
(4)	Ungerminated ... ..	3.9	24.1	33.6	43.4

*Tabulated Results (continued)*

No. of Expt.		Milligrams of Nitrogen digested after			
		(1) 0 hrs.	(2) 12 hrs.	(3) 24 hrs.	(4) 48 hrs.
<i>Series 16</i>					
(1)	Germinated: dried in hot sun for 1 day then at 30° for 4 weeks ... ..	5.9	54.0	66.4	78.1
(2)	Germinated: dipped into boiling water, dried at 30° for 4 weeks ... ..	3.9	38.1	47.6	58.0
(3)	Germinated: dried at 30° for 4 weeks	5.3	47.9	59.4	66.1
(4)	Ungerminated ... ..	3.9	32.2	41.7	47.6
<i>Series 17</i>					
(1)	Germinated in greenhouse for 2 days (30° in daytime) ... ..	8.4	61.9	82.6	96.0
(2)	Germinated in open air in warm weather in shade for 3 days ... ..	7.3	47.0	63.8	79.2
(3)	Germinated similarly to (2) ... ..	8.1	52.4	69.2	77.6
(4)	Ungerminated ... ..	4.5	35.0	44.5	50.1
<i>Series 18</i>					
(1)	Germinated by placing in funnels plugged with moist cotton wool. A little damp cotton wool was placed over the beans—kept at 27° for 24 hrs.	5.9	52.4	63.8	75.0
(2)	Germinated similarly to (1) but for 48 hrs. ... ..	7.6	68.0	88.5	101.9
(3)	Ungerminated ... ..	3.9	25.5	39.8	54.0
(4)	Germinated similarly to (2) ... ..	7.8	69.7	82.3	90.4
<i>Series 19</i>					
(1)	Germinated: dried in laboratory (at 15° in daytime) for 6 weeks ... ..	5.9	39.8	52.1	62.4
(2)	Germinated: dried in laboratory (at 15° in daytime) for 6 months ... ..	5.6	39.5	54.0	58.5
(3)	Germinated: dried in laboratory (at 15° in daytime) for 3 months ... ..	7.0	51.8	62.2	64.4
(4)	Ungerminated ... ..	3.9	28.3	38.1	44.5

I desire to express my thanks to Professor Benson of the Royal Holloway College, who suggested the work, for her continual interest and assistance, and to Professor Hopkins of the Biochemical Laboratory, Cambridge, for his advice and help.

## REFERENCE.

Chick and Hume (1919). *Biochem. J.* 13, 199.

## LVII. THE EFFECT OF PYRUVATES, ALDEHYDES AND METHYLENE BLUE ON THE FERMENTATION OF GLUCOSE BY YEAST JUICE AND ZYMIN IN PRESENCE OF PHOSPHATE.

BY ARTHUR HARDEN AND FRANCIS ROBERT HENLEY.

*From the Biochemical Department, Lister Institute.*

*(Received August 3rd, 1920.)*

OPPENHEIMER [1915] observed that the fermentation of glucose by maceration extract was greatly stimulated by the addition of a pyruvate or pyruvic acid and that acetaldehyde had a similar but less pronounced effect. The estimations were made by weighing at comparatively long intervals but it is obvious in the case of acetaldehyde, that the stimulation chiefly occurs at the commencement of the fermentation. The results with pyruvates are not always so clear and are apparently complicated by some unconsidered factor since in some cases the total  $\text{CO}_2$  evolved exceeds that obtainable from the glucose and pyruvate in the normal course of fermentation (e.g. Br<sub>1</sub>, Table III, p. 240 in which 316 milligrams of  $\text{CO}_2$  are obtained from 0.4 g. of glucose + 0.04 g. pyruvic acid which would together only yield in the normal course 220 mgms. or including the observed autofermentation 230 mgms., and Br<sub>2</sub> in the same table where 295 mgms. are obtained against 212 normally obtainable).

Neuberg somewhat later [1915] observed a similar stimulating action of pyruvates and other  $\alpha$ -ketoacids on the fermentation of glucose, mannose, fructose and saccharose and remarked that the activation was most pronounced at the commencement of the fermentation. Experiments continued for 19–20 hours (p. 82) showed little difference in the total fermentation in the presence and absence of pyruvate.

Neuberg subsequently examined the effect of a large number of aldehydes [1918] on alcoholic fermentation and found that they were all vigorous activators. He pointed out that the effect was most marked with glucose and mannose, less so with fructose and cane sugar and suggested that this fact might be related to the observation of Harden and Young [1909] that fructose under certain circumstances can stimulate the fermentation of glucose. The stimulation, like that produced by pyruvate, was most marked at the commencement of fermentation.

Meyerhof [1918] has made an interesting contribution to this subject in his study of the kinetics of cell-free fermentation. Lebedev [1912] observed that when maceration extract was mixed with a fermentable sugar a period of *induction* occurred during which no  $\text{CO}_2$  was formed and no change in rotation occurred, and this has frequently been confirmed. Meyerhof now finds that this induction is not observed when the extract contains even a trace (0.2 millimolecular concentration) of a hexosephosphate and hence has never been recorded for juice prepared by Buchner's process, in which recognisable amounts of hexosephosphate are present. The period is moreover shorter in presence of cane sugar than of fructose or glucose and is diminished when these two sugars are warmed for 4–6 hours at  $80^\circ$  with a neutral phosphate mixture. The induction period is also greatly lessened by grinding the dried yeast with glass powder before maceration.

Following on the induction period, the velocity of fermentation more or less gradually attains a maximum corresponding to the concentration of phosphate present. This is termed by Meyerhof the "Gäranstieg" and has been studied in some detail. The effects of the addition of excess of phosphate described by Harden and Young [1908], viz. a diminished maximum and a more gradual attainment of the maximum<sup>1</sup>, are found by Meyerhof to be also produced by the addition of salts such as sodium chloride or nitrate and hence the phenomenon is partly to be explained as a general salt effect. The rate of attainment of the maximum becomes greater as the hexosephosphate concentration increases, but this characteristic phenomenon cannot entirely be abolished by the addition of hexosephosphate. Meyerhof discusses the cause of the phenomenon and shows by an ingenious application of the effect of arsenates that it is probably not due to the production of a specially labile form of sugar from the hexosephosphate. The attainment of the maximum was also found to be more rapid the greater the concentration of the coenzyme, added in the form of boiled extract of yeast or muscle.

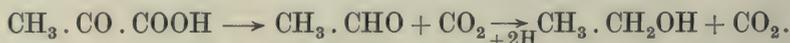
The facts that the activating effect both of  $\alpha$ -ketoacids and of aldehydes was chiefly manifested at the commencement of the reaction, that the experiments both of Oppenheimer and Neuberg were made with maceration extract which contains a large amount of mineral phosphate and that the effect was less marked with fructose than with glucose led us to enquire whether this action was a general stimulation of the fermentation process or a more specific acceleration of the reaction in presence of free mineral phosphate. The results show that when aldehyde is added to fermenting mixtures of yeast juice or

<sup>1</sup> Meyerhof [1918, p. 196] erroneously attributes to Harden and Young in explanation of this phenomenon the suggestion that the *sugar* forms with high and low concentrations of phosphate different esters of different stability, one of which, as the phosphate is used up, passes into the other. What they actually suggested was [Harden and Young 1908] that the phosphate is capable of forming two or more different unstable associations with the *fermenting complex* (by which was meant the complex of enzymes concerned, not the sugar). The alternative suggestion has also been made [see Harden 1914] "that the addition of increasing amounts of phosphate causes a progressive but reversible change in the mode of dispersion of the colloidal enzyme."

zymin (acetone yeast) with glucose no marked acceleration in the normal rate of fermentation occurs. If a suitable amount of phosphate be then added, sufficient to cause only a gradual rise of rate to the maximum in the control experiment with glucose, the effect of the presence of the aldehyde is greatly to diminish the time required for the attainment of the maximum, so that the volume of gas evolved in the period immediately following the addition of the phosphate is greatly increased. At the same time a considerably higher maximum is attained. On the completion of the esterification of the phosphate, the rate again diminishes both in the presence and absence of aldehyde and the total evolution is not greatly different in the two cases. Similar phenomena are produced by the addition of pyruvates. The effect varies with the concentration of aldehyde and is common, but in unequal measure, to the four aldehydes tested (formic, acetic, propionic and butyric). So far only glucose has been employed as the fermentable sugar, but experiments are in progress with fructose and cane sugar.

This striking effect of aldehydes, which are known to be readily reducible by yeast, strongly suggested that the cause of the delay in attainment of the maximum after the addition of phosphate was lack of an acceptor for hydrogen. In order to test this idea, methylene blue, which is also readily reducible by yeast, was substituted for the aldehyde, with the result that it was found to produce a very similar effect. When increasing amounts of methylene blue are added a point is soon reached at which the maximum, although it is more rapidly attained, is considerably lowered. This is probably due to the inhibitory effect of the dye on the enzyme complex. Even with the most favourable concentration of methylene blue however the rise of rate was not so rapid as with the aldehydes, and the maximum was unchanged.

According to the pyruvic acid theory, which may now be taken as established, the final stage of the alcoholic fermentation of sugar is the reduction of acetaldehyde (produced by the decomposition of pyruvic acid), a reaction which proceeds so rapidly that only an extremely small concentration of the aldehyde is present during normal fermentation.



Further, the production of the pyruvic acid from sugar appears only to be possible when some acceptor for hydrogen is available, this being normally supplied by the acetaldehyde produced in a later stage of the reaction.

On this view it would seem to follow that no rise in the rate of fermentation can occur without the provision of an additional quantity of a hydrogen acceptor. Some such acceptor is probably more or less rapidly formed and reduced during the period of delay, which follows on the addition of phosphate, this process being accompanied by a corresponding increase in the formation of pyruvic acid, until sufficient of this is being produced to provide the amount of acetaldehyde necessary for the maximum effect. When, however, the easily reducible aldehydes or methylene blue are added, these act as

acceptors and a much more rapid or even instantaneous attainment of the maximum becomes possible, as was actually observed in our experiments.

Whether this acceptor is the same substance as yields glycerol in the sulphite fermentation of Neuberg and Reinfurth, and is supposed by Neuberg to be methylglyoxal, is uncertain. It may be pointed out however that the precursor of glycerol assumed by Neuberg and Kerb [1913] must be much less rapidly reduced than acetaldehyde in the course of normal alcoholic fermentation since the ratio of glycerol to alcohol under these circumstances is only small. No experiments have yet been made to decide whether an enhanced glycerol production occurs during the period of delay.

It seems probable that the delay following the addition of phosphate when fructose is employed as the fermentable sugar is also due in part to lack of an acceptor. The facts that fructose yields a much higher maximum rate with phosphate and that the optimum concentration of phosphate is much higher than for glucose can accordingly be interpreted to mean either that fructose yields a hydrogen acceptor much more readily than glucose or that the acceptor formed is much more rapidly reducible. This question is at present under investigation. If this conclusion be granted, a simple explanation is afforded of the remarkable "induction" observed by Harden and Young [1909] when fructose was added to a mixture of yeast juice and glucose or mannose to which a considerable excess of phosphate had been added. Under these circumstances the rate of attainment of the maximum fermentation was greatly accelerated even when the phosphate concentration was kept constant and moreover the volume of  $\text{CO}_2$  evolved under these circumstances was much greater than could be obtained from the fructose added. In the light of the foregoing remarks it now appears that the function of the fructose under these conditions is probably to provide a hydrogen acceptor and this, once formed, enables the fermentation of the glucose to proceed rapidly, as explained above, even in the presence of a concentration of phosphate, which in the absence of an acceptor causes a prolonged delay.

It is further probable that the hydrolysis of the hexosephosphate, both that originally present and that slowly formed in the fermenting mixture, results in the formation of fructose, which in its turn yields a hydrogen acceptor and thus assists in the increase of the rate of fermentation. Meyerhof's observations on the marked effect of hexosephosphate on the rate of attainment of the maximum would thus receive a simple explanation.

Owing to the method of experiment employed by us, the full effect of the addition of hexosephosphate could not be observed as the fermenting mixtures always contained this substance formed from the phosphate originally present. The addition of a further quantity of hexosephosphate produced very little effect.

Whether the lack of acceptor combined with Meyerhof's "salt-effect" of the excess of phosphate provides a complete explanation of the delay in

attainment of the maximum after the addition of phosphate or whether time is required for some other change, such as transformation of the sugar into a fermentable form, as maintained by Euler, remains as a subject for further investigation.

### EXPERIMENTAL.

The yeast juice and zymin employed were both prepared from a brewery top-yeast. The acetaldehyde used in Exp. 2 was a preparation obtained from Kahlbaum. For the other experiments it was prepared from paraldehyde by distillation with dilute sulphuric acid. The formaldehyde was a dilution of formalin and the concentration was estimated by Ripper's method. The propionic aldehyde was prepared by heating a mixture of calcium propionate and formate and the butyric aldehyde by the oxidation of *n*-butyl alcohol with potassium dichromate and sulphuric acid. The methylene blue was Grübler's "Methylenblau med pur." The pyruvate solution was made by dissolving 1 g. of pyruvic acid in water, neutralising with *N* KHO and making to 100 cc.

#### *Effect of the addition of pyruvate and acetaldehyde to yeast juice in presence and absence of free phosphate.*

*Exp. 1.* 25 cc. Yeast-juice + 1 g. glucose. No toluene.  $T = 25^\circ$ .

	1% Pyruvate cc.	1% Acetaldehyde cc.	H <sub>2</sub> O cc.		0.3M Na <sub>2</sub> HPO <sub>4</sub> cc.	H <sub>2</sub> O cc.
1	5	0	0	and subsequently	5	0
2	5	0	0		0	5
3	0	5	0		5	0
4	0	5	0		0	5
5	0	0	5		5	0
6	0	0	5		0	5

Measurements were commenced at 2.20. The additions of phosphate and water were made at 3.20.

	Pyruvate		Aldehyde		Water	
	1	2	3	4	5	6
cc. CO <sub>2</sub> evolved before addition of phosphate in 55'	23.6	23.8	20.9	21.2	22.3	23.2
cc. CO <sub>2</sub> evolved after addition of phosphate in successive periods of 5'	Na <sub>2</sub> HPO <sub>4</sub>	H <sub>2</sub> O	Na <sub>2</sub> HPO <sub>4</sub>	H <sub>2</sub> O	Na <sub>2</sub> HPO <sub>4</sub>	H <sub>2</sub> O
1	17.8	1.9	29.8	2	5.2	1
2	24.0	2	12.8	2	7.8	3
3	6.7	4.6	7.4	3.7	28.1	4.2
4						
5	4.7	3.4	1.2	3.4	7.3	3.7
6						
Total in 30'	53.2	11.9	51.2	11.1	48.4	11.9

It will be seen that the addition of pyruvate or aldehyde did not appreciably alter the normal rate of fermentation, as in the 55' preceding the addition of phosphate all six flasks gave approximately the same amounts of gas. Further,

the three flasks 2, 4 and 6 to which H<sub>2</sub>O was subsequently added continued to ferment at equal rates, giving almost exactly equal amounts of gas in 30' (11.9, 11.1 and 11.9 cc.).

On the addition of phosphate, flask 6, containing no aldehyde or pyruvate, showed the normal behaviour, the rate gradually rising to a maximum which was attained in 10-15' and amounted to 15 per 5' (calculated by plotting the figures given in the table). In presence of aldehyde the rate rose to its maximum of 29.8 in the first 5', whilst in presence of pyruvate an intermediate result was obtained the evolution being 17.8 in the first 5' and 24 in the second.

*Exp. 2.* 25 cc. Yeast-juice + 1 g. glucose. No Toluene. T = 25°.

	1% Pyruvate cc.	Water cc.		0.3M Na <sub>2</sub> HPO <sub>4</sub> cc.	H <sub>2</sub> O cc.
1	5	0	and subsequently	5	0
2	5	0		0	5
3	0	5		5	0
4	0	5		0	5

Measurements were commenced at 3.10 and the additions of phosphate and water were made at 3.40.

	Pyruvate		Water	
	1	2	3	4
cc. CO <sub>2</sub> evolved before addition of phosphate between 3.10 and 3.30.	6.9	7.1	7.2	7
cc. CO <sub>2</sub> evolved after addition of phosphate in successive periods of 5'.	Phosphate	Water	Phosphate	Water
1	3.4	0.3	0.3	0.5
2	5.6	0.5	1	0.7
3	4.4	0.4	0.8	0.4
4 } 5 }	10.2	0.8	1.6	0.8
Total in 25'	23.6	2	3.7	2.4
Total in 140'	49	9.7	31.2	9.2
Total in 17 hrs.	88.6	49.5	76.2	46.1

This is an example in which a quantity of phosphate largely in excess of the optimum was added. In the absence of added phosphate (Nos. 2 and 4) the fermentation was substantially the same with and without pyruvate. After addition of phosphate in the presence of pyruvate the rate rose much more rapidly than in its absence and in the latter case, probably owing to the continued action of excess of phosphate on the enzyme complex, the total was considerably less.

*Effect of varying concentrations of acetaldehyde on the fermentation of glucose by zymin in presence and absence of phosphate.*

Exp. 3. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0.2 cc. Toluene. Acetaldehyde 1% solution (by weight).  
T = 25.8°.

	1% Acetaldehyde	
1	0	} and subsequently 10 cc. 0.15M Na <sub>2</sub> HPO <sub>4</sub> to each.
2	1.2	
3	6	
4	12	

Incubated for 1 hour and the phosphate then added.

	1	2	3	4
	Acetaldehyde			
	0 cc.	1.2 cc.	6 cc.	12 cc.
cc. CO <sub>2</sub> evolved before addition of phosphate in 45'.	34.4	33.6	20.7	3.4
cc. CO <sub>2</sub> evolved after addition in successive periods of 5'.				
1	2.7	3.4	12.5	5.5
2	3.6	4.6	12.8	8.4
3	5.3	5	12.3	9.7
4	6.2	7.2	11.7	9.4
5	7.7	7.7	9.8	9.0
6	9.1	9.6	9.3	7.9
7	9.0	9.3	7.2	7.5
8	8.0	7.5	3.6	7.4
Total in 40'	51.6	54.4	79.1	64.8
Total in 110'	113.6	118.8	119.6	103.1

The characteristic effect is here well marked. In the control (No. 1) the maximum of 9.1 cc. is slowly reached in 30-35' and the rate then as gradually diminishes. 1.2 cc. of 1% aldehyde [1 in 1667] produce practically no effect, 6 cc. on the other hand [1 in 334] produce a very marked effect, the maximum is much higher (12.8 cc.) and is very rapidly attained (5-10'). The total evolved in 110' is however only about 5% greater than that of the control. A point of considerable interest is that both 6 cc. and 12 cc. of 1% aldehyde diminish the normal rate of fermentation although they both produce a considerable acceleration in the rate at which the maximum is attained after the addition of phosphate. This is especially marked in the case of the 12 cc. of aldehyde solution, which diminished the normal rate of fermentation to 1/10 of that of the control. This effect is probably due to a specific inhibition of the hexosephosphatase, thus diminishing the liberation of phosphate on which the normal rate depends.

*Effect of formic, acetic, propionic and butyric aldehydes on the fermentation of glucose by yeast-juice in presence of phosphate.*

Exp. 4. 25 cc. Yeast-juice + 1 g. Glucose + 0.2 cc. Toluene. T = 25°.

1	5 cc. Water	} and then 10 cc. 0.3M K <sub>2</sub> HPO <sub>4</sub> .
2	0.78 % Formaldehyde	
3	1 % Acetaldehyde	
4	1.3 % Propionic Aldehyde	
5	1.6 % Butyric Aldehyde	

Incubated for 50' before addition of phosphate.

	1	2	3	4	5
	Water	Formaldehyde	Acetaldehyde	Propionic Aldehyde	Butyric Aldehyde
cc. CO <sub>2</sub> evolved in 30' before addition of phosphate	7.3	4.1	7.0	7.7	7.2
cc. CO <sub>2</sub> evolved after addition of phosphate in successive periods of 5'.					
1 } (10')	15.5	23.6	57.1	58.1	53.7
2 }					
3	12.5	22.4	16.4	16.6	16.1
4	15.3	20.7	2.7	2.7	2.8
5	18.4	7.0	1.6	1.5	1.7
6	11.9	1.4	1.2	1.6	1.4
7	2.5	1.5	1.8	1.6	1.4
	76.1	76.6	80.8	82.1	77.1

Exp. 5. As above, but 15 cc. of 0.3M K<sub>2</sub>HPO<sub>4</sub> added. Incubated 25'.

	6	7	8	9	10
	Water	Formaldehyde	Acetaldehyde	Propionic Aldehyde	Butyric Aldehyde
cc. CO <sub>2</sub> evolved after addition of phosphate in successive periods of 5'.					
1 } (10')	10.3	22.9	56.8	55.0	48.1
2 }			{ 28.4	{ 26	{ 23
			{ 28.4	{ 29	{ 25.1
3	5.5	22.3	27.6	27.2	30.1
4	6.6	23.9	20.5	19.9	22.4
5	8.0	21.1	4.8	7.9	3.8
6	10.3	15.7	1.6	2.1	1.8
7	11.3	2.4	1.3	1.6	1.5
8	15.3	1.4	1.4	1.4	1.2
9	17.8				
10	13.9				
11 } (10')	4.5				
12 }					

With this sample of juice very high maxima were attained, but as the first readings were made 10' after the addition of phosphate the exact maximum could not be directly observed. They were obtained by plotting the total fermentations and reading the evolutions per 5' from the curves, with the following approximate results.

Exp. 4.

	1	2	3	4	5
Maximum ...	18.4	22.4	34	35	27
Minutes after addition when attained ...	20-25	30-15	0-5	0-5	0-5

Exp. 5.

	6	7	8	9	10
Maximum ...	17.8	23.9	25.4	29	30.1
Minutes after addition when attained ...	40-45	15-20	0-5	5-10	30-15

The effect here is very marked and moreover in several cases the maximum is reached in the first 5' of fermentation. The results with the acetaldehyde in No. 8, Exp. 5, are plotted in Fig. 1.

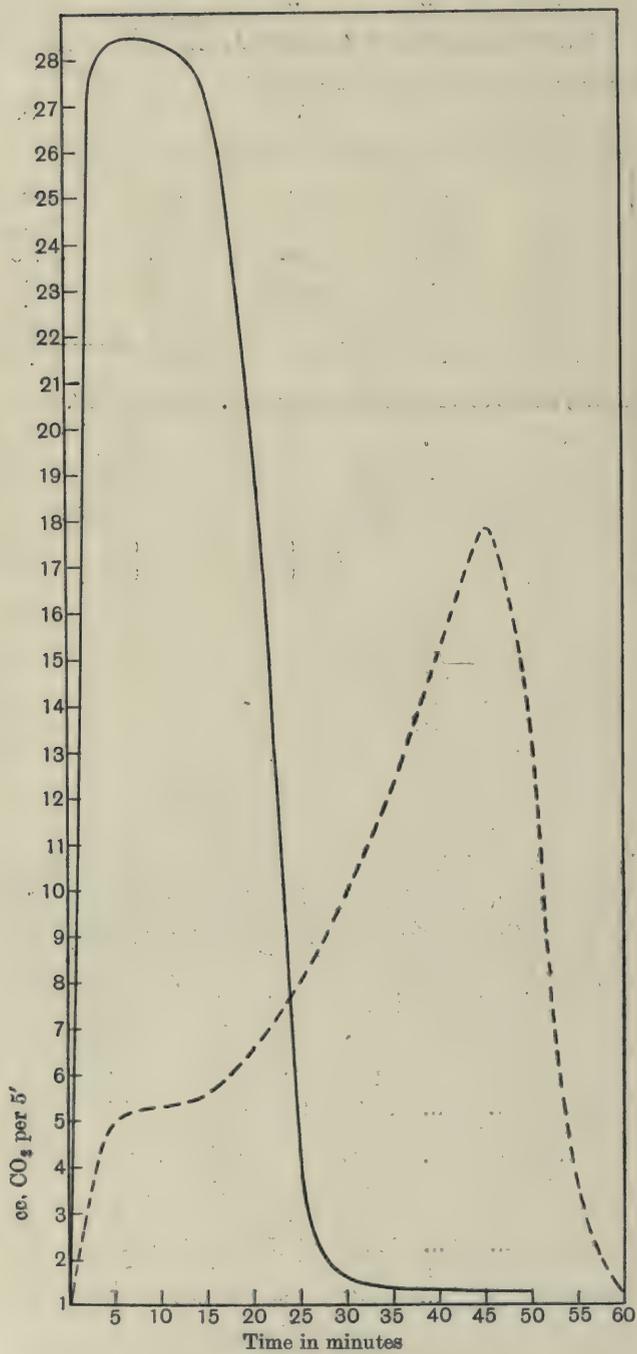


Fig. 1.

*Effect of (a) Acetaldehyde, (b) Na Hexosephosphate on the fermentation of glucose by zymin in presence of phosphate.*

Exp. 6. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0.2 cc. Toluene. T = 25.8°.

1% Acetaldehyde

1	0	} and subsequently	1	} 10 cc. 0.3M K <sub>2</sub> HPO <sub>4</sub>	
2	1.2 cc.		2		
3	6 cc.		3		
4	0		4		10 cc. H <sub>2</sub> O + 10 cc. 0.3M K <sub>2</sub> HPO <sub>4</sub>
5	0		5		Na Hexosephosphate from 0.2 g. Basalt + 0.52 g. K <sub>2</sub> HPO <sub>4</sub> in 20 cc. (equivalent to 10 cc. 0.02M Hexosephosphate and 10 cc. 0.3M K <sub>2</sub> HPO <sub>4</sub> ).

Incubated for 65' before the additions were made.

	Water	1.2 cc. Acetaldehyde	6 cc. Acetaldehyde	4	5
	1	2	3		
cc. CO <sub>2</sub> evolved before additions in 50'	39.2	33.8	28	38.9	38.4

Additions made at 1.05.

cc. CO<sub>2</sub> evolved after addition in successive periods of 5'.

	Phosphate	Phosphate	Phosphate	Phosphate and water	Phosphate + Hexose-phosphate
1	2.7	3.2	13.6	0.3	3.5
2	2.9	2.7	15.6	1.8	3.8
3	4.3	4.1	15.4	3.0	4.4
4	5.0	5.4	15.6	4.3	6.1
5	7.3	7.7	13.4	5.0	} 15.6
6	9.1	9.5	12.1	8.1	
7	10.5	10.7	11.1	9.2	8.8
8	11.4	12.3	10.0	10.3	8.8
9	11.0	10.4	8.0	10.3	8.6
10	10.9	10.8	6.4	9.2	8.0
11	9.6	9.1	3.6	8.9	} 14.6
12	8.4	8.1	3.4	8.6	
Total in 60'	93.1	94	128.2	79.0	82.4
Total in 120'	157	169.2	169.3	152.3	144.9

(a) The result with acetaldehyde confirms that obtained in Exp. 3, but the total in 2 hrs. is slightly larger in presence of acetaldehyde than in its absence.

(b) The addition of hexosephosphate has only a small effect in accelerating the attainment of the maximum. The total in 2 hours is slightly less than that in the control.

*Effect of varying concentrations of methylene blue on the fermentation of glucose by zymin in the presence of phosphate.*

Exp. 7. 4 g. Zymin + 2 g. Glucose in 20 cc. 0.2 cc. Toluene. T = 25.5°.

1	Methylene Blue 0	} and subsequently 10 cc. 0.3M K <sub>2</sub> HPO <sub>4</sub> to each.
2	" 0.1 g.	
3	" 0.2 g.	
4	" 0.3 g.	

The results of 1 and 2 are shown in the curves (Fig. 2) in which A and B represent the evolutions per 5' in absence and presence of methylene blue respectively.

In presence of 0.2 g. and 0.3 g. of methylene blue the course of the reaction was almost the same as with 0.1 g. but the maximum attained was in each case slightly lower (0.1 g., 13.2 cc.; 0.2 g., 12.5; 0.3 g., 12.7). The methylene blue in (2) became colourless in about 1 hr. and in (3) in about 2 hours.

The totals evolved in 2hr. 15 min. in the 4 flasks were almost identical, as will be seen from the following statement.

No.	M.B. g.	Max. attained (cc. in 5')	Total in 2 hrs. 15 min. cc.
1	0.0	13.2	181.2
2	0.1	13.2	184.5
3	0.2	12.5	176.8
4	0.3	12.7	178.9

When a still larger concentration of methylene blue is employed, the maximum is considerably lower than in its absence but is more quickly attained. The dye apparently partially inhibits the enzyme complex.

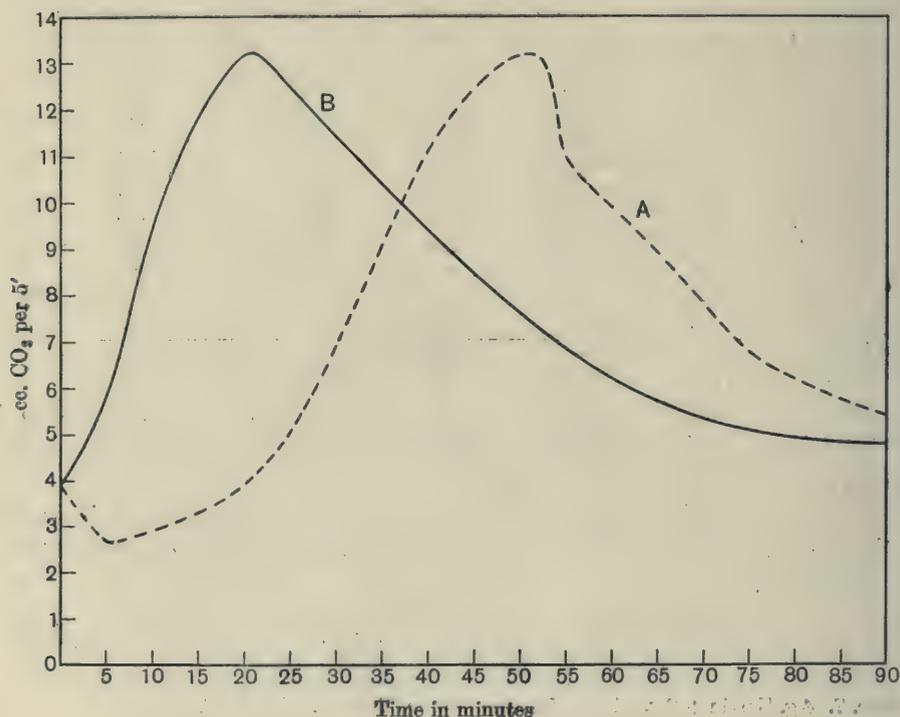


Fig. 2.

Exp. 8. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0.2 cc. Toluene. T = 26°.

1	Methylene Blue 0	} and subsequently	} 6 cc. 0.3M K <sub>2</sub> HPO <sub>4</sub>
2	" 0.5 g.		
3	" 0		
4	" 0.5 g.		

*Result.* After the addition of phosphate.

	Phosphate added 0.3M	M.B. g.	Max. attained	Time required to attain maximum	Total evolved in 1 hr. 35 min.
1	6	0	11	30-35'	107.0
2	6	0.5	8.8	0-5'	97.4
					in 1 hr. 55 min.
3	10	0	12.2	30-35'	147.1
4	10	0.5	10.6	5-10'	149.7

## REFERENCES

- Harden (1914). *Alcoholic Fermentation* (Longmans) 2nd ed. p. 72.  
 Harden and Young (1908). *Proc. Roy. Soc. B.* **80**, 299.  
 — (1909). *Proc. Roy. Soc. B.* **81**, 336.  
 Lebedev (1912). *Ann. Inst. Past.* **26**, 16.  
 Meyerhof (1918). *Zeitsch. physiol. Chem.* **102**, 85.  
 Neuberg (1915). *Biochem. Zeitsch.* **71**, 75 and 83.  
 — (1918). *Biochem. Zeitsch.* **88**, 145.  
 Neuberg and Kerb (1913). *Biochem. Zeitsch.* **58**, 158.  
 Oppenheimer (1915). *Zeitsch. physiol. Chem.* **93**, 235.

# LVIII. THE DISTRIBUTION OF INORGANIC IRON IN PLANT AND ANIMAL TISSUES.

BY HENRY WALLACE JONES.

(Received August 8th, 1920.)

## EXPERIMENTAL METHODS.

IN carrying out tests for the detection of inorganic iron in the tissues, two points must be borne in mind, firstly, the previous preparation of the tissue, and, secondly, the application of the reagents so that false results are not obtained.

With plant tissues when the question is not the structural arrangement but rather whether this or that cell-constituent contains iron, it is better to work with finely teased or broken-up tissues. Glass rods, drawn to a point, were used for this purpose, and also in order to break up some of the green cells and set free the chloroplasts, a portion of the tissue in each case was still more broken up by turning upon it the blunt end of the glass rod and grinding it between this and the microscope slide on which it was being mounted.

In tissues which contained a large quantity of chlorophyll, or where it was desired to stain the stroma of the chloroplast for inorganic iron, the tissues were first boiled in absolute alcohol several times until they became colourless, the greenish extract being poured off with each fresh quantity of alcohol.

In the case of animal tissues, organisms of small size can be mounted direct, but with larger tissues sections have to be cut. The tissues for this purpose were hardened in absolute alcohol for 24 hours, and then embedded in paraffin.

The sections were mounted on slides and after removing the paraffin by xylene, were "taken down" the alcohol-water series before staining.

Preliminary observations were made upon plant tissues, with the ammonium sulphide and ferrocyanide methods.

## AMMONIUM SULPHIDE METHOD.

This method was introduced by Vogel in 1845. The solution used was one containing ammonium hydrogen sulphide, prepared by passing sulphuretted hydrogen through dilute ammonia having a specific gravity of 0.96. The teased tissues were placed in this solution (which must be freshly prepared) for three or four hours, and then mounted in the stain, ringing the coverslip with gold size.

The inorganic iron in the tissues is stained brownish-black, while the rest of the tissues are stained a lighter brown.

The chief disadvantages of the method are: firstly, the difficulty of recognising the iron when present in small quantities, because, the rest of the tissue being stained brown, it is only by very careful comparisons in tissues of equal thickness that the presence of iron can be detected with certainty; and secondly, permanent sections can only be made with difficulty.

#### FERROCYANIDE METHOD.

This method was first used by Perls [1879] and later by Molisch [1893], who was the first to differentiate between inorganic and "masked" iron.

The tissues are placed in a freshly prepared solution of 1.5 % potassium ferrocyanide and a 5 % solution of hydrochloric acid, for three or four hours, and then mounted in the stain and the coverslip ringed with gold size.

It is advisable to use ferrocyanide solution rather than ferricyanide, as the iron is chiefly present in the ferric form, and consequently then gives "Prussian blue" at once. When treated in this way the tissues tend to vary in their depth of staining, this becoming more intense during the first few days, especially if exposed to day-light, and then gradually fading to a much lighter colour.

Another disadvantage of this method is, that if permanent sections are made in Canada balsam, they fade rapidly, this being probably due to the reduction of the stain by means of the balsam forming a compound analogous to  $\text{Fe}_2\text{Fe}(\text{CN})_6$ , which is white in the absence of oxygen [Mann, 1902].

#### HAEMATOXYLIN METHOD.

This method, which was introduced by Macallum [1897], depends on the fact that when a solution of haematoxylin in pure distilled water is mixed with a very dilute solution of an ordinary iron salt, such as ferric chloride, a deep blue-black coloration is immediately produced.

If, instead of a solution of an ordinary iron salt, a solution of highly colloidal iron or dialysed iron hydroxide be mixed with the stain, a brownish colour is produced, while if organic iron is used, no change in colour takes place. This fact has been explained by Roscoe and Schorlemmer as due to the organic iron compounds having all their possible valences united to carbon atoms.

This staining as a test for iron is quite different from the ordinary use of haematoxylin as a nuclear stain, in which case a mordant is always used, either preceding the haematoxylin, as for example, the iron-alum mordant in Heidenhain's haematoxylin-iron method, or simultaneously, as in the use of the haem-alum stain.

In Macallum's process no mordant whatever is used, but only a solution of haematoxylin in pure distilled water. This gives the colour change only where

a mordant, *e.g.* iron, is naturally present in the tissues. In using it not only must even minutest traces of iron in the water and other fluids be avoided, but also all traces of alkali and acid, since these interfere with the delicacy of the reaction—alkali gives a rose-red colour with the haematoxylin, and acid inhibits the development of the blue-black colour when the amount of iron is small.

To make the staining solution 0.3 gram of pure haematoxylin is dissolved in 50 c.c. of twice distilled water, and kept in Jena glass flasks, since the alkali dissolved out from ordinary glass rapidly turns the solution pink.

Plant tissues, after being prepared, were stained for 12 hours in this solution, while sections of animal tissues were stained for 48 hours.

This was found to be by far the most satisfactory method for the detection of the inorganic iron, and was used in all the animal tissues, and nearly all the plant tissues investigated. Sections so stained can readily be made permanent by dehydrating with alcohol, and mounting in Canada balsam.

## EXPERIMENTAL RESULTS.

### *Section A. Plant Tissues.*

In examining plant tissues, it was found that taken as a whole they gave the iron reactions more rapidly and intensely than animal tissues.

In the various grades of plant life it was found that the lower organisms gave a more intense reaction than those higher in the scale. The staining occurred in three different places—in the nuclei, in the chloroplasts, and in large masses scattered throughout the cytoplasm. Leaves of the wall-flower did not show any definite iron staining. Leaves of ordinary water-cress, gave a very definite reaction for iron; large masses, stained bluish-black, were scattered irregularly throughout the leaves, and many of the chloroplasts were also stained.

Descending lower in the scale, sections of various types of sea-weed were examined. The majority of these showed definite iron staining, particularly in the nuclei and the chloroplasts. The staining in the nuclei was specially well marked, while in some of the sections there were also large deeply-stained masses irregularly situated in the cytoplasm.

The reactions for inorganic iron were most marked with the lower plants, such as unicellular green plants, isolated or bound together in delicate alga threads. The algae observed were *Vaucheria*, *Spirogyra* *Ulva*, and *Ulothrix*. The nuclei and chloroplasts took on a very deep bluish-black colour very readily; the rest of the cytoplasm was usually unstained, but sometimes patches giving the brown colour of colloidal iron were observed.

Diatoms readily took on a dark blue colour, the central part of the cell more intensely than the rest.

*Section B. Animal Tissues.*

In this series the maximum amount of staining occurred in the lower organisms, *e.g.* Crustacean and Molluscan types, while the Mammalia gave only slight reactions.

In adult *Mammalian tissues* taken from guinea-pigs, sections of the liver showed only very slight iron-staining. This occurred chiefly in the nuclei of the cells in the form of minute, darkly-stained granules, readily visible with the  $\frac{1}{13}$ " objective.

In sections of the stomach, the epithelial cytoplasm was unstained, but the granules in the nuclei, specially the nuclei of the muscle fibres, were well stained.

The spleen shows very definite staining, in the form of small irregular granules scattered in large numbers throughout the tissue; granules in the nuclei are also stained.

Sections of the kidney, testicle, and ovary show only the granular staining in the nuclei, except that in the kidney there is also some slight staining of the glomeruli.

In all the mammalian tissues the staining is more marked in the cells which immediately surround the blood vessels.

In *foetal tissues*, taken from the guinea-pig, the reaction was in most of them much more readily obtained than in adult tissues.

In the foetal liver, both the granules scattered throughout the cytoplasm and those in the nuclei were more deeply stained than in the adult liver.

In the foetal kidney the staining was similar to that found in the adult.

The spleen gave very definite reactions, both the granules in the nuclei and the granules irregularly scattered throughout the cytoplasm being more deeply stained than in adult tissues.

*Placental tissues*, also taken from the guinea-pig, showed very definite staining in patches in the chorionic villi, while most of the nuclei were also stained.

In *human blood smears* only a slight blue coloration of the red cells took place, not sufficient to indicate the presence of inorganic iron.

In *avian tissues*, taken from the sparrow, inorganic iron could be very readily detected. In the liver the sections showed large numbers of darkly stained granules scattered throughout the cytoplasm and also in the nuclei. The cytoplasm (when using a high magnification) also shows very minute smaller granules which give an intense reaction for inorganic iron.

In the kidney, the glomeruli and the lumina of the convoluted tubules are well stained.

In the spleen, no staining could be detected, and in the ovary staining was confined to the nuclei.

These avian tissues on the whole, specially the liver, gave a much more definite reaction for iron than did the mammalian tissues.

In *amphibian tissues*, taken from the frog, the reactions were still more marked. In the liver, there were large numbers of small darkly-stained granules, scattered throughout the cytoplasm, in addition to the well-known pigment masses which occur in unstained sections of frog's liver.

In the kidney, the chief staining took place in the granules of the nuclei.

The spleen showed small darkly-stained granules scattered throughout the cytoplasm, in addition to large masses of pigment like those found in the liver.

The cytoplasm of the red corpuscles was stained a brownish colour, suggesting the presence of colloidal iron, while inorganic iron was present in small granules in the nuclei. The nuclei of the leucocytes were stained dark blue.

In tissues taken from *gold-fish* the hepato-pancreas treated with haematoxylin showed two or three deeply-stained small round granules in the cytoplasm of each cell—these granules were independent of the nucleus and were found in unstained sections to be highly refractile and colourless. They were also strongly stained by the ferrocyanide method. Sections of the kidney and ovary showed similar granules, but not to the same degree. In its blood films the red corpuscles were stained brown, presumably owing to the presence of colloidal iron; the rest of the film was unstained.

In the *cray-fish* the hepato-pancreas gave a very well-marked reaction, the granules in the nuclei being more deeply stained than in any other animal tissue examined. There were also well-stained granules scattered throughout the cytoplasm.

In the *gonidia*, the ova showed a well-marked reaction.

The staining was more intense in cray-fish tissues generally than in any of the higher animals studied.

In the *oyster*, the hepato-pancreas and the gills showed numerous granules scattered through the cytoplasm, and well-stained nuclei.

In the *earth-worm*, the granules in the cell nuclei were extremely well shown and the cuticle was also well stained.

In *hydra* the chloroplasts in many of the cells show a well-marked bluish stain.

In *swimming prawns* and "*plankton*," the swimmerettes are stained uniformly deep blue.

In *sagitta*, the body and tail are also stained deep blue.

#### CONCLUSIONS.

1. Inorganic iron is more widely distributed throughout animal and vegetable tissues than is generally realised.

2. The lower plants and animals give the reaction for inorganic iron much more strongly than do the higher ones.

3. Granules containing inorganic iron are present in almost all the nuclei of plants and animals.

4. Aquatic animals, either marine or fresh water, contain more inorganic iron than those living on land.

5. Foetal tissues contain more inorganic iron than do adult tissues.

## REFERENCES.

- Macallum (1897). *J. Physiol.* **22**, 92.  
Mann, (1902). *Physiological Histology*, 292.  
Molisch (1893). *Ber. deutsch. bot. Ges.* **12**, 73.  
Perls (1879). *Virchow's Archiv*, **39**, 42.

# LIX. THE NOMENCLATURE OF THE SO-CALLED ACCESSORY FOOD FACTORS (VITAMINS).

BY JACK CECIL DRUMMOND.

*From the Institute of Physiology, University College, London.*

*(Received August 12th, 1920.)*

IN 1912 Hopkins published his classical paper in which he described the important influence of certain dietary constituents on the processes of growth and nutrition. These substances he termed the "accessory factors of the diet." At about the same time Funk, who was working on the subject of experimental beriberi, coined the name "Vitamine" for the same class of substances. Since then the literature has been a good deal confused by the great variety of names which have been utilised to denote these or similar dietary constituents (auximones, Bottomley; nutramines, Abderhalden, etc.). The criticism usually raised against Funk's word Vitamine is that the termination "-ine" is one strictly employed in chemical nomenclature to denote substances of a basic character, whereas there is no evidence which supports his original idea that these indispensable dietary constituents are amines. The word has, however, been widely adopted, and therefore until we know more about the actual nature of the substances themselves, it would be difficult and perhaps unwise to eliminate it altogether. The suggestion is now advanced that the final "-e" be dropped, so that the resulting word Vitamin is acceptable under the standard scheme of nomenclature adopted by the Chemical Society, which permits a neutral substance of undefined composition to bear a name ending in "-in." If this suggestion is adopted, it is recommended that the somewhat cumbrous nomenclature introduced by McCollum (Fat-soluble A, Water-soluble B), be dropped, and that the substances be spoken of as Vitamin A, B, C, etc. This simplified scheme should be quite sufficient until such time as the factors are isolated, and their true nature identified.

# LX. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. III: TECHNIQUE FOR CARRYING OUT FEEDING TESTS FOR VITAMIN A (FAT-SOLUBLE A).

BY JACK CECIL DRUMMOND AND KATHARINE HOPE COWARD.

*From the Institute of Physiology, University College, London.*

*(Received August 12th, 1920.)*

IN a recent communication Osborne and Mendel [1920] referred to the experiments of Steenbock, Boutwell and Kent [1918], and of Drummond [1919, 1] on the destruction of the fat-soluble accessory factor by heat. They were unable to confirm these observations, and in other respects there were also discrepancies for which the reason was not apparent. We have made a careful examination of these discrepancies, and believe that satisfactory explanations are available. Of the difference between the opinions on the effect of heat on the nutritive value of butter we have little to say in this paper, because a later communication will be devoted entirely to that subject; it may, however, be said in passing that we have evidence that the destruction of fat-soluble A is an oxidation process, which confirms what has already been stated by Hopkins [1920]. It is discrepancies of another type which we wish to discuss in this paper, such for instance as the considerable differences noted between the amounts of butter fat required to supply the fat-soluble vitamin for growing rats, to which attention was drawn by Osborne and Mendel [1920]. At first our tendency was to ascribe such differences to variations in the food value of the samples of butter used by the separate investigators, but after giving the matter more careful attention, we have formed the opinion that this undoubted source of disagreement is frequently accompanied by one of a more serious nature, and one, fortunately, that can be readily eliminated. We refer to the very different types of basal diet used in various laboratories. In a previous paper of this series [Drummond, 1919, 2], an experimental method was described by which substances may be tested for the presence of the fat-soluble factor. This method was used for some time with success, but we were led to introduce certain improvements which are described here, and which may be helpful to other investigators who encounter the many difficulties which surround this type of research.

There appears to be little or no need to emphasise the importance of employing carefully selected animals for feeding tests of this nature, for one gathers from the published results that the majority of investigators fully

appreciate this point. In our opinion, however, it is frequently the composition of the basal dietary which is responsible for many of the misleading and contradictory statements which tend to confuse the literature on the vitamins at the present time.

Evidence has been produced which tends to show that the requirements of the growing rat for vitamin A become less as the animal approaches maturity [Drummond, 1919, 2], and our experience leads us to believe that the amount of vitamin which must be supplied to a rat in order to restore growth which has been inhibited by feeding on the deficient basal diet, will be inversely proportional to the weight of the animal.

Should this be confirmed, we think many of the discrepancies in the literature will be accounted for.

#### PREPARATION OF PURIFIED BASAL DIETS.

##### i. *Preparation of Pure Protein.*

Up to the present we have made a practice of using only highly purified caseinogen as a source of purified protein in our basal dietaries. We have done this following the general scheme used by many other investigators, but we are now considering whether it would not be advisable to employ another protein of equal or superior tissue building value, and one less prone to be contaminated with the fat-soluble factor. A search for such a protein is being made. Commercial caseinogen contains relatively large amounts of the fat-soluble vitamin, and should never be used for experiments relating to that factor without having been carefully purified. We have encountered numerous cases in which prolonged growth of young animals was observed in young rats which were fed upon a diet supposedly free from fat-soluble A, but which were in reality obtaining considerable supplies of the vitamin from the insufficiently purified caseinogen.

We heat our caseinogen for 24 hours or more in shallow dishes to a temperature of 102° C., after which it is subjected to a prolonged and continuous extraction with alcohol and ether.

##### ii. *Purity of Carbohydrate.*

In the past we have gone to the expense and trouble of preparing a highly extracted form of wheat starch to use as a source of carbohydrate in the basal diets. Later experiments have shown us however that rice starch in the crude form is almost entirely devoid of fat-soluble A, and may be employed without any lengthy and costly preliminary extraction.

##### iii. *Purity of Fat.*

In the selection of a fat to include in the basal fat-soluble-free diet the greatest care must be employed. As will be shown in the following paper the natural oils and fats which are usually supposed to be free from vitamin A,

are not always so, and disconcerting variations in a single oil may be encountered. As a consequence of many such conflicting results we have carefully reinvestigated this question, and have ascertained that no hard and fast line can be drawn between the animal and vegetable oils and fats, when their growth-promoting powers are being considered. We therefore think it advisable that no natural oil or fat should be used as a source of fat in a diet intended to be free from fat-soluble A. To overcome this difficulty we employ a fully hardened (hydrogenated) and refined vegetable oil, usually cotton seed oil. Such oils consist very largely of tristearin, and are, so far as we can ascertain from carefully controlled feeding tests, entirely devoid of vitamin A. It is possible that even greater security might be obtained by excluding fat entirely from the basal diet, in view of the fact that rats appear to be able to dispense with the presence of pure fats in their diet [Drummond, 1919, 2; 1920].

#### iv. *Purity of other Constituents of Basal Diet.*

The orange juice and salt mixture which we include in the basal ration have been proved by direct tests to be devoid of vitamin A. The yeast extract is also thought to be equally inactive in that respect, although definite results have not yet been obtained to make quite sure of this point.

The composition of the purified basal ration which we employ in all our routine tests is given below:

Purified caseinogen...	...	...	18 parts
Purified rice starch...	...	...	52 ,,
Refined hydrogenated vegetable oil			15 ,,
Yeast extract	...	...	5 ,,
Orange juice	...	...	5 ,,
Salt mixture	...	...	5 ,,

Rats fed upon this diet behave in somewhat different manner according to their age [Drummond, 1919, 2]. Small rats of 50–70 g. (4–5 weeks old) should show very little growth at all, and should remain stable for a week or two after the slight initial growth. Any considerable increase of body weight in rats of this age when fed upon a purified diet of this composition is interpreted by us as an indication that the basal ration is insufficiently purified. Animals considerably over 100 g. are in our opinion unsatisfactory for testing for vitamin A, and as far as possible we attempt to test all fractions on rats the growth of which has been suspended for 10–14 days, and which are not heavier than 80–120 g.

#### TESTING SUBSTANCES FOR VITAMIN A.

As far as possible we make it a routine in this laboratory to test all substances for the presence of vitamins by administering a definite weight of the substance directly to the animal before the day's ration of the basal food is

given. In the large majority of cases this method, which is the only one permitting of an accurate quantitative measure of intake, is practicable. In cases where it is found to be difficult to carry this out, the supplement of foodstuff or fraction must be incorporated in the diet, and the intake judged by records of the total consumption of food.

#### RESULTS.

Even with the employment of the greatest care, this method occasionally gives results of doubtful significance. The enormous amount of routine work which is entailed by feeding large numbers of experimental animals by such a process necessitates in an average laboratory some restriction in the size of the experimental groups, a fact which tends to increase the error due to individual variation. Such errors are frequently encountered, and are sometimes disconcerting, but one usually obtains a definite result from the majority of the animals in a particular group.

As we have previously remarked, we are of the opinion that insufficient purification of the basal diet is responsible for many misleading results.

In the curves given by Osborne and Mendel [1920], it will be observed that some of the young rats grew for a considerable time, and attained a fairly heavy weight before they showed the typical decline due to deficiency of vitamin A. This means that when the rats are fit to use for feeding tests they are often considerably over 100 g. in weight. Such animals would presumably recover health and recommence growing on receiving a much smaller amount of the missing vitamin than would be necessary to restore a declining rat of 60–90 g. Further, the former animal, although it had ultimately declined on a deficiency of fat-soluble A, would still be obtaining some fat-soluble factor in the impure basal diet in addition to that contained in the supplement. The observed result might therefore lead one to ascribe a higher food value to the supplement than was justifiable.

Our own experience has given us many examples of how the presence of very small amounts of the factor A in an insufficiently purified basal diet may confuse the issue of the experiment.

#### SUMMARY.

In testing foodstuffs for the presence of the fat-soluble vitamin the greatest care should be devoted to ensuring that the basal dietary is rendered as free from that vitamin as possible. Details for the preparation of a highly purified ration are given. Failure to work with a sufficiently pure diet may lead to conflicting and misleading results.

#### REFERENCES.

- Drummond (1919, 1). *Biochem. J.* **13**, 81.  
— (1919, 2). *Biochem. J.* **13**, 95.  
— (1920). *J. Physiol.* (in the press).  
Hopkins (1920). *Brit. Med. J.* **ii**, 147.  
Osborne and Mendel (1920). *J. Biol. Chem.* **41**, 549.  
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **35**, 517.

# LXI. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. IV: NUTS AS A SOURCE OF VITAMIN A.

By KATHARINE HOPE COWARD AND JACK CECIL DRUMMOND.

*From the Institute of Physiology, University College, London.*

*(Received August 12th, 1920.)*

THE examination of a large number of animal and vegetable fats and oils for the presence of the fat-soluble A accessory food factor suggested to the authors the desirability of investigating raw nuts, known to be rich in fats, for the same vitamin. Certain nut butters had already been examined by Halliburton and Drummond [1917], who had come to the conclusion that they were not equal to butter in this respect, but that there were "certain indications which made it probable that the nuts themselves do in some cases contain the accessory factors in small amount." The literature has not provided accounts of any similar work on the subject, although the vitamin B has for some time been known to occur in large quantities in the coconut and pea-nut [Daniels and Loughlin, 1917; Johns, Finks and Paul, 1919], and, more recently, has been found in the almond, English walnut, Brazil nut and chestnut by Cajori [1920].

## EXPERIMENTAL METHOD.

The method adopted for the investigation was the one usually followed in this laboratory. Rats of 50-70 g. weight were fed on an unlimited allowance of a basal diet consisting of

Purified caseinogen	...	18	grs.
Purified starch	... ..	52	"
Salt mixture	... ..	5	"
Yeast extract (marmite)	... ..	5	"
Hardened cotton seed oil	...	15	"
Orange or lemon juice	...	5	cc.

mixed with sufficient cold water to form a stiff paste. Fresh water for drinking and cleaning purposes was supplied daily. The rats were weighed twice a week and when they had ceased growth and their weights had remained practically stationary for four successive weighings, they were fed daily with

approximately 1 g. per rat of the nut to be tested. The eagerness with which the rats came to eat their nuts made this a comparatively simple matter, most of them consuming their ration immediately, and in only one experiment, that of the almonds, were any portions left uneaten on the following morning.

### RESULTS.

The weights in g. of the rats on the nuts tested are given in the following table.

Nuts		Number of days after commencement of nut supplements									
		0	4	7	10	14	18	21	25	28	32
Pea-nuts 38.6 % fat	Rat 80 ♀	110	120	118	117	114	109	109	110*	110	110
	" 81 ♀	80	86	81	86	92	91	91	100	101	100
	" 82 ♀	78	82	79	79	81	78	80	77*	80	74
Brazil nuts 66.8 % fat	" 83 ♂	107	120	120	99	102	118	120	120		
	" 85 ♀	92	100	97	99	99	100	99	102*		
	" 84 ♀	80	90	89	90	95	94	95	98*		
Butter nuts 60 % fat	" 55 ♀	72	77	71	67	70					
	" 54 ♀	88	92	93	92	90					
	" 56 ♀	86	104	104	107	104					
Barcelona nuts	" 80 ♀	110	120	118	125	123					
	" 81 ♀	100	100	107	109	109					
	" 82 ♀	74	62	70*							
Almonds 55 % fat	" 83 ♂	122	124	130	140	130*					
	" 85 ♀	106	101	100	101	98					
	" 84 ♀	93	94	96	91	89					
Walnuts 62 % fat	" 55 ♀	76	79	78	81	81					
	" 54 ♀	100	102	105	101	102					
	" 56 ♀	113	115	122	115	112					

Rats 80, 82, 84, 85, 83 developed a bad form of the characteristic eye disease associated with a deficient supply of vitamin A at points marked \*.

### DISCUSSION.

The results are interesting in view of the opinion which is steadily gaining ground that vitamin A is found most abundantly in actively assimilating green plant tissues and to a much smaller extent in resting tissues, such as occur in all seeds, and the special food storehouses in the roots and stems of certain plants. The nuts used were chosen somewhat at random from those usually considered desirable by vegetarians, many of them, as shown in the table, having a high fat content. Structurally, they show some variation. The almond, walnut, pea-nut and Barcelona nut, although not strictly speaking all true "nuts," are true seeds and show well-defined plumule and radicle, while the mass of the seed is filled with cotyledons containing the reserve food store. The Brazil nut has a mass of endosperm in which are embedded the growing points, not fully enough developed to be visible to the naked eye; and the butter nut has probably a similar structure. As the results

showed an almost complete absence of vitamin A from all the six kinds of nuts, no distinction can be drawn between tissues which are purely reserve tissues and those which have potentially another function, such as assimilation, as have the cotyledons of the almond. This suggests that the vitamin must be developed at some stage subsequent to germination, and the authors hope shortly to publish results of work which support this view.

In view of the recent work on the thermostability of vitamin A, another point had to be investigated. Inquiry in one direction showed that pea-nuts are "roasted" before being sent to the market, though at what temperature or for what length of time, no exact information could be obtained. On the other hand another source of information denied any roasting of the nuts previous to their appearance on the market. English walnuts are dried in the open air, but there are no grounds for thinking that this process or that which Brazil nuts, Barcelona nuts, butter nuts and almonds normally undergo before they are sold as food causes any destruction of vitamin A.

The nuts were tested for the presence of the lipochromes, carotene and xanthophyll, by the method described in the previous paper on fats and oils by the authors. In no case was more than a mere trace of pigment found, and this failed to give characteristic lipochrome reactions with strong sulphuric acid, nitric acid, and iodine in potassium iodide solution.

#### CONCLUSIONS.

1. The nuts examined, Brazil, Barcelona, pea, walnuts, almonds and butter nuts, although containing large percentages of fats, possess a relatively low food value as sources of the vitamin A often associated with fats.
2. These results furnish additional evidence for the theory that vitamin A is formed in the green part of the living plant and is not stored to any appreciable extent as such in the seed and other resting tissues.

#### REFERENCES.

- Cajori (1920). *Proc. Soc. Exp. Biol. Chem.* **17**, 65.  
Daniels and Loughlin (1917). *J. Biol. Chem.* **33**, 295.  
Halliburton and Drummond (1917). *J. Physiol.* **51**, 235.  
Johns, Finks and Paul (1919). *J. Biol. Chem.* **37**, 497.

## LXII. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. V: THE NUTRITIVE VALUE OF ANIMAL AND VEGETABLE OILS AND FATS CONSIDERED IN RELATION TO THEIR COLOUR.

BY JACK CECIL DRUMMOND AND KATHARINE HOPE COWARD.

*From the Institute of Physiology, University College, London.*

*(Received August 12th, 1920.)*

MORE than one investigator has recently called attention to the apparent relationship between the presence of certain yellow pigments in foodstuffs and the occurrence of the fat-soluble accessory factor.

Steenbock [1919] traced this association in a number of natural foodstuffs, but also pointed out that certain exceptions occurred in which the presence of the fat-soluble factor was not associated with yellow pigments. To cover such exceptions he suggested that the colouring matters might be present in the form of a leuco-compound. This theory was criticised by Palmer and Kempster in an interesting series of papers [1919, 1, 2, 3], in which they showed that the natural yellow pigment of fowls, which is derived from the xanthophyll of the foods, bears no important relation to growth, or to the functions of fecundity or reproduction, at least for one generation.

Rosenheim and Drummond [1920] showed that the experiments of Palmer and Kempster were open to some criticism, and that certain of their results might be interpreted as evidence that the lipochrome pigments played a rôle in nutrition. In this paper some support was given to the theory advanced by Steenbock, but it was definitely shown that fat-soluble A is not identical with either of the two chief lipochromes, carotene and xanthophyll.

In one criticism advanced by Palmer [1919] against the association theory he pointed out that certain vegetable oils (*e.g.* cottonseed oil), which are supposed to be rich in lipochromes, are destitute of the vitamin.

This criticism appeared to be a reasonable one and stimulated us to re-examine the nutritive values of certain animal and vegetable oils and fats. It was obvious that a useful index of the food value of such fats would be in our possession, if it were definitely found that the presence of the fat-soluble factor is associated with certain yellow pigments. In order to test the accuracy

of Palmer's criticism we decided to investigate the growth-promoting power of a vegetable oil which is deeply coloured with lipochrome pigments, namely, palm oil.

Several young rats which were in failing health, as a result of continued maintenance on a diet deficient in vitamin A, were given a diet containing 20 % of a deeply pigmented sample of palm oil. The change of diet brought about an immediate and striking recovery; in fact, one rat of this series showed a more rapid recovery of health and clearing up of the well-known eye disease than we had ever before encountered.

As soon as this result was confirmed, it was obvious that the whole question of the relative food value of the animal and vegetable oils must be reinvestigated. The earlier experiments, made before the technique of conducting such feeding tests was as good as it is now, had led us to consider that the vegetable oils as a class contain little or none of the factor A, whereas animal fats, with the exception of lard, are good sources of this indispensable substance [Experiments and bibliography, Halliburton and Drummond, 1917].

Accordingly a large number of animal and vegetable oils, several of which had not been previously investigated, were tested, both for the presence of the growth-promoting factor A, and for the presence and nature of any accompanying pigments. The results, which are given in tabular form in Table I (and in Curves 1-22, Figs. 1, 2 and 3), demonstrate clearly that we have not been justified in drawing a hard and fast line between animal and vegetable oils and fats, when considered from the point of view of their vitamin content.

Many of the vegetable oils contain appreciable amounts of the A factor, although as a class they are considerably inferior to the majority of animal fats, such as butter or cod liver oil. Our experiments make it clear, however, that unless we accept the suggestion advanced by Steenbock, of the existence of a leuco-compound of the pigment, to account for the exceptions, the theory of the association of yellow pigments of the lipochrome class with the presence of vitamin A fails to hold good. This is particularly well seen in the case of one or two animal fats, such as the pigment-free dog fat, which we found to be a relatively rich source of A (Curves 11 and 12, Fig. 1).

#### THE PRESENCE OF THE FAT-SOLUBLE ACCESSORY FACTOR IN CERTAIN VEGETABLE OILS.

Our early results with palm oil naturally caused us to devote considerable attention to the vegetable oils. It has been suggested by McCollum, Simmonds and Pitz [1916] that the fat-soluble factor may exist in certain plant tissues in the form of a complex, which must be decomposed before the fat-soluble factor can be obtained in a truly "fat-soluble" form, a process which appears to be brought about very readily, as by treating the crushed seed with alcohol. The amount of the fat-soluble factor in oils derived from seed will therefore depend upon the amount originally present in the seeds, and

the treatment which the seeds have undergone prior to the extraction of the oil. Thus, in considering our results with palm oil, it must be remembered that the majority of the samples at present obtainable have been prepared by the crude native process. This consists in throwing the broken fruit into pits dug in the ground and lined with leaves, where they are left for some days until the flesh softens, so that the kernels may be readily separated. During this time the flesh undergoes fermentation processes, and the oil is finally

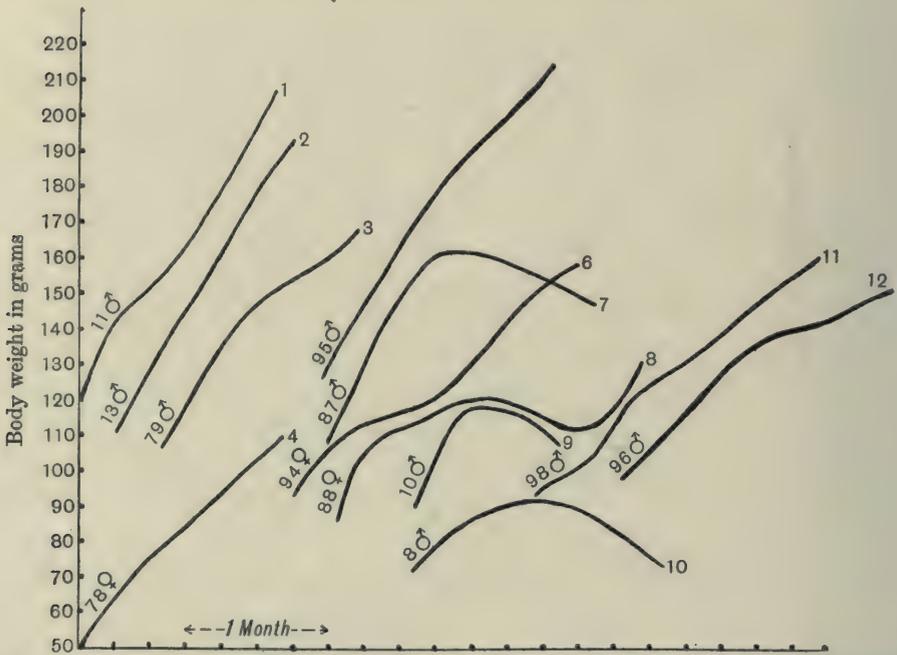


Fig. 1. In every case the curves show only the behaviour of the animal after the supplement has been given. The preliminary period during which growth was inhibited on a deficiency of vitamin A is omitted for simplicity.

Curves 1 and 2. Rapid recovery of growth by addition of stable-fed horse fat equivalent to 10 % of the diet.

Curves 3 and 4. Slightly less rapid recovery on receiving the same amount of a deeply pigmented sample of grass-fed horse fat.

Curves 5 and 6. Recovery of growth on supplement of 15 % abdominal fat of pig (there was one marked failure to recover in this series).

Curves 7 and 8. Evidence that mutton fat contains small amounts of vitamin A. Results of feeding a supplement of 15 %.

Curves 9 and 10. Failure to recover on 15 % subcutaneous fat of pig.

Curves 11 and 12. Fairly good recovery maintained by a supplement of 15 % of dog-body fat; a pigment-free fat.

separated by beating the residue to a pulp and straining. Whether the vitamin exists free in the flesh of the palm nut, or whether it is present as a complex, from which it is liberated during the fermentation, is not known, but experiments to determine this point are contemplated.

The pigment of palm oil is very largely carotene, accompanied by a small

amount of xanthophyll. Of the other vegetable oils examined, only maize and linseed gave definite tests indicating the presence of appreciable quantities of carrotene and xanthophyll.

Table I.

Oil or fat	Remarks	Rough approximation of value as source of vitamin A	Lipochrome pigments		
			Total	Carro- tene	Xantho- phyll
Butter ... ..	Average sample ... ..	10	10	8	2
„ ... ..	Dark sample (Darlington) ... ..	10	30	24	6
„ ... ..	Pale „ „ ... ..	10	15	12	3
„ ... ..	“Strawberry,” 1 week winter feed	10	10	8	2
„ ... ..	„ 2 weeks „ „	8	2.5	2	0.5
„ ... ..	„ 2 weeks grass ... ..	10	11	8	3
Cod liver oil ... ..	Average sample ... ..	10	—	—	—
Dog-body fat ... ..	Subcutaneous ... ..	6-7	0	—	—
Beef fat ... ..	„ ... ..	6-8	—	—	—
Mutton fat... ..	Average ... ..	2	0	—	—
Pig fat ... ..	Subcutaneous ... ..	1	0	—	—
„ ... ..	Perinephritic ... ..	5-6?	0	—	—
Lard ... ..	Refined ... ..	0	0	—	—
Horse fat ... ..	Stable fed ... ..	6-8	1	1	0
„ ... ..	Grass fed ... ..	6-7	65	50	15
Linseed oil... ..	Average sample ... ..	1-2	16	6	10
Hardened linseed oil	„ ... ..	0	0	—	—
Palm oil ... ..	Very dark ... ..	3-4	76	57	19
Maize oil ... ..	Bright yellow ... ..	2-3	46	23	23
Cottonseed oil ... ..	Average sample ... ..	1	0	—	—
Hardened cottonseed oil	„ „ ... ..	0	0	—	—
Peanut oil ... ..	„ „ ... ..	1	0	—	—
Sesame oil ... ..	„ „ ... ..	0	0	—	—
Olive oil ... ..	„ „ ... ..	0-1	0	—	—

*Note on Table I.* The figures which are given to represent the approximate food values must be regarded as very empirical. It is obvious that our technique is as yet insufficiently accurate to enable us to allocate relative food values. These numbers, based on butter as 10, are given, however, to indicate very roughly the order of the differences in the nutritive values of the various oils and fats, so that it can be seen that they do not run proportional to the amount of lipochrome pigments, and only refer to the actual samples examined.

The figures representing the amount of pigments present in the oils are derived from colorimetric estimations made on the unsaponifiable fraction from 10 g. of the oil. This fraction dissolved in 25 cc. of light petroleum was compared with a standard solution of potassium dichromate in a Hellige colorimeter. This method is one given by Wilstätter, and gives good results for estimations of carrotene and xanthophyll, when standard curves have been prepared from pure solutions of those substances. The proportions of carrotene and xanthophyll present were judged approximately by colour comparisons of the respective fractions obtained by the well-known phase-test with 80 % alcohol and light petroleum.

The pigments of cottonseed oil, sesame oil and peanut oil appear to be of a different class.

## THE FAT-SOLUBLE FACTOR IN ANIMAL FATS.

Following our observation of the presence of the vitamin in highly-pigmented palm oil, we decided to test the food value of certain pigment-free animal fats. As will be seen from Curves 11 and 12, a sample of dog-body fat, which was devoid of pigment, showed considerable growth-promoting activity. This led us to re-examine the food value of lard. We again confirmed the absence of the fat-soluble factor from a highly-refined sample of lard, which was also pigment-free. When, however, we examined the fresh pig-body fat, purchased from a neighbouring butcher's shop, we obtained rather conflicting results, which indicated that this fat may contain the fat-soluble factor, particularly in the case of a sample from the abdomen (Curves 5, 6, 9, 10). Both samples were devoid of pigment.

As we knew nothing of the origin of these samples we felt that the results were insufficiently conclusive to enable us to arrive at any decision on what is obviously a most important point. We are, however, carrying out a number of feeding experiments on pigs, in conjunction with Capt. J. Golding, of University College, Reading, and Dr S. S. Zilva, of the Lister Institute, the results of which will shortly be published, and which we hope will provide a solution to the much debated question of the food value of lard.

## FOOD VALUE AND PIGMENTATION OF ANIMAL FATS.

The valuable researches of Palmer and Eccles [1914] have taught us much concerning the origin of the lipochrome pigments of the body fats and milk fats of animals. When we were examining the possibility of vitamin A being associated with the lipochrome pigments, we paid careful attention to their work. It will be remembered that by feeding a cow on a diet deficient in lipochrome pigments, they were able to obtain a butter almost devoid of pigment. As a result of the very kind co-operation of Dr L. Hamilton, Warden of Studley Agricultural College, Warwickshire, we were enabled to make a similar observation. A shorthorn cow which had been out at grass since the early part of the year was placed in stall on May 12th, and was given a winter feed, consisting of 80 lb. of mangolds, 50 lb. hay, 8 lb. cottonseed cake and 5 gallons of water daily. After seven days indoors the milk was collected for four days, and the cream made into butter. It was of approximately the same depth of colour as that made from the milk of the cows still out at pasture. After 14 days indoors the milk was again collected for a few days and made into butter. This sample was much paler than the previous one and of a much more granular consistency. The cow was then turned out of doors, and after two weeks more her milk again collected separately and made into butter. This had regained its normal colour and consistency. Quantitative measurements of the pigments present in each sample of butter (Table I) and of its growth-promoting activity were made. The latter were carried out by the technique described in the previous paper, substituting 3 % of the butter for

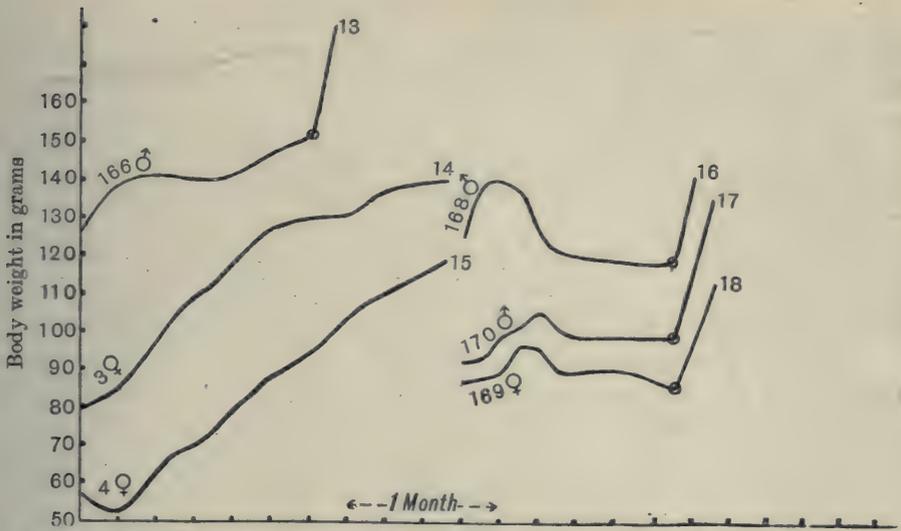


Fig. 2. Preliminary period on basal diet is not represented, all curves begin at point at which supplement was given.

Curves 14 and 15. Show prompt recovery with a supplement of 20% of a deeply-coloured sample of palm oil. One rat of this series, before it was given palm oil, was declining in weight and showed very severe external eye disease. This improved rapidly when the palm oil was given.

Curves 13 and 16. A supplement of 15% cottonseed oil has no marked beneficial effect on growth. From point  $\odot$  10% butter given instead of cottonseed oil.

Curves 17 and 18. Failure of supplement of 15% of sesame oil to promote growth. From point marked  $\odot$  10% butter given.

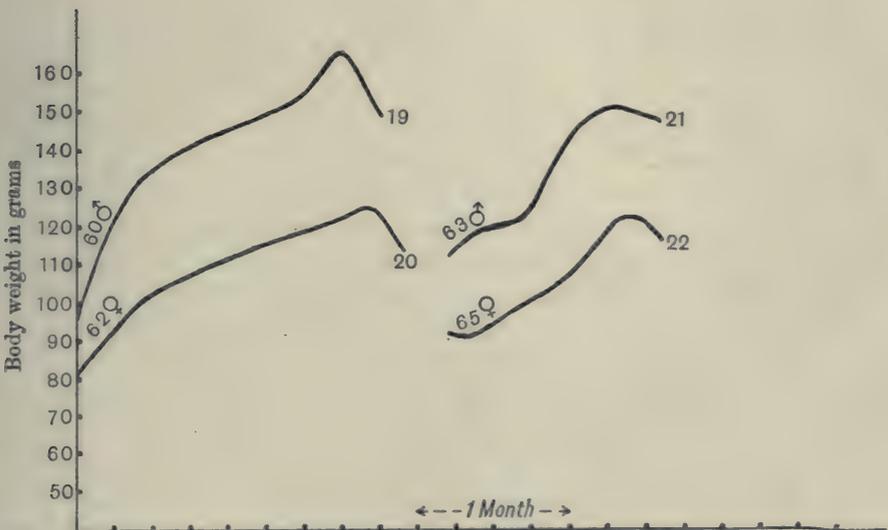


Fig. 3. The preliminary period on fat-soluble-free basal ration is omitted.

Curves 19 and 20. Some recovery, although temporary, on adding a supplement of 20% of maize oil (highly pigmented).

Curves 21 and 22. Same type of recovery on 15% of linseed oil.

the larger quantity of the hardened fat in the basal diet, this quantity being selected because experiments with an average sample of mixed butter had shown that it was just appreciably above the minimum at which growth is obtained, and therefore any falling off in the food value would be quickly observed. The control, in which 3 % of one of the butters was used in addition to 12 % of the hardened fat, showed no better growth than was given by the 3 % butter series alone.

It was found that corresponding with the large decrease in the amount of pigment in the butter there was a slight but appreciable falling off in the growth-promoting power. When put back on grass the cow gave a richly coloured butter, and there was at the same time a rise in its food value (Fig. 4). These results at first appeared to lend some support to the pigment theory, for they were obtained before the experiments with the colourless active animal fats were completed, but we observed a lack of proportion between the decrease of pigmentation and that of food value. This was confirmed by our experience with two samples of butter fat sent to us by Capt. J. Golding from the Royal Agricultural Show held in June this year at Darlington. By his kindness we obtained samples of the darkest and palest butters from the butter-making competitions held there. Neither sample contained added pigment. Our feeding tests showed that the growth-promoting power of the lighter coloured sample, which had a pale cream tint, appeared to be as good as, if not slightly better than, that of the darker sample, which possessed an unusually deep orange yellow colour (Table I). We were not able to obtain information as to the feeds of the cows from which the respective butters were made.

Similar results were given by two samples of horse fat, which were obtained from the carcasses of stable-fed and grass-fed horses respectively (Curves 1, 2, 3 and 4, Fig. 1). The results obtained by Palmer and Eccles [1914, 1, 2, 3, 4], and by Palmer [1915, 1916], are of value in an attempt to interpret these results. The latter paper showed that a definite physiological relation exists in all species of animals between the pigmentation of tissue fat with lipochrome pigments and the presence of these pigments in the blood serum. Those species, such as the cow, horse, hen, and man, the tissue fat of which is coloured with lipochrome, transport these pigments in their blood serum. Species, the tissue fat of which is colourless, such as the pig, sheep, and goat, carry only insignificant traces of lipochrome pigments in the serum. Certain unpublished experiments, made in conjunction with Dr O. Rosenheim, have shown us, however, that in both classes of animals considerable amounts of lipochromes are normally to be found in the liver. It therefore appears probable that in the digestion and absorption of plant foods such as green vegetables, which contain not only lipochromes, but also fat-soluble accessory factor, both vitamin and pigment pass to the liver, and may be held there in considerable amounts. What significance these observations may have is as yet uncertain.

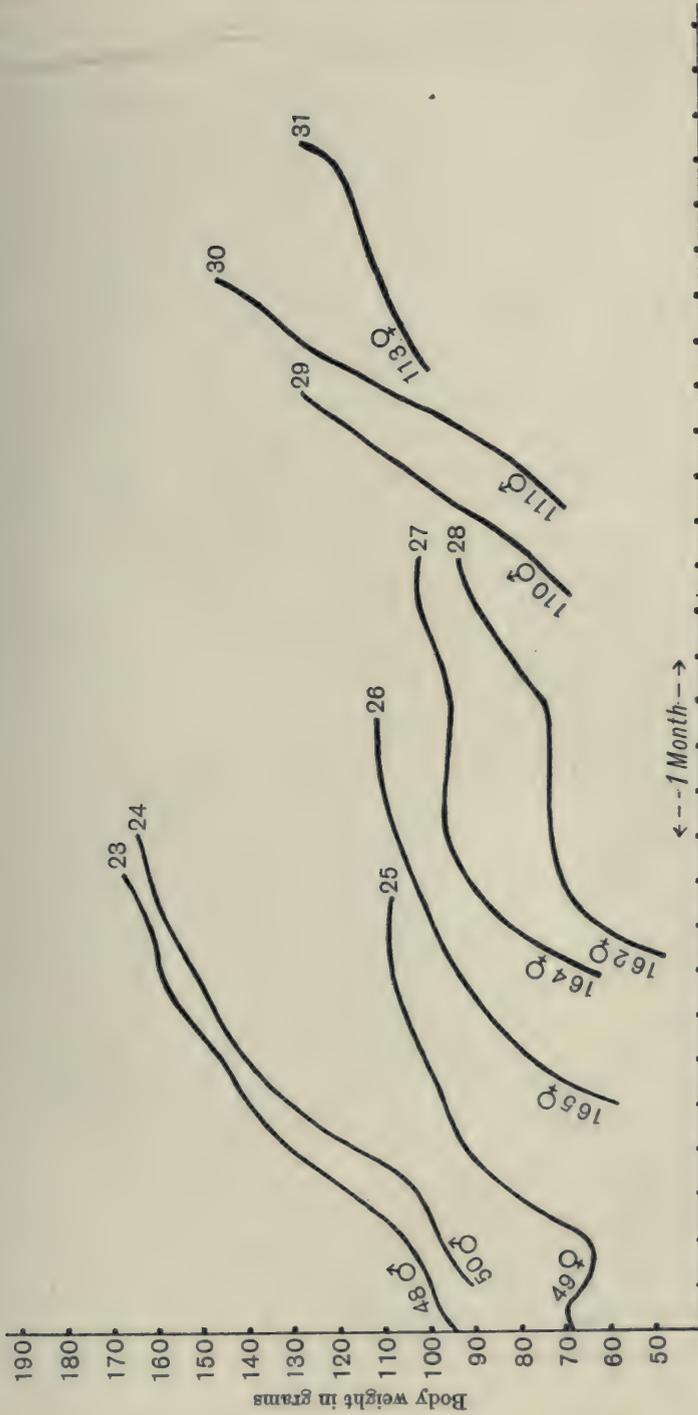


Fig. 4. The preliminary period of inhibited growth on the basal ration is omitted.

Curves 23-25. Recovery on being given supplement of 3% of butter from shorthorn cow, "Strawberry," after one week's winter feeding.

Curves 26-28. Recovery somewhat less complete on butter from "Strawberry," after 14 days' winter feeding.

Curves 29-31. Recovery more rapid than in either above cases on supplement of 3% of butter from "Strawberry," after 14 days' grass feeding.

Whether there will be any transference of the pigment to the body fat and subsequent storage there, will depend, as Palmer has shown, on the species of the animal, but probably all mammals are able to store up the fat-soluble factor to a greater or less extent in the fat depôts of their body. In other words, the fat-soluble vitamin and the lipochromes do not appear to follow the same paths after absorption into the animal organism. The experiments on pigs now in progress, to which reference has been made, will, it is hoped, provide more definite information on this point. No success has followed attempts to cause storage of lipochromes in the body fat of swine by feeding them on a diet rich in green fodder, and it remains to be seen whether such fats contain vitamin A.

The experiments outlined in this communication make it apparent that considerable caution must be observed in allocating a food value to an oil or fat, unless the sample has been tested by direct quantitative feeding experiments. On the whole the edible animal fats, such as butter and cod liver oil, appear to be of greater value in assisting growth than the common vegetable oils. But the idea that the latter are as a class nearly or completely devoid of fat-soluble A must be modified. What appears to us to be of primary importance is the appreciation of the fact that the nutritive value of a fat of animal origin is dependent on the diet of the animal from which it is derived, and that the food value of either animal or vegetable oils and fats may be considerably affected by the methods of preparation and refining. In agreement with the observations of Rosenheim and Drummond [1920], who demonstrated that fat-soluble A is not identical with either carotene or xanthophyll, we have now found that the presence of these lipochrome pigments in animal or vegetable oils and fats is neither a measure, nor even a reliable indication, of growth-promoting powers, as may be seen by comparing the estimations of pigments given in Table I with the growth curves. It remains to be seen whether there are any grounds for believing that the fat-soluble accessory factor is a pigment which may in some cases exist in a leuco-form, as suggested tentatively by Steenbock.

The research also gives us a preliminary result on the important question of the influence of winter feeding of cows on the food value of the butter they yield. In the short space of three weeks it was found that a diet low in vitamin A brought about a distinct fall in the nutritive value of the milk secreted. Experiments on a larger scale are already in progress, from which it is hoped to obtain more accurate and definite information on this important point.

We are indebted to Dr Rosenheim of King's College, London, for the loan of a Hellige colorimeter, and for standard curves which he had prepared. The expenses of this research were defrayed by a grant from the Medical Research Council, which the authors wish gratefully to acknowledge.

## SUMMARY.

1. No hard and fast line can be drawn between the animal and the vegetable oils and fats when their value as a source of vitamin A is being considered.

2. Taken as a class the animal fats possess a growth-promoting power superior to that of the vegetable oils, but we have observed that one or two members of the latter class (*e.g.* palm oil) may show considerable activity in that respect.

3. Unless we assume the existence of a leuco-form, it does not appear probable that the fat-soluble vitamin is a member of the lipochrome class of pigments. The frequent association of the growth factor with pigments of that type must therefore be regarded as accidental.

4. The nutritive value of an animal oil or fat would appear to be influenced considerably by the diet of the animal. One preliminary experiment shows that the winter feeding of cows may have the effect of lowering the food value of the milk unless considerable care is exercised in the selection of the animal's diet.

Probably the nutritive value of both animal and vegetable oils and fats is influenced by the processes of preparation and refining which they may undergo.

## REFERENCES.

- Halliburton and Drummond (1917). *J. Physiol.* **51**, 235.  
McCollum, Simmonds and Pitz (1916). *Amer. J. Physiol.* **41**, 361.  
Palmer (1915). *J. Biol. Chem.* **23**, 261.  
— (1916). *J. Biol. Chem.* **27**, 27.  
— (1919). *Science*, **50**, 1.  
Palmer and Eccles (1914, 1). *J. Biol. Chem.* **17**, 191.  
— (1914, 2). *J. Biol. Chem.* **17**, 211.  
— (1914, 3). *J. Biol. Chem.* **17**, 223.  
— (1914, 4). *J. Biol. Chem.* **17**, 237.  
Palmer and Kempster (1919, 1). *J. Biol. Chem.* **39**, 299.  
— (1919, 2). *J. Biol. Chem.* **39**, 313.  
— (1919, 3). *J. Biol. Chem.* **39**, 331.  
Rosenheim and Drummond (1920). *Lancet*, *i*, 862.  
Steenbock (1919). *Science*, **50**, 352.



## LXIII. A MODIFICATION OF THE BARCROFT AND WINTERSTEIN MICRORESPIROMETERS.

BY NEIL KENSINGTON ADAM<sup>1</sup>.

*From the Biochemical Laboratory, Cambridge.*

*(Received September 31st, 1920.)*

THE apparatus described in the present paper was designed for taking observations on the rate of absorption of oxygen by an isolated frog's muscle in any desired atmosphere, provision being made for simultaneous measurements of tension. The principle of the gas measurements is the same as that commonly employed in microrespirometric work, namely, the observation of the contraction in volume in a chamber containing the tissue, together with a little alkali, whose function is to keep the carbon dioxide tension negligible. To record the tension, a fine wire is led from the upper end of the muscle through a fine capillary plugged with vaseline. This vaseline seal appears to be quite gastight and to cause no measurable error in the gas measurements.

The apparatus consists essentially of two suitably shaped glass vessels (*A* the respiration, and *B* the compensation, chamber) in communication with a capillary manometer showing the pressure difference between the chambers: together with a calibrated gas burette by means of which the necessary measured volume of gas may be forced into the respiration chamber to bring the manometer to equality on the two sides. Readings of the burette give directly the volume of gas absorbed by the tissue, the correction for pressure and temperature being that for the conditions prevailing at the moment of closing the apparatus.

The instrument thus consists essentially of a Barcroft differential manometer with the addition of the burette of the Winterstein microrespirometer [Winterstein, 1912]. The use of the burette has several advantages; that calibration of the chamber volume is unnecessary, that the pressure may be maintained constant in the respiration chamber during a prolonged experiment, and that the range of the instrument is increased, so that greater changes of volume may be measured than with a simple differential manometer of equal sensitiveness. The manometer was adopted instead of the oil drop in a horizontal tube used by Winterstein, Thunberg and others, since it possesses a much greater range for equal compactness, and it should not be subject to the curious spontaneous movements described by some who have used the latter arrangement [Winterstein, 1912; Haberlandt, 1911].

<sup>1</sup> This work was carried out during the tenure of the Benn W. Levy studentship.

*Details of construction.*

The scale of centimetres in *D* gives the scale of the drawing (Fig. 1). The respiration chamber (*A*) has an opening at the bottom, closed with a ground stopper, through which the tissue is introduced. One platinum electrode passes through the stopper, which is continued in the form of a bent tube reaching above the level to which the instrument is immersed in the water-bath. This tube is filled with salt solution and serves to conduct the stimulating current to the muscle without short-circuiting through the water-bath. There is space at the bottom of each chamber for about 1 cc. of the CO<sub>2</sub> absorbent. Each chamber is provided with a tube and tap, which serves as an exit for the gas when filling. The compensation chamber (*B*) is made of similar dimensions to the other, but is not provided with the opening at the bottom nor with the capillary at the top. Exact correspondence in volume is unnecessary, but the chamber walls should be of the same thickness.

The chambers are connected with the manometer and burette by accurately ground joints, and are held in place by elastic bands passing over the hooks shown.

The manometer and burette, *C* and *D* (Fig. 1), with the necessary connections for filling, are made in one piece of glass and are clamped upon a glass scale mounted on a board: for this purpose ordinary spring wooden clothes-pegs have been used. The manometer is of about  $\frac{1}{2}$  mm. bore and is fused at each end to the three-way stopcocks 1 and 2. The burette and its connections have the form shown in the drawing. The portion 4-5-6 is calibrated before being built into the instrument: it is provided with one mark at 5 on the glass which gives a means of fixing the burette during an experiment over the same part of the scale as at the time of calibration: a precaution which is only necessary if the calibration shows that the inequalities are great enough to affect the accuracy of an experiment. Below 6, the calibrated tube is fused to a bent tube ending in a thick walled rubber tube of suitable length (three to five inches according to bore), closed with a glass stopper at the top and provided with a number of ordinary spring pinch clips for adjusting the mercury level. A stopcock, 3, is fused on to the burette at the top for filling purposes.

The manometer with its taps, and the burette with its connections, may be made and fitted to the board separately, then clamped to the board in their correct positions over the scale and the final joint made by fusing the glass at 7 when the parts are on the board. It is well to strengthen the apparatus by securing the mercury tube to the manometer at the point 8 where the tubes cross.

*Cleaning and filling.*

All parts of the apparatus are filled with a chromic acid solution and the whole is either immersed in, or heated over (if a sufficiently large basin is not available), a basin of the same fluid. Frequently many hours' heating at or near the boiling point is necessary. The acid is washed out, finally using

distilled water, and at this stage insufficient cleaning may be detected by the presence of greasy unwetted places on the glass. Drying is simply effected by warming on or over a sand-bath while air is drawn through all parts of the apparatus. Care must be taken that all parts are thoroughly dried in turn.

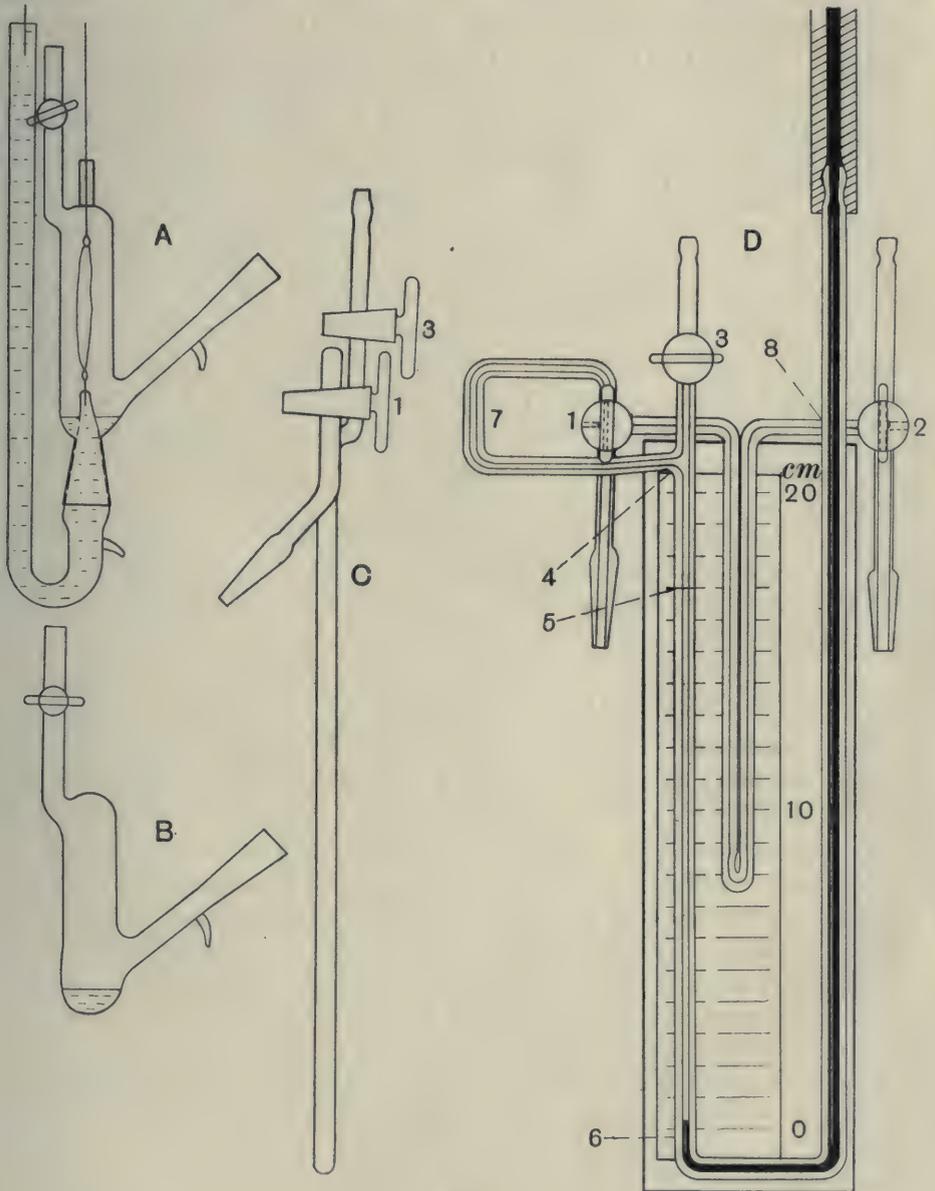


Fig. 1.

The tap grease employed was a mixture of white vaseline and paraffin wax (2 : 1) and was used as sparingly as possible: this was selected in preference to the usual lubricants on account of its smaller power of absorbing oxygen.

*Filling the manometer.*

Paraffin oil boiling between  $160^{\circ}$  and  $190^{\circ}$  approximately is suitable, having a low vapour pressure and high mobility, so that equilibrium is reached at once. Clove oil, which is occasionally recommended for capillary manometers, is much too viscous. The barrel of the tap, 1 (or 2), is greased and inserted: the apparatus is held in a stand with the tap, 2 (or 1), from which the barrel has been removed, above the other, and a small amount of oil is introduced in a pool above the opening of the manometer: by cautiously opening the lower tap the correct quantity is allowed to run in, avoiding air bubbles. The excess in the tap seating is cleaned away with filter paper, and the column allowed to run down into place. It should fill the two limbs slightly more than half full. No difficulty is found in adjusting the length of the oil column if it is run back again to the empty tap and excess removed with filter paper or more added with a capillary pipette as may be required. Taps 2 (or 1) and 3 may now be inserted. Except when the instrument is in use it is well to leave both taps 1 and 2 closing the ends of the manometer (as in the diagram), since if the oil column is broken or allowed to come into contact with the tap grease the apparatus will probably require cleaning again.

The mercury burette is filled from the rubber tube: it is well to fill it only to within a few centimetres of the zero mark, since the mercury in the long vertical column may descend slightly, owing to the contraction of the rubber or to leaks.

*Details of manipulation.*

The compensation chamber, containing a little potash ( $2\frac{1}{2}\%$ ), is attached. The excised tissue is tied to the platinum loop on the stopper of the respiration chamber and the other end to the fine wire: the wire is threaded through the capillary, and the capillary dipped into molten vaseline till the grease runs up to within about 2 mm. of the chamber proper. It must not be allowed to spread over the inside of the chamber or errors due to the grease absorbing oxygen will occur. Finally about 0.5 cc. of the potash is introduced by means of a capillary pipette and the respiration chamber attached to the remainder of the apparatus, which is placed in a well-stirred water-bath, preferably provided with a thermostat. The taps on the chambers are opened and the gas mixture required is passed in through taps 2 and 3 and out through the chambers. If the mercury in the burette is raised to the level of the T-piece when the gas is first passed, and is quickly lowered to the zero before discontinuing the stream, the whole of the apparatus is washed out except the manometer, which is a negligible volume. The incoming gas stream is then shut off at 2 and 3, tap 2 turned so as to connect the compensation chamber with the manometer (through  $90^{\circ}$  clockwise from the position of the diagram), tap 1 to connect the respiration chamber with the manometer and burette

(through 180°), and the taps on the chambers closed. The barometer and temperature are read.

Reliable readings can usually be taken 15 minutes after closing the taps. To take a reading, the screw clips are opened or closed so as to alter the mercury level until the manometer shows equal pressures in the respiration and compensation chambers. The height of the mercury is then read, parallax being avoided by the silvering on the back of the glass scale. When the mercury reaches the top of the calibrated tube the gas must be renewed, and the mercury adjusted to zero during the passage of the gas; this need not interrupt the readings for more than 20 minutes. With my two instruments the volume of one centimetre on the burette is 0.02266 and 0.01231 cc. respectively, so that the length of 20 cm. suffices for several hours' continuous readings at a rate of respiration of the order 0.03 cc. per hour.

#### *Limits of accuracy.*

The stability of the zero of this instrument is the most important factor determining the accuracy with which rates of oxygen absorption can be measured. In blank experiments without any tissue but with the potash in the bulbs, it has been found that, neglecting the first 20 or 30 minutes after passing the gas, the variations in zero are appreciable though small, not usually exceeding 0.5 cubic millimetres per hour for the first hour, and falling considerably later. The cause of these variations has not been definitely ascertained; they may be due to absorption of gas by the grease used for the joints and taps, or possibly to very slight movements at the ground joints. In any case the minimum possible amount of tap grease should be employed and this should consist of substances as saturated chemically as possible, consistent with proper lubrication of the surfaces. The figures are given for the approximate amount of zero change I have observed with the grease made of paraffin wax and white vaseline: mixtures containing rubber, and ordinary vaseline, appear to give a much larger spontaneous change. An apparatus similar in principle to this, but with joints of rubber pressure tubing instead of ground joints, was found to have a very much larger zero change, comparable in amount with the actual consumption of small muscles, and since this tendency to change zero was either abolished or much reduced when the apparatus was filled with hydrogen instead of oxygen, it appears to be due to the absorption of oxygen by the rubber connections. The errors with the present apparatus are of the order 1 in 20,000 of the volume of the chambers: it may be of interest to note that I have observed similar slow changes of the zero of an ordinary Barcroft blood gas apparatus.

The oxygen consumption of a single resting sartorius of the frog is of the order 20 cubic millimetres per hour, so that this quantity may be determined to an accuracy of 2 or 3 %.

Errors due to a possible imperfect absorption of CO<sub>2</sub> appear to be very small. During a period of constant respiration the apparatus will reach a

steady state, and any small amount of  $\text{CO}_2$  unabsorbed will remain constant so that no error is introduced if the volume diminution is taken as the oxygen absorption. When a change of rate of respiration, say an increase, occurs, there will be a slight accumulation of unabsorbed  $\text{CO}_2$ , so that the diminution in volume will be less than the true absorption of oxygen. The amount of the increase in the unabsorbed  $\text{CO}_2$  will be proportional to the increase in the rate of production of  $\text{CO}_2$ , and may be calculated approximately from the dimensions of the apparatus and the constant of diffusion of  $\text{CO}_2$  into air. The increase in the quantity of  $\text{CO}_2$  escaping absorption which must be present in order to raise the concentration gradient of  $\text{CO}_2$  between tissue and absorbent till the steady state is again reached at the increased rate of production, for an increase in rate of  $\text{CO}_2$  production of 1 cubic millimetre per minute, may be calculated to be as follows. It is assumed for simplicity that the chambers are cylindrical and that the  $\text{CO}_2$  is all evolved at the same distance from the absorbent (column 1). Column 2 gives the amount of the increase in the volume of unabsorbed  $\text{CO}_2$  in cubic millimetres.

Distance cm.	Total increase of unabsorbed $\text{CO}_2$ cmm.
1	0.05
2	0.2
2.5	0.3
3	0.45
3.5	0.6
4	0.8

These amounts are several times larger than any at all likely to be met with, since the assumed rate of increase in the  $\text{CO}_2$  production is several times larger than is probable. It is clear that the difference between the volume diminution and the oxygen absorption is so small as to be usually within the error of reading the scale with the naked eye, and is negligible unless extremely rapid changes of rate are to be followed with great accuracy.

A third condition, the effect of which must be considered, is the progressive change in the composition of the gas in the respiration chamber, for this will have an effect on the rate of respiration of the tissue, for which allowance may have to be made. The chambers used have a volume of about 10 cc., so that the absorption of 0.05 cc. of oxygen (a probable amount for work with large muscles in one hour) will cause a diminution of the oxygen concentration of about 0.5%. A diminution in the volume corresponding to 20 cm. of the larger burette mentioned will reduce the oxygen tension by 4.5% of an atmosphere. In cases where this alteration might have an important effect, larger chambers would be required, as recommended by Krogh [1915, p. 526].

## SUMMARY.

An instrument, modified from Barcroft's blood gas apparatus and Winterstein's microrespirometer, is described, for determining the oxygen consumption of frog's muscle or other small amounts of tissue in any desired atmosphere. The instrument permits of simultaneous observations on the tension of contraction.

I am indebted to the Royal Society for a grant covering the cost of this work.

## REFERENCES.

- Haberlandt (1911). *Arch. Anat. Physiol.* 424.  
Krogh (1915). *Abderhalden's Handbuch der Biochemischen Arbeitsmethoden*, 8, 519.  
Winterstein (1912). *Biochem. Zeitsch.* 46, 440.

## LXIV. THE FERMENTATION OF CELLULOSE IN THE PAUNCH OF THE OX AND ITS SIGNIFICANCE IN METABOLISM EXPERIMENTS.

BY AUGUST KROGH AND HANS OLUF SCHMIT-JENSEN.

*From the Laboratory of Zoophysiology, University of Copenhagen.*

*(Received September 25th, 1920.)*

It is well known that in herbivorous animals and especially in ruminants the fermentation processes taking place in the gut are so intensive that they must be taken into account in metabolism experiments if serious errors are to be avoided, but the best methods of measuring the fermentation and of utilising the measurements for correcting the energy balance are still matters of doubt and controversy, though they have been the object of several investigations. We have therefore made a series of experiments which, though they do not solve the problem, will at least make it a little less obscure.

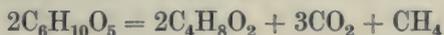
It has long been recognised that the fermentation in the paunch of the ox is mainly a methane fermentation of cellulose. The value of the fermentation from the point of view of the animal's economy lies in the fact, that the wholly indigestible cellulose is converted into substances which can be absorbed and utilised, but this gain is obtained only at the cost of a certain proportion of the energy available in the cellulose. The problem in which we are interested can be formulated as the determination of the loss of energy involved in the fermentation, though the way in which we propose to attack it is an indirect one.

It was shown by Markoff [1913] that in the fermentation cellulose is split up into fatty acids of the average composition of butyric acid, carbon dioxide and methane. From many of Markoff's experiments small quantities of hydrogen are also reported as being present, but their reality appears to us to be extremely doubtful. In many metabolism experiments on cattle [H. Möllgaard and A. C. Andersen, 1917; A. C. Andersen, 1920] and in all our own experiments on the fermentation of material from the paunch no hydrogen was produced, and we must point out that the routine of combustion analysis used in Zuntz's laboratory and adopted by Markoff is very liable to show positive, but spurious hydrogen percentages<sup>1</sup>. We are convinced,

<sup>1</sup> The combustion of methane requires a comparatively high temperature, but if the platinum wire in the combustion pipette is too hot methane is dissociated into carbon and hydrogen, and part of the carbon will generally escape combustion. The contraction upon combustion will in such a case be more than double the volume of carbon dioxide formed, and the surplus is set down as hydrogen.

therefore, that hydrogen is not a product of the cellulose fermentation in the paunch.

When the products formed by the fermentation are always the same, viz.  $\text{CO}_2$ ,  $\text{CH}_4$  and  $\text{C}_4\text{H}_8\text{O}_2$ , there is reason to believe that we have to do with a definite reaction and constant proportions between the products. The reaction might be imagined for instance to take place according to the equation



involving a proportion between the volumes of  $\text{CO}_2$  and  $\text{CH}_4$  produced as 3/1. The chief object of our experiments has been to study the proportion between the  $\text{CO}_2$  and  $\text{CH}_4$  formed by the fermentation, to find out whether it is constant or not, and to determine it numerically.

Such a study has been undertaken before by Boycott and Damant [1907] and especially by Markoff [1913], but the results were very disappointing, the relation  $\frac{\text{CO}_2}{\text{CH}_4}$  being extremely variable and almost always very high. It can be shown, however, that this is to a great extent due to methodical errors. The fermenting mass in the paunch contains large quantities of carbonates and bicarbonates, especially from the saliva with which the fodder is mixed during rumination. The fatty acids formed will liberate  $\text{CO}_2$  from the carbonates, and unless means are found to determine separately the  $\text{CO}_2$  formed by the fermentation from organic sources, the ratio of  $\text{CO}_2$  to  $\text{CH}_4$  will be higher than that demanded by the equation of the reaction. Boycott and Damant made no attempt to distinguish between fermentation  $\text{CO}_2$  and carbonate  $\text{CO}_2$ . Markoff tried to determine the carbonate separately, but the methods adopted were not sufficiently accurate<sup>1</sup>, and nothing definite is known therefore regarding the proportion of  $\text{CO}_2$  formed by the fermentation to the  $\text{CH}_4$ .

#### METHODS.

We have used the following technique.

The samples of material were collected by one of us in the Copenhagen public slaughter-houses immediately after the animals had been killed. The cows slaughtered had been fed for one or more days on hay and straw only, and we were likely therefore to find the cellulose fermentation comparatively unmixed with other fermentation processes which might take place in the paunch. In some cases the samples were carried to the laboratory in Dewar flasks, so as to maintain their temperature, but usually they were allowed to cool, as this did not apparently make any difference. In the laboratory the samples were mixed with such an amount either of water or of a suitable buffer mixture ( $\frac{1}{7}$  molar phosphates) that it became possible to take out small representative samples. Generally the larger particles of straw had to

<sup>1</sup> In most experiments the samples were sterilised by boiling before the  $\text{CO}_2$  was determined. As the contents of the paunch have a high  $\text{CO}_2$  tension a very considerable loss could not possibly be avoided.

be removed before taking samples. After thorough stirring, and while stirring was still going on, the samples were taken by means of a small vessel (30 cc. crucible) which was dipped into the mixture. The samples were transferred to fermentation flasks of about 50 cc. capacity, and the exact quantity taken determined by weighing.

The fermentation flasks were provided with a side tube reaching down to the bottom of the vessel and closed by means of a tap. (See Fig. 4, no. 1, in the paper by Krogh [1914, 2].) After weighing, each fermentation flask was connected with a manometer of the type used for microrespiration (Fig. 1, Krogh [1914, 2]), but with somewhat wider tubes, and filled with mercury instead of oil, and put into a water-bath at body temperature, in which arrangements for shaking them vigorously were provided. Before the fermentation experiment could begin, the air in the flasks was washed out by a current of pure nitrogen<sup>1</sup>. About 100 cc. of nitrogen were introduced through the side tube, and after bubbling through the mixture taken out through the top of the manometer and collected over mercury in a gas receiver. During this process, which occupied 5-10 minutes, the bottles were shaken vigorously to establish equilibrium between the gas current and the fermenting mixture and especially to drive out all the oxygen. Finally a slight surplus pressure was established, the tap on the side tube closed, the shaking continued for one or two minutes more, a sample of the gas drawn out at the top of the manometer for subsequent micro-analysis [Schmit-Jensen, 1920]<sup>2</sup>, the manometer closed, read and noted together with the barometer, temperature of bath and time of beginning.

The fermentation could now be followed on the manometer, and when a suitable pressure had been reached, the flasks were again vigorously shaken. Immediately afterwards the manometer was read<sup>3</sup> and recorded together with the time and the temperature of the water-bath, whereupon a sample of the air in the flask was collected after washing out the manometer tubing by means of part of the excess pressure.

After the sampling of the gas 2 cc. of 20 % hydrochloric acid were introduced through the side tube to stop fermentation and liberate the CO<sub>2</sub> from the mixture, and while a vigorous shaking was kept up, the CO<sub>2</sub> was driven off by a current of air, taken up in baryta solution and determined by titration as described by Krogh [1914, 1]. For the air current the nitrogen (+ CO<sub>2</sub>)

<sup>1</sup> We have used nitrogen from air in a gasometer with pyrogallate solution. The generator described by C. van Brunt [1914] is certainly much better.

<sup>2</sup> This analysis is necessary, because the fermenting mixture always possesses a more or less considerable CO<sub>2</sub> tension. In some cases a trace of oxygen has been left, and in others a slight amount of methane was also present.

<sup>3</sup> While the fermentation flask is in a water-bath at 37° moisture is apt to distil up into the narrow tubes of the manometer and collect there in short columns of water, which by their capillary resistance prevent the uniform movement of the column of mercury. To prevent this the fermentation flask was in the later experiments shut off by a clip from the manometer during the whole of the experiment, and connection established only when it was desired to take a reading.

stored since the beginning of the experiment was first used and afterwards supplemented by a suitable amount of CO<sub>2</sub>-free air. In this manner the baryta would absorb all the free and combined CO<sub>2</sub> present in the fermenting mixture before fermentation + the quantity formed during fermentation— the minute quantities present in the gas samples. These were added as a correction.

The quantity of CO<sub>2</sub> present before fermentation was determined in the same manner on separate samples treated just as the others, put into fermentation bottles and placed in the water-bath, but acidified with HCl immediately afterwards.

The gas samples taken at the beginning and end of the fermentation experiment were analysed by the micro-method worked out by Schmit-Jensen [1920] for CO<sub>2</sub>, H<sub>2</sub>, CH<sub>4</sub> and occasionally O<sub>2</sub>, while the remainder was taken to be nitrogen. From the analyses and the pressures and temperatures, the quantity of each gas at the beginning and end of the fermentation was calculated.

We reproduce as an example the protocol and calculation of Exp. 8 B.

Material. From paunch of a single cow, fresh contents of brownish yellow colour, on the whole finely triturated, but containing straw particles of about 3 cm., rather dry, not foaming, alkaline towards litmus, acid to phenolphthalein.

Emulsions for experiments:  $\left\{ \begin{array}{l} \text{A. 250 g. material + 500 cc. secondary phosphate } (\frac{1}{4} \text{ molar}). \\ \text{B. 250 g. + 500 cc. phosphate (7 primary, 3 secondary)}. \end{array} \right.$

Specific gravity of emulsion 1.034.

Sample B. 22.88 g. = 22.14 cc., B<sup>1</sup> (for CO<sub>2</sub> determination) 23.81 g., B<sup>2</sup> 23.81 g.

CO<sub>2</sub> determinations:

B<sup>1</sup> 5.02 cc. or 0.211 per g., B<sup>2</sup> 4.76 cc. or 0.200 per g. Average per g. 0.206 cc. B after fermentation 11.83 cc. According to the average of B<sup>1</sup> and B<sup>2</sup> it should have contained 4.72 cc. before fermentation. CO<sub>2</sub> produced by fermentation therefore 7.11 cc.

Volume of fermentation flask B

59.26 cc. + manometer tubing 0.27 cc. — glass beads for shaking 0.20 cc.

Volume of 1 mm. manometer tubing 0.0009 cc. Volume of compensating bottle 19.95 cc.

Manometer readings.

19'	4 <sup>30</sup> p.m.	138.3	142.8	Diff. $d_1 = 4.5$ . T. 37.0°. Barometer, 733 mm.
	8 <sup>00</sup> p.m.	122.5	159.0	
	10 <sup>35</sup> p.m.	115.0	166.5	
20'	11 <sup>00</sup> a.m.	101.0	182.0	Diff. $d_2 = 117.3$ . T. 37.5°.
	1 <sup>30</sup> p.m.	97.5	186.0	
21'	12 <sup>00</sup> m.d.	83.2	200.5	

## Analyses (corrected for systematic error).

%	Initial sample		Final sample		Average	
	1	2	1	2	Initial	Final
CO <sub>2</sub>	2.0	2.1	5.4	5.5	2.05	5.45
O <sub>2</sub>	0	—	0	—	0	0
H <sub>2</sub>	—	—	—	0	0	0
CH <sub>4</sub>	0	—	8.1	8.4	0	8.25
N <sub>2</sub>	—	—	—	—	97.95	86.3

The total quantity of gas in the apparatus at the beginning and end of the experiments has been calculated as follows: at the beginning we have the gas volume  $A = 59.26 + 0.27 - 0.20 - 22.14 = 37.19$  cc. By reducing this to 0° and 760 mm. we get

$$A \frac{273}{273 + t_w} \cdot \frac{P - f_w + d_i}{760} = 37.19 \frac{273}{273 + 37.0} \cdot \frac{733 - 46.5 + 4.5}{760} = 29.75 \text{ cc.}$$

At the end of the experiment we have the total gas volume

$$A_1 = A + \frac{A + C}{2C} v d_j = 37.19 + \frac{37.19 + 19.95}{2 \cdot 19.95} 0.0009 \times 117.3 = 37.19 + 0.15 = 37.34$$

and reduced to 0° and 760 mm.

$$A_1 \frac{273}{273 + t_w} \cdot \frac{P - f_w + d_j}{760} = 37.34 \frac{273}{273 + 37.5} \cdot \frac{733 - 48 + 117.3}{760} = 34.67.$$

The initial and final quantities of each gas are found by multiplying the volumes with the corresponding percentages.

cc.	Initial	Final	Produced	Relation $\frac{\text{CO}_2}{\text{CH}_4} = \frac{7.11}{2.86} = 2.49$
CO <sub>2</sub>	(0.61)	(1.89)	7.11	
CH <sub>4</sub>	0	2.86	2.86	
N <sub>2</sub>	29.15	29.9	0.75	

*Accuracy and sources of error.* The accuracy of our determinations is not very great. It is very difficult to obtain good average samples of materials like the contents of the paunch, and the method finally adopted is the outcome of a number of trials. Even this method, however, is not so accurate as we should desire.

The samples for determining the initial quantity of CO<sub>2</sub> have always been taken and analysed at least in duplicate. Special trials have shown that our CO<sub>2</sub> determinations are not vitiated by fatty acids or HCl being driven over into the baryta. A statistical treatment of the determinations shows that the dispersion of the results from single samples is  $\pm 3\%$  with a maximum of 5%. The differences between the initial and final quantities of CO<sub>2</sub>, which are affected by these errors, are of the same order of magnitude as the initial quantities themselves, and the mean error on the determination of CO<sub>2</sub> production is in most experiments about  $\pm 4\%$ .

The gas analyses have been corrected for the systematic errors found by Schmit-Jensen. According to the double determinations made in the course of the present investigation, the mean error of a double determination of CH<sub>4</sub> works out as  $\pm 0.12\%$ . As the average CH<sub>4</sub> percentage in the samples

is about 7, the percentage error on the quantity of  $\text{CH}_4$  works out as 1.7 %. There is a further slight error, due to the determination of total pressure. The mean error on the quantity of  $\text{CH}_4$  is therefore about 2 %.

The relation  $\frac{\text{CO}_2}{\text{CH}_4}$ , which has been the main object of our determinations, is generally found to lie about 2.5. It will of course be influenced by the errors on the  $\text{CO}_2$  and  $\text{CH}_4$  determinations, and the resulting mean error can be calculated. We have

$$\frac{\text{CO}_2}{\text{CH}_4} = \frac{2.5 \pm 0.1}{1 \pm 0.02}$$

and hence the error

$$\pm \sqrt{2.5^2 \times 0.02^2 + 0.1^2} = \pm \sqrt{0.0125} = \pm 0.11.$$

The determination of total gas pressure has in most of the experiments been rather inaccurate, because drops of condensed water obstructed the tubing between the flasks and the manometer. The error may have amounted to 4 or even 6 mm. or 0.5 to 0.8 % of the total pressure. Such an error has very little influence upon the determination of  $\text{CH}_4$ , because the percentage of this gas is always small, but the influence on the nitrogen determination is considerable, and as this determination is further influenced by the sum of the gas analytical errors, the mean error on the difference between the initial and final quantity of nitrogen becomes considerable, viz. about  $\pm 0.25$  cc.

#### EXPERIMENTAL RESULTS.

The results of our experiments are summarised in Table I.

*The constancy of the gaseous nitrogen.* In Exps. 1, 2, 3, 4, 6 and 7, the gaseous nitrogen remains constant within the limits of error. The average production in one of these experiments is  $-0.06 \pm 0.18$  cc. In Exp. 8 A we have no determination of the nitrogen, and the production of 0.75 cc. found in 8 B is probably due to some undetected error. In Exps. 5 A and B we have observed a slight, but probably real, production of gaseous nitrogen. Krogh [1916, p. 53] has found that the presence of nitrates in the fermenting contents of the coecum of rabbits will cause a liberation of free nitrogen. Nitrates or nitrites may, perhaps, have been present in the paunch in this case, though we failed to detect macroscopic remains of beet root, which contains considerable quantities of nitrate<sup>1</sup>. In any case we can conclude from our experiments that free nitrogen is neither produced nor absorbed in appreciable amount by the fermentation of cellulose in the paunch.

*The production of methane.* In column 6 of Table I we have given the average rate at which methane has been produced. This has been figured out per 24 hours and for 100 kg. of undiluted material from the paunch in order to facilitate comparison with the quantities of methane produced in metabolism

<sup>1</sup> According to determinations made in the Danish "Forsøgslaboratorium" beet root may contain from 0.05 to 0.15 g.  $\text{HNO}_3$  in 100 g. fresh substance. Turnips do not contain any nitrates.

Table I.

No.	Material	Emulsion in	Duration of exp. hours	Nitrogen produced cc.	CH <sub>4</sub> produced per 100 Kg. in 24 h.	Rate of ferment. mm. per hour			CO <sub>2</sub> / CH <sub>4</sub>
						1st day	2nd day	3rd day	
1	Contents of paunch, finely triturated	Secondary phosphate	24	0	54	7, 3.5, 3.5	—	—	2.85
2	Fermentation in paunch very strong. Material moist, fairly well triturated, alkaline to litmus, acid to phenol, phthalein	Water Secondary phosphate	5.5 25	-0.2 -0.2	79 19	18, 19, 17 2.3, 2	—	—	2.95 2.7
3	Fermentation not very pronounced. Very finely triturated, alkaline to litmus, acid to p.p. Used after 6 hours	Secondary phosphate	25	0	18	2.3, 2.4	—	—	2.7
4	Fermentation rather weak. Coarse, straw and turnip. Neutral to litmus	7 sec. phosph. + 3 prim. phosph.	14	0	63	10, 17	—	—	2.95
5	Fermentation weak. Finely triturated, very moist. Alkaline to litmus, acid to p.p.	7 sec. phosph. + 3 prim. phosph. 7 sec. phosph. + 3 prim. phosph. 7 sec. phosph. + 3 prim. phosph.	17 15.5 63	-0.2 0 +0.6	74 75 9	12, 8, 11 6, 10, 2.5 1.9, 2.1	—	—	2.2 2.2 (2.8)
6	From 5 animals, rich in larger particles, alkaline to litmus, acid to p.p.	7 sec. phosph. + 3 prim. phosph. Water	63 23	+0.6 +0.1	10 28	2.7, 2.2 8, 6, 8	0.8	0.7	(2.2) 2.4
7	From 13 animals, coarse moist, contains turnips. Turns p.p. slightly red. Used after 6 hours	Water Water + CaCO <sub>3</sub>	24 65	-0.4 0	28 6	7, 6, 7 2.2	—	—	2.4 (4.9)
8	From single animal. Fermentation weak. Finely triturated, rather dry. Alkaline to litmus, acid to p.p.	Water + CaCO <sub>3</sub> Secondary phosphate	66 45	+0.2 ?	6 17	2.1 6, 5, 2	—	1.2	(4.0) 2.65
		7 sec. phosph. + 3 prim. phosph.	43	+0.7	21	8, 6, 2.5	1.2	—	2.5

experiments on cattle. Since a cow, the paunch of which contains between 50 and 100 kg. of food, will produce from 100 to 300 l. methane in 24 hours, it is evident that the intensity of the fermentation has even in the best of our experiments been considerably below that observed in natural conditions, and in most of our determinations it has fallen far short of this ideal. In Markoff's experiments the intensity of fermentation has also, generally, been lower than in the living animal. This is ascribed to the cooling and to the influence of oxygen in the interval between the taking out of the material and removal of the oxygen in the water-bath at 37°. We believe that the contact with oxygen is the factor chiefly responsible.

In column 7 we have given some figures indicating roughly the variation in fermentation rate in the course of each experiment. The figures give the increase in pressure in the fermentation flasks in mm. mercury per hour. They are comparable only within the same experiment and to a certain extent in experiments made with the same buffer mixture. When, for instance, the rate is much higher in Exp. 1 B than in 1 A, this means only that far more CO<sub>2</sub> is evolved as gas in B than in A, owing to the greater acidity of the fermenting mixture.

In Exps. 1 B, 2, 4 A, 5, 6 and 7 the rate remains practically constant during the first 24 hours. In 1 A and 8 it decreases, and in 3 and 4 B it increases materially. In Exps. 5, 7 and 8, which have been continued beyond 24 hours, the rate has decreased on the second day and still more on the third. We are unable to give any explanation of the variations in rate.

The relation  $\frac{\text{CO}_2}{\text{CH}_4}$  given in the last column varies between 2.95 and 2.2, except in Exp. 7, in which the fermentation was extremely slow and the CaCO<sub>3</sub> added had probably made it abnormal. Except in this case it does not seem to be in any way influenced by the varying intensity of the fermentation. The double determinations agree very closely, except in the two experiments 5 and 7 lasting over 48 hours and showing the slowest fermentation. The differences between different experiments are so much larger than the mean error of a determination that they must be considered as real, and it must be admitted, therefore, that in the conditions obtaining the relation  $\frac{\text{CO}_2}{\text{CH}_4}$  is *not* constant, though the variations found are very much smaller than formerly supposed.

The experiments show that within the range of hydrogen ion concentrations which have been found in the paunches of cows (P<sub>H</sub> between 7 and 8) the reaction of the fermenting mixture has no influence upon the character or intensity of the fermentation.

The average for the experiments 1, 3 and 4, which show a fairly rapid rate of fermentation, is  $\frac{\text{CO}_2}{\text{CH}_4} = 2.6$ , and the average for all the experiments, except the abnormal nos. 5 and 7, is likewise  $2.6 \pm 0.08$  with a dispersion of  $\pm 0.27$ .

*The application of the result in metabolism experiments on cattle.*

The figure 2.6 can be utilised to correct the results directly derived from determinations of the gaseous metabolism. A certain caution is necessary, however, since the relation  $\frac{\text{CO}_2}{\text{CH}_4}$  is not quite constant, and may possibly show larger variations in cows fed upon a more varied fodder.

To illustrate the application of our result, we have taken an experiment made by A. C. Andersen [1920], in which both the gas exchange and all solid ingesta and excreta were determined and calculated on a 24 hour basis. The metabolism of nitrogen-free substances was found to require 1451 l. oxygen and produce 1583 l.  $\text{CO}_2$  and 127 l.  $\text{CH}_4$ . The apparent R.Q. is  $\frac{1583}{1451} = 1.091$ . According to our result  $2.6 \times 127 = 330$  l.  $\text{CO}_2$  should be due to fermentation, and the true R.Q. for the organism itself should be  $\frac{1583 - 330}{1451} = 0.864$ .

The energy metabolism is calculated from the oxygen intake by means of Zuntz's formula. We have

$$E = \text{O}_2 \{4.686 + (\text{R.Q.} - 0.707) 1.23\}.$$

In the present instance the uncorrected heat production would be  $1451 (4.686 + 0.384 \times 1.23) = 7487$  Cal. and the corrected only 7079 Cal.

The summary calculation is evidently inadmissible. Against the application of the correction it might be argued that Zuntz's formula has been elaborated on the assumption of a metabolism of ordinary fats and carbohydrates only, and cannot be used when the lower fatty acids formed by the fermentation are catabolised. If Markoff is right, that the fatty acids correspond on an average to butyric acid, the caloric value of the oxygen used for the catabolisation of these substances will correspond closely to that obtained from the formula. Butyric acid has a respiratory quotient of 0.8 with a caloric value of the oxygen of 4.74 Cal. per l., while the formula gives 4.80.

It is of special interest to compare our calculation with the elegant solution of the problem given by A. C. Andersen himself. Andersen's reasoning is as follows: if the methane produced by the fermentation is burned the total (non-protein) metabolism (methane included) is one of carbohydrate and fat, and Zuntz's formula must be strictly applicable to find a total heat production, and if the heat of combustion of the methane is afterwards subtracted, the rest is the metabolism of the animal.

Methane burns according to the equation  $\text{CH}_4 + 2\text{O}_2 = \text{CO}_2 + 2\text{H}_2\text{O} + 213$  Cal. The volume of oxygen used is double that of the methane and carbon dioxide, and we have consequently in the above example

Total  $\text{CO}_2 = 1583 + 127 = 1710$  l. Total  $\text{O}_2 = 1451 + (2 \times 127) = 1705$  l. R.Q. = 1.003. Hence the total heat production 8610 Cal. and, by subtraction of the heat of combustion of 127 l. methane = 1207 Cal., the metabolism of the animal itself 7403 Cal.

This figure is 324 Cal. or 4.6 % higher than that obtained by our calculation. That it should be higher was to be expected, since in Andersen's calcula-

tion the amount of heat produced by the fermentation in the paunch is included, while our calculation aims at having it excluded. The heat of fermentation cannot strictly be reckoned as due to the metabolism of the animal and is practically lost from the point of view of the animal's economy, even though it is generated inside its body<sup>1</sup>.

According to the provisional formula of fermentation given above (p. 687) the heat of fermentation per g.-molecule of methane would be equal to the heat of combustion of 2 g.-molecules of  $C_6H_{10}O_5$  (1356 Cal.) minus the heat of combustion of 2 g.-molecules of  $C_4H_8O_2$  (1054 Cal.) and 1 of methane (213 Cal.), that is 89 Cal. or 3.97 Cal. per l. methane. In the experiment under discussion the loss by fermentation would therefore, on this assumption, amount to 505 Cal. When Andersen's figure is reduced by this amount it becomes 6898 Cal., 181 Cal. or 2.6 % lower than ours.

Our experiments show that the provisional formula does not represent the fermentation exactly, and it is probable, moreover, that part of the energy liberated by the fermentation is utilised for synthetic processes (building up of organisms?) within the fermenting mixture. The loss is therefore probably somewhat lower than indicated by the formula.

It would be desirable to get direct bio-calorimetric determinations on the fermenting mass combined with determinations of the  $CH_4$  evolved. The making of such determinations has been attempted by Markoff [1913, p. 63], but so far as we are aware the results have not been published. We have also made some attempts, but have not been able to overcome satisfactorily the technical difficulties. So far as they go, our experiments indicate a heat of fermentation of about 50 Cal. per g.-mol. of  $CH_4$ . When corrected by means of this figure Andersen's results would become 7119 Cal., 40 Cal. or 0.6 % higher than ours. The closeness of this agreement has of course no real significance, but it would seem, nevertheless, that our method of calculating the energy expenditure of the ruminating organism itself cannot be very far wrong.

#### SUMMARY.

Methods are described for measuring the gaseous products of fermentation processes and especially the  $CO_2$  and  $CH_4$  evolved by the cellulose fermentation taking place in the paunch of the ox.

Usually gaseous nitrogen is neither absorbed nor produced by this fermentation.

The relation between the  $CO_2$  and  $CH_4$  formed by the fermentation appears to be independent of the rate of fermentation and, within the limits investigated, of the hydrogen ion concentration. The relation  $\frac{CO_2}{CH_4}$  works out

<sup>1</sup> In the calculation made by Andersen it is perfectly legitimate to include this amount of heat, since he compared the heat production found by respiration experiments (+ urine analysis) with the heat computed from weighings and analysis by means of the calorimetric bomb of all the ingesta and excreta of the animal.

as on an average 2·6, but real variations occur. The limits observed are 2·2 and 2·9 respectively.

A method is suggested by which the observed respiratory exchange of cattle can be corrected, when the production of methane is measured.

The expenses of this research have been defrayed out of a grant from the Carlsberg foundation.

#### REFERENCES.

- Andersen (1920). *D. Kgl. Veterinær og Landbohøjskole. Aarsskrift* (Kjöbenhavn).
- Boycott and Damant (1907). *J. Physiol.* **36**, 283.
- van Brunt (1914). *J. Amer. Chem. Soc.* 1448.
- Krogh (1914, 1). *Zeitsch. allg. Physiol.* **16**, 178.
- (1914, 2). *Biochem. Zeitsch.* **62**, 266.
- (1916). "The Respiratory Exchange of Animals and Man," *Monographs of Biochemistry* (Longmans, Green and Co., London).
- Markoff (1913). *Biochem. Zeitsch.* **57**, 1.
- Möllgaard and Andersen (1917). *D. Kgl. Veterinær og Landbohøjskole. Aarsskrift* (Kjöbenhavn).
- Schmit-Jensen (1920). *Biochem. J.* **14**, 4.

# LXV. A METHOD FOR OBTAINING UNCONTAMINATED SPECIMENS OF URINE FROM THE BILLY GOAT ; WITH SOME NOTES UPON THE NORMAL METABOLISM OF THIS ANIMAL.

BY RUDOLPH ALBERT PETERS.

*From Physiological Department, Royal Engineers' Experimental Station, Porton.*

*(Received September 25th, 1920.)*

THE method described in this communication<sup>1</sup> was applied during the war to a study of the normal urine of the goat. By its means some data upon the normal metabolism of the goat were collected, and although by no means complete, it has been thought worth while to put them upon record as they are in general agreement with the work of others. Steenbock, Nelson and Hart [1914] showed that the calf used ammonia for the purpose of neutralising acid given in the diet. Winterberg [1898] had previously shown that the rabbit used ammonia under similar circumstances. Some previous observations upon the goat have been made by Baer [1906], who showed that under conditions of starvation, the output of ammonia was increased.

The notes dealt with in this paper are concerned with the urine of the goat upon different diets, during starvation, and after feeding with acid and alkali. For this purpose the titratable alkalinity of the urine and the hydrogen ion concentration have been studied, as well as the ammonia excretion, and in some cases the excretion of chlorides. The reaction of the urine is expressed throughout in the usual Sørensen notation. For the estimation of the titratable alkalinity,  $P_H = 7.45$  has been used as the titration end point.

## I. METHODS.

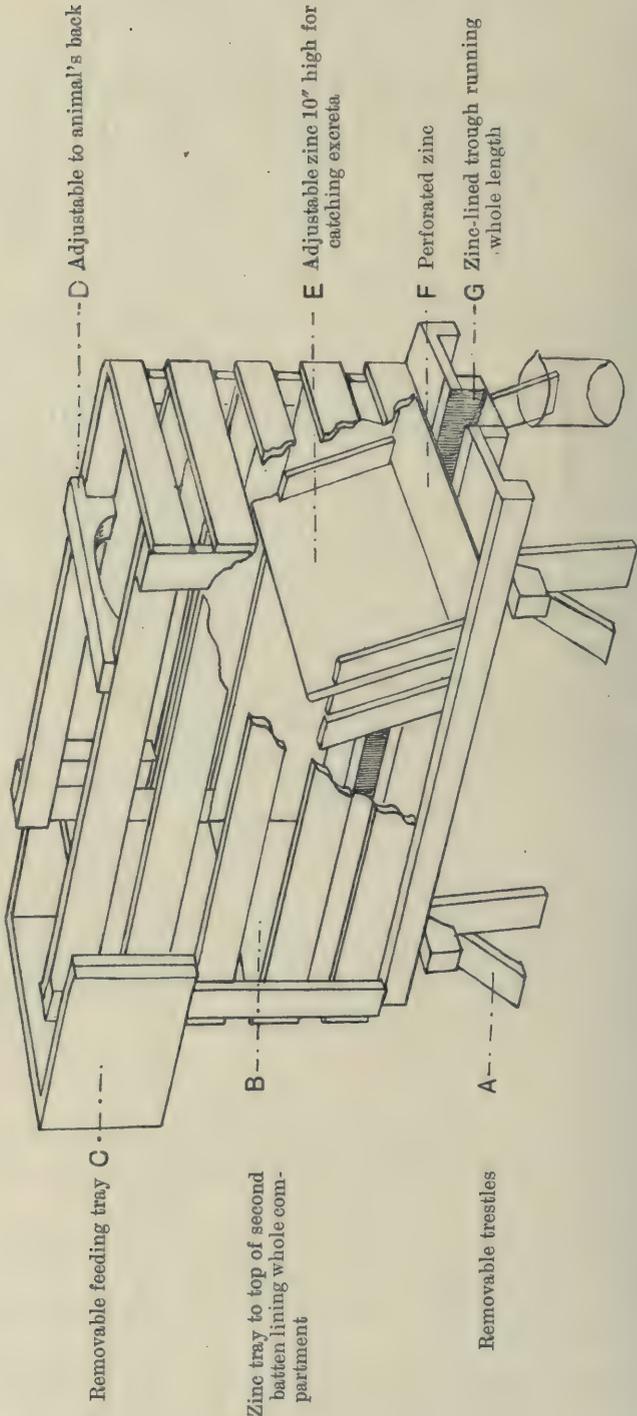
For the preservation of the samples of urine, chloroform and toluene were used. No precautions were taken to avoid loss of  $CO_2$  during the collection of the samples.

1. *The Collection of Samples of Urine.* A crate of design and dimensions shown in Fig. 1, made of wood, was used for the goat in all experiments. It has a three-inch zinc lined bottom from which the urine runs out and is collected in a suitable vessel.

<sup>1</sup> Published by permission of the Controller, Chemical Warfare Department. This work was done two years ago. It was hoped that an opportunity might arise to clear up some of the points, but as none is likely to appear at present, I have published these notes in the hope that they may be useful.

GOAT CRATE

Length [without extension for trough] .....	37½"
Breadth .....	13½"
Depth [without trestles] .....	26½"
Feeding tray .....	13½" x 17"
Battens .....	3"



Removable feeding tray C

Zinc tray to top of second batten lining whole compartment

Removable trestles

Fig. 1.

In order to avoid contamination with faeces billy goats were used. A bag was devised by J.c.-Cpl. Morgan, which is attached to the goat and into which it passes its faeces. This is shown in Fig. 2.

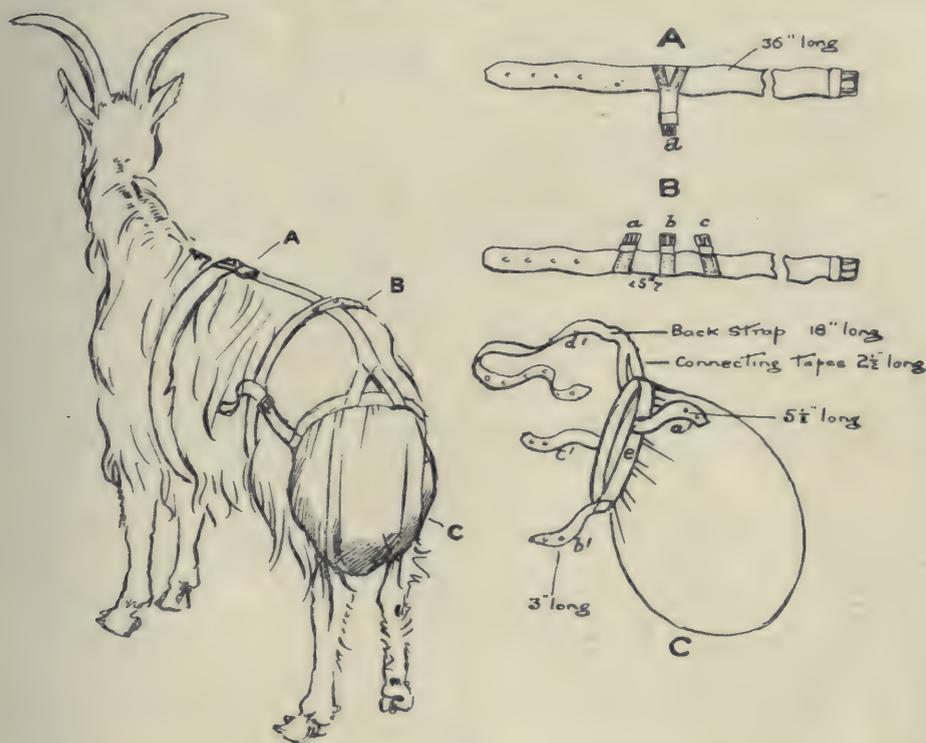


Fig. 2.

*A* is chest-band made of two-inch webbing, 3 feet in length. *d* in centre buckles to strap *d'* on bag in Fig. 2 *C*.

*B* is girth-band made of elastic 1 inch in breadth by 2 feet 6 inches in length, *a*, *b*, *c*, in centre fixed on slant, buckled to tapes *a'*, *b'*, *c'* in Fig. 2 *C*.

*C* is faeces bag of waterproof jaconet; sizes vary with animals. Medium size, flat, length 12 inches, width 8 inches. For a 56 lb. animal width 4 inches is sufficient. A band of elastic (*e*) 12 inches long is fixed round the opening. On this are fixed tapes *a'*, *b'*, *c'*, *d'*, made of elastic to prevent chafing. *a'* and *c'* are 5½ inches, *b'* is 3 inches and *d'* 18 inches in length. *a'*, *b'*, *c'* buckle to *a*, *b*, *c*, in Fig. 2 *B*

To fix.

1. Put on chest-band *A*. If it tends to slip put it between the forelegs.
2. Connect *C* to *B* by the small tape *b'* and buckle *b*. Buckle round the girth-band, and buckle *d'* to *d*; then adjust the other small straps *a'* and *c'*. The back strap *d'* (Fig. 2 *C*) is fastened to girth-band by a large safety pin. The bag is removed easily by undoing the safety pin, and slipping the whole over the beast's legs.

2. *Determination of Hydrogen Ion Concentration.* This has been studied colorimetrically, using a comparometer and indicators similar to those developed by the American workers [see Clark and Lubs, 1917] on the subject. Pheno and cresol red were the indicators used practically throughout. A three-tube comparometer was used similar to that of S. W. Cole.

Standard phosphate, borate and potassium chloride mixtures were used for the comparison.

3. *Titratable Alkalinity.* 10 cc. samples of urine are titrated with acid ( $N/10$  HCl) or alkali ( $N/10$  NaOH) according as the urine is more acid or more alkaline than  $P_H = 7.45$ . The estimations are made in a comparometer. The control urine tubes receive similar dilutions to the sample that is being studied.

The titration end point has been taken as  $P_H = 7.45$  which is approximately the reaction of normal blood.

Determinations of titratable alkalinity using phenolphthalein, instead of phenol red or cresol red and boiling off the  $CO_2$  formed, were done on some goats. The results in the main run hand in hand with those obtained by using the  $P_H = 7.45$  standard, as shown in the following table.

Table I.

Determinations of titratable alkalinity using (1)  $P_H = 7.45$  as an end point, and (2)  $P_H = 9.0$  (phenolphthalein) as an end point.  
 + = cc.  $N/10$  alkali (NaOH).  
 - = cc.  $N/10$  acid (HCl).

Goat	$P_H = 7.45$	$P_H = 9.0$ (phenolphthalein)
A.	+ 43.5	+ 101.5
24 hours sample	+ 85.1	+ 639.4
	+ 43.5	+ 375
	+ 25.5	+ 196.4
	- 21.8	- 29
	- 227.5	- 214.5
B.	+ 27.0	+ 66.6
24 hours sample	+ 49.5	+ 253.0
	+ 31.8	+ 206.5
	+ 33.7	+ 266.8
C.	- 12.3	- 24.5
	- 5.7	- 37.1
	+ 3.6	+ 244.0
	+ 15.5	+ 49.6
	- 4.6	- 41.4

The results are parallel on the whole, though there are larger increments in the titration value with phenolphthalein. The comparison was made by Lt. T. W. Wilson, U.S.A.

4. *Estimation of ammonia (amino-acid).* Samples have been tested by Sørensen's formaldehyde method with the following modification by Lieut. Wilson for the analysis of alkaline urines. The alkaline urine is acidified to  $P_H = 7.0$ , raised to the boiling point and cooled quickly. After this the estimation is proceeded with in the standard way.

5. *Estimation of chlorides.* The chlorides were estimated by Volhard's method.

## II. CALCULATION OF RESULTS AND EXPLANATION OF DIAGRAMS.

As a general rule 24 hourly specimens have been collected. The results have been expressed as cc. of  $N/10$  alkali per 24 hours, and grams  $NH_3$  per 24 hours.

In a few instances the urine was collected in periods shorter than 24 hours. As the rate of excretion is of most interest here, the results are calculated in times of 24 hour periods, *i.e.* the average hourly rate of excretion during a period over which the specimen is collected is multiplied by 24.

Suppose a 15 hour sample of urine measured 170 cc., the result for the cc. of alkali per 24 hours has been calculated on a volume of 272 cc.

$$(= 170 \times \frac{24}{15} \text{ cc.} ).$$

In this way the comparison of the rate of excretion in irregular periods is possible.

The results have been plotted on diagrams. In the diagrams the results given for a definite day say 6. viii. 18 represent the amounts excreted, the  $P_H$  for the same urine etc., for the 24 hours including the night 6. viii. 18 to 7. viii. 18.

## III. THE NORMAL URINE AND ITS VARIATION WITH DIFFERENT DIETS.

As in other animals the acidity and the ammonia excretion in the urine of the goat vary considerably with the diet.

Most of the goats used in these experiments, when taken from the goat pen, excreted an acid urine. They were placed on the experimental clover hay diet. The equilibrium reached by the urine tended to run roughly parallel to the H-ion concentration of an infusion of the hay or grass sample. The infusion was made by heating the hay gently in water and allowing it to stand for two hours.

Good clover hay was alkaline, about  $P_H = 8.5$ . A goat's urine on this diet has also a  $P_H$  of about 8.5.

Poorer samples of meadow hay and grass, the latter especially, had a more acid infusion value. Meadow hay infusion had a  $P_H$  of 8.0, and fresh grass of between 6.0 and 7.0. The goat's urine on this latter diet always showed a more acid value than on the former. On fresh grass in fact, the urine tended to be actually acid. The time taken to reach this equilibrium was between two and three days. A delay of two or three days would be expected from the fact that fresh food is not received into an empty stomach in the goat, but into a stomach that contains normally a large quantity of food in process of digestion.

The results given in Tables II-IV are typical of those obtained when the normal goat is changed from a diet of grass to one of hay.

Generally speaking, a goat which has been on a grass diet in a pen must have a urine of low hydrogen ion concentration, for at the time of removal from the pen the  $P_H$  usually lies between 5.0 and 6.0. In two to three days

on a good clover hay diet the  $P_H$  rises and tends to become constant at a value of 8.5. While this  $P_H$  remains constant, the titratable alkalinity shows large daily variations.

The excretion of ammonia (and amino-acid) varies from 0.05–0.3 gram ammonia per 24 hours. Amounts larger than 0.6 g. per 24 hours have not so far been found. As would be expected the chlorides show large variations.

Table II. (Goat No. 2403, weight 38 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	$P_H$	cc. acid 24 hrs
16. iii. 18	22	1½	—	375	1040	8.4	53
17. iii. 18	24	½	—	265	1040	8.1	26.5
18. iii. 18	24	1½	—	195	1035	8.5	41
19. iii. 18	24	1½	—	310	1042	8.5	106
20. iii. 18	24	1½	—	185	1042	8.5	44
21. iii. 18	24	1½	—	260	1030	8.5	68
22. iii. 18	24	1½	500	215	1040	8.5	99
23. iii. 18	24	1½	500	200	1032	8.5	36
24. iii. 18	24	1½	250	200	1033	8.4	38

No ammonia or chloride estimations.

Table III. (Goat No. 3144, weight 41 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	$P_H$	cc. acid 24 hrs	NH <sub>3</sub> g. per 24 hrs	NaCl g.	Faeces
16. v. 18	24	1	nil	315	1033	8.5	59.8	0.147	—	F
17. v. 18	24	1½	1000	345	1034	8.5	63.8	0.111	—	F
18. v. 18	24	1½	900	475	1031	8.5	73.6	0.464	—	F
19. v. 18	24	1	500	485	1032	8.5	48.5	0.181	—	F
20. v. 18	24	1½	1500	455	1033	8.5	50.0	0.092	2.13	F
21. v. 18	24	1½	1050	570	1031	8.5	51.3	0.198	2.66	F

Table IV. (Goat No. 3520, weight 52 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	$P_H$	cc. acid 24 hrs	NH <sub>3</sub> g. per 24 hrs	NaCl g.	
6. vi. 18	24	1½	2000	520	1022	8.7	72.8	0.0265	1.946	
7. vi. 18	24	1½	500	395	1036	8.7	98.7	0.026	2.22	
8. vi. 18	24	1	1000	230	1040	8.7	37.9	0.027	1.05	
9. vi. 18	24	1	500	190	1041	8.7	38.0	0.029	0.111	
Goat taken out of the crate with diarrhoea.										
18. vi. 18	24	1	500	205	1042	8.1	8.2	0.070	1.99	
19. vi. 18	24	1	1000	235	1043	8.7	44.6	0.05	1.69	
20. vi. 18	24	1	1000	230	1040	7.45	nil	0.18	1.99	
21. vi. 18	24	1½	1000	300	1037	8.1	15.0	0.107	1.20	
22. vi. 18	24	1½	1000	375	1032	8.5	77.9	0.26	1.50	
23. vi. 18	24	1½	1000	325	1040	8.5	52	0.064	2.54	
24. vi. 18	24	1½	1000	300	1042	8.5	66	0.051	3.28	

The effect on the urine of a change from rich to poor hay is shown in the case of goat No. 3128 (Table V). From 11. v. 18-17. v. 18 it was fed on a good clover hay diet. The  $P_H$  value started low, when it came out of the grass pen, and then rose. It was taken out of the crate from 17-28th. Beginning on 28th it was fed for three days on a poor sample of meadow hay (infusion value  $P_H = 6.2$ ), on 31. v. 18 the normal clover hay diet was given to it again, when it reached a proper equilibrium  $P_H = 8.5$ .

Table V. (Goat No. 3128, weight 66 lb.)

Date	Hours	Diet lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	$P_H$	cc. acid 24 hrs	$NH_3$ g. per 24 hrs	NaCl g.
11. v. 18	24	1 $\frac{1}{4}$	nil	210	1027	7.2	-4.2	0.117	—
12. v. 18	24	1	500	160	1030	6.4	-8.0	0.129	—
13. v. 18	24	1 $\frac{1}{2}$	1350	195	1030	8.1	17.5	0.061	—
14. v. 18	24	1 $\frac{3}{8}$	nil	385	1042	8.5	113.5	0.065	—
15. v. 18	24	1 $\frac{5}{8}$	875	175	1035	8.1	20.1	0.035	—
16. v. 18	24	1 $\frac{7}{8}$	1000	330	1041	8.1	21.4	0.072	—
17. v. 18	24	1 $\frac{1}{2}$	1500	280	1040	8.3	49	0.053	—
28. v. 18	24	2	1000	275	1034	5.0	-50.9	0.225	3.410
29. v. 18	24	2 $\frac{1}{2}$	445	445	1017	5.2	-46.7	0.287	3.09
30. v. 18	24	1	750	—	—	6.0	(not free from faeces)		
31. v. 18	24	2 $\frac{1}{4}$	1000	480	1035	8.3	38.4	0.497	6.25
1. vi. 18	24	1	1000	950	1023	8.5	195	0.274	3.44
2. vi. 18	24	1 $\frac{1}{8}$	500	655	1028	8.5	81.9	0.155	2.60
3. vi. 18	24	1 $\frac{1}{2}$	1000	500	1037	8.5	107.5	0.042	1.81

Clover hay diet. Infusion value  $P_H = 8.5$

Poor meadow hay diet. Infusion value  $P_H = 6.2$

Clover hay diet. Infusion value  $P_H = 8.5$

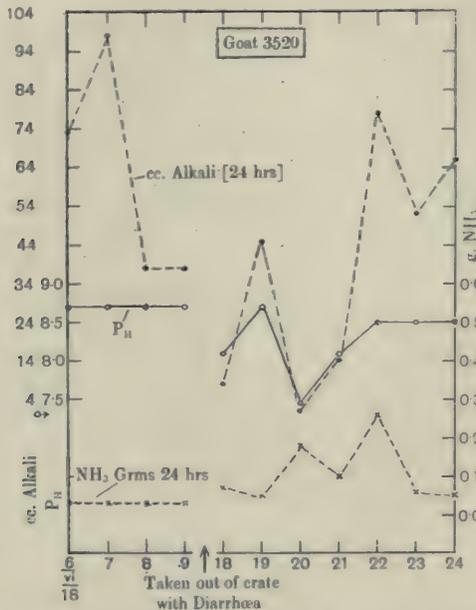


Fig. 3. Showing titratable alkalinity,  $P_H$  and ammonia content of urine of Goat No. 3520, normal, fed on clover hay diet.

The case is not quite so simple as it was thought at first, as can be seen from goat No. 4360 (Table VI). This goat was fed throughout on a diet of freshly-cut grass giving an infusion value of  $P_H = 5.5-5.7$ . For the first three days the  $P_H$  dropped, and then rose again, showing some form of compensation. Owing to the urgency of other work further experiments could not be done to settle this point.

Table VI. (Goat No. 4360, weight 58 lb.)

Date	Hours	Diet	Water cc.	Vol. of urine cc.	Sp. Gr.	$P_H$	cc. acid 24 hrs	Ammonia g. per 24 hrs
5. viii. 18	24	not weighed	nil	875	1016	8.1	+175.5	—
6. viii. 18	24	—	500	750	1024	6.7	- 11.2	—
7. viii. 18	24	—	500	920	1022	6.3	- 18.4	—
8. viii. 18	24	—	500	575	1036	7.3	- 2.8	—
9. viii. 18	24	—	nil	690	1036	8.1	+ 27.6	0.22
10. viii. 18	No specimen							
11. viii. 18	24	—	500	885	1028	8.1	44.2	0.27
12. viii. 18	24	—	300	650	1036	8.1	26	0.14
13. viii. 18	24	—	200	565	1044	8.1	22.6	0.11

Grass diet.  
Infusion value  
 $P_H = 5.5-5.7$

Table VII. (Goat No. 2697, weight 39 lb.)

(The urine was free from protein and sugar.)

Date	Vol. of urine cc.	Sp. Gr.	$P_H$	cc. N/10 acid	Protein	Sugar	Auton
9. iv. 18	245	1028	6.3	+12.3	—	—	—
10. iv. 18	285	1030	7.3	+ 5.7	—	—	—
11. iv. 18*	400	1026	8.4	-36.0	—	—	—
12. iv. 18	310	1026	8.1	-15.5	—	—	—
13. iv. 18	230	1030	7.1	+ 4.6	—	—	—

\* Starved 24 hours.

Table VIII. (Goat No. 2720, weight 57 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	$P_H$	To $P_H$ 7.45		Faeces
							cc. acid 24 hrs	$NH_3$ g. per 24 hrs	
9. iv. 18	24	1	—	240	1040	8.4	25.2	—	F
10. iv. 18	24	1½	500	260	1050	8.5	65	0.022	F
11. iv. 18	24	1½	500	190	1060	8.1	12.3	0.0014	F
12. iv. 18*	24	nil	500	320	1025	8.3	17	0.032	F
13. iv. 18	24	2	350	270	1034	6.7	-7.81	0.1	F
14. iv. 18	24	1½	500	300	1048	8.5	75.5	0.038	C
15. iv. 18	24	1½	500	210	1050	8.3	21	0.032	F
16. iv. 18	24	1½	1250	1975	1048	8.1	13.8	0.028	F
17. iv. 18	24	2½	1500	150	1048	7.5	7.5	0.04	F

16th and 17th, traces of albumin.

\* Starved 24 hours. F=Urine free of faeces. C=Urine contaminated with faeces.

The data for the urine of goat No. 2720 are shown graphically in Fig. 4.

*Effect of Starvation.*

Tables VII, VIII and IX show the effect of starvation on the urine of the normal goat. When this period of starvation was 24 hours, the maximum acidity was reached between the 24th and 48th hour after the commencement of starvation; when the period of starvation was 48 hours, the maximal acidity occurred between the 48th and 72nd hour after the last food was taken. In the four cases observed the period of maximal acidity was not during the period of starvation, but after it had ceased in each case.

Table IX. (Goat No. 2879, weight 52 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P <sub>H</sub>	To P <sub>H</sub> 7.45		Faeces	Sugar	Albu- min
							cc. acid 24 hrs	NH <sub>3</sub> g. per 24 hrs			
25. iv. 18	24	¾	—	300	1036	6.7	-13.5	.232	—	—	—
26. iv. 18	24	1¼	800	210	1045	6.9	-6.3	.132	F	—	—
27. iv. 18	24	1	500	195	1048	8.3	+11.7	-.071	F	slight trace	—
28. iv. 18	24	1	800	155	1048	8.5	+41.07	-.022	F	"	—
29. iv. 18	24	2	1250	225	1043	8.3	+16.87	-.049	F	—	—
30. iv. 18	24	1¼	1250	255	1047	7.8	3.82	-.142	F	—	—
1. v. 18	24	1¼	1000	225	1039	8.3	14.62	-.070	F	slight trace	—
2. v. 18	24	nil	1250	530	1017	8.3	18.55	-.072	—	—	—
3. v. 18	24	nil	125	517	1015	7.6	2.58	-.105	F	—	—
4. v. 18	24	1¼	1000	255	1020	6.9	-6.37	-.166	F	slight trace	—
5. v. 18	24	1¼	1250	230	1040	8.3	+13.8	-.168	F	—	—
6. v. 18	24	1	1100	305	1040	8.3	+3.81	-.067	F	—	—

*The Effect of feeding with alkali.*

Table X shows the effect of feeding two goats on alkali. In each case 10 g. of sodium bicarbonate was given in a single dose. As in other cases the animal had been kept in the house on a hay diet sufficiently long for the titratable alkalinity of the urine to be constant for a couple of days before the dose. In each case the urine showed a considerable rise in alkalinity on the day of the meal, which had entirely passed off on the day subsequent to it.

Table X. (Goats Nos. 2441, 2453.)

Alkali Goat No.	Days	Titratable alk.	Remarks
2441	1st	62	10 g. NaHCO <sub>3</sub> fed
	2nd	110	
	3rd	97	
	4th	169	
	5th	107	
	6th	27	
2453	1st	5	10 g. NaHCO <sub>3</sub> fed
	2nd	69	
	3rd	75	
	4th	154	
	5th	27	
	6th	49	

The course of this experiment on goat No. 2453 is shown in Fig. 5.

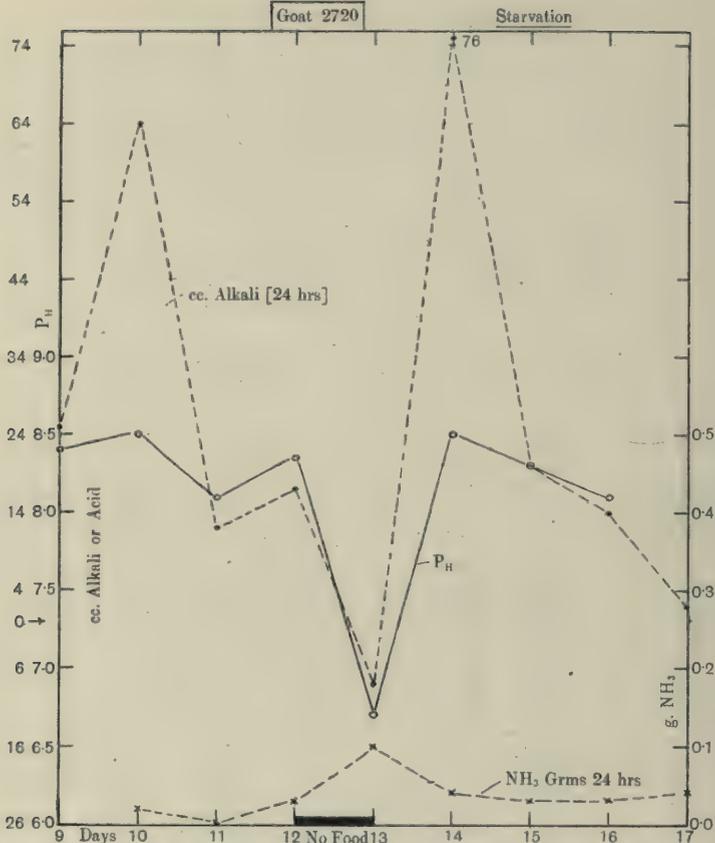


Fig. 4. Showing the effect of starvation on the titratable alkalinity, the hydrogen ion concentration and the ammonia content of the urine of a normal goat (No. 2720, see text).

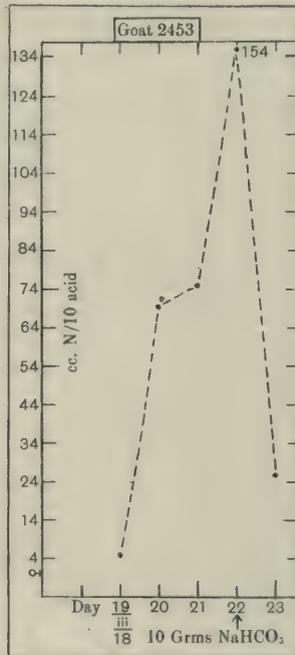


Fig. 5. Effect of feeding with NaHCO<sub>3</sub> on titratable alkalinity of goat urine.

IV. EFFECT OF FEEDING WITH ACID.

Table XI shows the effect of the administration of a dose of hydrochloric acid to a goat. The urine became acid to litmus in the first 24 hour period, the maximum acidity was reached in the period 24-48 hours after administration of the acid. As the urine became acid the excretion of ammonia rose, reaching a maximum in the same period as the maximal acidity and fell as the alkalinity became re-established.

The data which were obtained for the urine of goat No. 2904 are represented in Fig. 6.

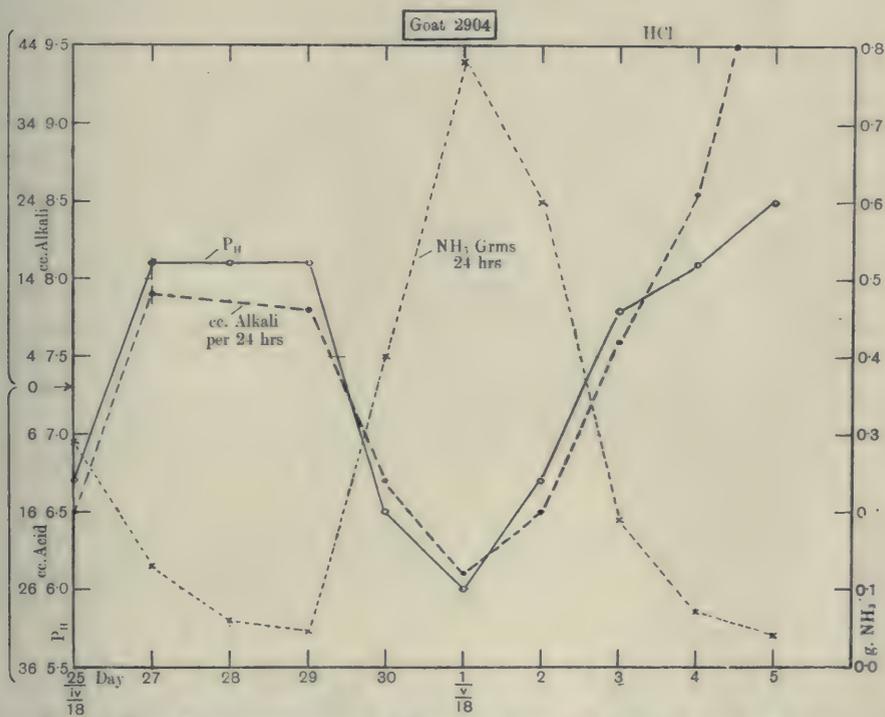


Fig. 6. Effect of administration of HCl on the titratable alkalinity, hydrogen ion concentration and ammonia content of goat's urine.

Table XI. (Goat No. 2904.)

Date	Hours	Diet, hay	Water	Vol. of urine	Sp. Gr.	P <sub>H</sub>	cc. N/10 acid per 24 hrs	NH <sub>3</sub> g. per 24 hrs	Faeces
26. iv. 18	24	1½	500	210	1036	6.7	-15.7	0.287	F
27. iv. 18	24	1	250	235	1042	8.1	+11.7	0.125	F
28. iv. 18	24	1	520	220	1038	8.1	14.3	0.057	F
29. iv. 18	24	1½	1000	285	1035	8.1	9.97	0.046	F
30. iv. 18*	24	1	1250	340	1033	6.5	-11.9	0.407	F
1. v. 18	24	1½	1000	375	1033	6.0	-24.3	0.784	F
2. v. 18	24	1½	1500	370	1035	6.7	-16.6	0.600	F
3. v. 18	24	1½	500	305	1033	7.8	+ 6.1	0.194	F
4. v. 18	24	1	1000	330	1040	8.1	24.7	0.072	F
5. v. 18	24	1	1250	335	1037	8.5	68.7	0.039	F

\* At 11 a.m. on 30th 30 cc. of strong HCl in 200 cc. water were given by stomach tube.

## DISCUSSION.

The use of ammonia by the goat for neutralisation of acid ingested is another case in which the original distinction between the herbivorous and carnivorous animal has broken down. It is in agreement with the work of Steenbock, Nelson and Hart [1914] upon the production of ammonia for neutralisation purposes by the calf. Fitz, Alsberg and Henderson [1907] showed that rabbits after the ingestion of acid excreted an increased amount of phosphates in the urine. Their paper gives no recorded analyses of the ammonia output, and as Winterberg has shown that rabbits fed on oats utilise ammonia for neutralisation, the rabbit would not seem to be really exceptional.

## SUMMARY OF RESULTS.

1. A method is described of obtaining urine specimens uncontaminated by faeces from the billy goat for the study of its metabolism.
2. The results which have been obtained show that the goat's urine responds to alterations in the acidity of the diet. With the addition of acids to the diet, the  $P_H$  of the urine gets less, and the titratable alkalinity falls. As the urine passes the neutral point and becomes actually acid there is a tendency for ammonia to be excreted.
3. The goat though herbivorous uses ammonia for the neutralisation of ingested acid, and in this respect confirms the conclusions of others that there is no real distinction between the herbivorous and carnivorous animal in this respect.
4. The goat readily excretes alkali when this is administered by the mouth.
5. The goat's urine reacts to a period of starvation by becoming more acid. In one or two days' starvation the urine reaches its most acid value after the starvation is stopped.

I am indebted to Mr Barcroft for his help, and to Lieut. T. Woodward Wilson, U.S.A. for advice and criticism, and for having performed some of the estimations quoted in the paper. I am also grateful to Lc.-Cpl. Morgan, R.E. for his invaluable assistance.

## REFERENCES.

- Baer (1906). *Arch. expt. Path. Pharm.* **54**, 153.  
Clark and Lubs (1917). *Amer. J. Bact.* **2**, 1.  
Fitz, Alsberg and Henderson (1907). *Amer. J. Physiol.* **18**, 113.  
Steenbock, Nelson and Hart (1914). *J. Biol. Chem.* **19**, 399.  
Winterberg (1898). *Zeitsch. physiol. Chem.* **25**, 202.

# LXVI. THE ACTION OF SEA WATER ON COTTON AND OTHER TEXTILE FIBRES.

BY CHARLES DORÉE.

*From the Chemical Department, Borough Polytechnic Institute, London, S.E.*

*(Received October 11th, 1920.)*

THE rapid disintegration of textile fabrics when exposed to sea water is well known. Under the condition of complete immersion most textile fibres become completely rotten in three to five weeks. The scientific interest attaching to the decomposition of such resistant materials as cotton and linen by the action of sea water is considerable. It is also of great importance to the marine biologist and others to discover the cause of this destructive action, leading either to a method of protecting fabrics, or to the production of a resistant material. The present paper contains a description of some experiments made with these objects in view.

The general effect of sea water is well illustrated in the following Table I which contains the results of trials carried out in the large tank of the Brighton Aquarium for which I am indebted to Dr J. Lyster Jameson.

Table I. *General Effect of Sea Water on various Textiles.*

+ indicates no obvious sign of weakening. ± visible deterioration. - completely rotten.

	Description	Examined after		
		Three weeks	Five weeks	Ten weeks
1.	Cotton fabric ... ..	-	...	...
2.	" " treated spermaceti ... ..	-	...	...
3.	" " treated spermaceti and ironed	+	-	...
4.	" " tanned with cutch ... ..	-	...	...
5.	Linen fabric ... ..	+	-	...
6.	" " treated spermaceti and ironed	+	+	-
7.	" " tanned with cutch ... ..	+	-	...
8.	Worsted fabric ... ..	+	+	±
9.	" " tanned valex* ... ..	+	+	+
10.	Silk fabric ... ..	+	-	...
11.	" " treated spermaceti ... ..	+	+	-
12.	Viscose silk ... ..	-	...	...

\* This gave way completely in the next four weeks.

The fabrics were mounted in square frames and weighted so as to float vertically with the top edge just awash. After a week nearly all the materials showed a flocculent deposit, apparently bacterial and diatomaceous in

character, which readily shook off, leaving the fabric with a greasy feel which suggested the presence of organisms closely attached to the fibres. Green and rusty brown spots then appeared and gradually the fabrics disintegrated.

As will be seen cotton will not endure an immersion of three weeks, and linen and silk stand less than five. The spermaceti treatment (used by fishermen for preserving their lines) makes very little difference, an interesting result which may be due to the fact that spermaceti, as an ester, is possibly decomposed by micro-organisms. A better mixture would no doubt be paraffin wax combined with a softening agent. The tanning process mentioned consisted of a treatment with 5 % dichromate solution at 60°-65° for one minute. The material was then worked in a 3 % tanning solution (valex or cutch) for one minute and the process repeated several times. The spermaceti treatment was carried out by immersion for 15 minutes in a bath composed of spermaceti two parts, liquid paraffin B.P. 1 part at about 65°.

The tanning of vegetable fibres, however, has little influence, but the author has recently obtained a better result by tanning cotton for one hour at 70° in a 0.05 % tannin solution and then fixing by immersion in solutions of metallic salts the metals of which are toxic to bacteria. With copper a "life" of nine weeks was secured, though antimony and zinc made little difference.

#### *The Chemical Changes produced in Cellulose by the action of Sea Water.*

To ascertain the chemical changes produced in cellulose fibres a purified madder bleach calico was used. Test pieces were exposed<sup>1</sup>, P in the light, Q in the dark. P soon became covered with pink growths which then disappeared to a considerable extent leaving rusty stains. In three weeks P was falling to pieces while Q, though whole, was quite rotten. P gave the following results:

	%
(a) Loss of weight in boiling 1 % NaOH (5 mins)	17.44
(b) Loss of weight in boiling 1 % NaOH (60 mins)	17.70
(c) Loss of weight in cold 17.5 % NaOH (20 mins)	19.15
(d) Hygroscopic moisture	6.09
(e) Copper reduced	1.42

The original madder bleach cotton gave under (a) 0, under (b) 0.6 % loss, (d) and (e) are normal figures. Of the part soluble under (c) only 20 % was reprecipitated by acid.

It is obvious that a very profound change is produced in the cellulose, as 17 % has become soluble in dilute sodium hydroxide solution. The fact that practically nothing more dissolves on further boiling points to the action being superficial and progressive.

<sup>1</sup> These and all subsequent trials were made for me at the Marine Biological Station, Plymouth.

*The respective influences of Oxygen, Light and Micro-organisms.*

The variables involved in the action of sea water on cellulose are oxygen, light, and bacteria or mould growth. To study their influence a series of laboratory experiments was set up in which all or none of these conditions were present and each was excluded in turn. Pieces of the standard madder bleach calico were placed in large flasks or jars full of sea water obtained from Lowestoft. Suitable devices were used to ensure freedom from oxygen or bacteria, and the immersion was continued in each case for nearly two years except in that of Exp. E, which was removed at the end of one year.

In Table II, which follows, will be found details of these experiments and in Table III the analytical constants of the pieces exposed.

Table II. *Influence of Oxygen, Light and Bacteria: Experimental details.*

Exp.	Weight in grams	Time in months	Exposed to			Notes on Experiments
			Oxygen	Light	Bacteria	
A	22.68	21	○	○	○	5 litres of sea water in flask, boiled with the cotton air-free and allowed to fill completely from a second flask permanently attached. Added HgCl <sub>2</sub> about 1 part in 2000. Conditions well maintained throughout. Cotton yellowish at end.
B	54.66	20	+	+	○	8 litres in covered glass jar. Water kept covered with toluene and oxygenated frequently by blowing purified air.
C	47.00	21	○	+	+	5 litres; oxygen removed by blowing hydrogen for 3 hours. Large bolt-head flask completely filled, with pipette through cork for expansion, etc. Freedom from air well maintained—fabric brownish at end.
D	27.94	22	○	+	○	A duplicate of A; fabric brownish at end.
E	40.46	12	+	○	+	8 litres; large stone jar exposed to the air and oxygenated frequently.
F	35.00	22	+	+	+	5 litres; oxygenated frequently and kept in good light. Growths appeared and disappeared from time to time.

All weights given in this paper are "dry" weights calculated at water-oven temperature. The moisture was always determined on a separate piece of fabric weighed simultaneously.

Table III. *Analytical Constants of the Cotton after immersion.*

Expt.	Exposed to			Relative strength of warp threads	Change in weight %	Ash %	Loss of weight % on boiling with 1% NaOH		Copper reduced %
	Oxygen	Light	Bac-teria				5 mins.	60 mins.	
Original cotton	...	...	...	100*	...	0.13	0.00	0.50	2.4
A	○	○	○	126	+0.3†	0.21	0.07	0.42	2.4
B	+	+	○	124	-0.4	0.17	0.16	0.21	1.7
C	○	+	+	107	-0.6	0.16	0.10	0.65	2.3
D	○	+	○	105	+0.8†	0.12	0.52	0.64	1.9
E	+	○	+	85	-2.4	0.21	1.80	1.90	2.8
F	+	+	+	79	-2.0	0.11	0.83	0.97	2.4

\* The actual breaking load was 123 grams.

† Due no doubt to the property of cellulose of fixing mercury from mercuric chloride (Vignon). This was present in Exps. A and D.

These results show clearly that if bacterial growth is prevented sea water as such has no action on cotton. Exps. A and B show practically the same values as the normal cotton, light and oxygen by themselves appearing to be without effect (Exp. B). The curious increase in strength shown by the first four samples is possibly due to shrinkage. On the other hand Exps. E and F, in which micro-organisms and oxygen were present, show marked deterioration comparable in kind, though not in degree, with Exps. P and Q made in the sea. They show a distinct loss in strength and in weight and in addition the remaining cellulose exhibits a solubility of nearly 2 % in sodium hydroxide which is not increased by more prolonged boiling. Light seems to have a slightly accelerating influence on the rate of destruction.

This series of experiments points strongly to the conclusion that bacterial or mould growth is the cause of the destruction of cotton fibres by sea water and that the presence of oxygen is essential.

This conclusion was supported by microscopic evidence. The investigation of a somewhat similar problem, viz. the deterioration of cotton on wet-storage, by Fleming and Thaysen [1920] led to the result that cotton stored damp is not affected unless micro-organisms are present, and not then if the moisture content of the cotton is reduced below 9 %. Finding it impossible to study microscopically the changes in the structure of the fibre brought about by the micro-organisms, these authors applied the modification of the viscose reaction suggested by Balls.

After treatment with the alkali-bisulphide mixture the fibres immersed in water swell to five times their normal size bursting the cuticle if it remains uninjured. In the paper mentioned, photographs are given showing the result of this test.

(a) With the normal fibre, presenting the appearance of a series of bulbs connected by rings of cuticle.

(b) With the fibres damaged by a streptothrix which has destroyed the cuticle and also perforated the fibre in many places causing it to break up into short pieces (fly).

(c) With the fibres damaged by a cellulose-decomposing schizomycete which eats away the cuticle over large areas and causes a progressive destruction from the surface to the interior.

The cotton fabrics E and F which were most affected by exposure to sea water when submitted to similar tests showed of course a large number of normal fibres. A considerable proportion, however, gave evidence of cuticular damage, and in each case the appearance was exactly that assigned by Fleming and Thaysen to the cellulose-decomposing schizomycete. This was abundantly proved by an examination of specimens P and Q (17 % soluble in NaOH). In this case practically every fibre showed evidence of the schizomycete—the streptothrix action was not observed.

*The Nature of the Change produced in Cotton by the action of Sea Water.*

Some conclusions as to this can be drawn from the figures obtained in these experiments. Numerous trials as with P and Q show that complete disintegration of the cotton takes place. On drying, the piece readily breaks up into a dusty powder. Yet except in the important matter of solubility in alkalis, the material shows very little difference from the normal cellulose. The pieces dyed with methylene blue and benzopurpurin to the same extent as the original fabric. The formation of a reducing substance such as oxycellulose which is almost invariably the result of chemical attack is excluded by the fact that the copper numbers in Table III are identical with the normal value<sup>1</sup>. The action of the micro-organisms would seem to be mechanical and in this connection it is of interest to recall the observations of Cross and Bevan [1918] on the destructive breakdown of cotton fibre under the mechanical process of beetling. A "cellulose dust" is formed which in its proximate composition, copper reducing power, dyeing and mercerising qualities etc., resembles normal cellulose. On treatment with 17.5 % sodium hydroxide in the cold, however, only 44 % remains undissolved and of the 56 % in solution only 48 % is reprecipitated by acids. These curious changes are being critically studied, and the results obtained in the present paper certainly show a similarity which is worth recording. Some marked change takes place in the cellulose complex which results in an entirely new direction of cleavage under the influence of alkali and yet permits of the retention of other characteristic properties.

Hess and Wittelsbach [1920] regard cellulose as a complex built up of "cellulose" (hydrocellulose) molecules united by the residual valencies of the hydroxyl groups. They suggest that the physical characteristic of cotton as a hollow thread is reproduced in the arrangement of the cellulose molecules in the complex, and that this complex can be broken down by mechanical means producing changes similar to those resulting from moderate chemical treatment.

It is exceedingly probable that the change whatever it may be is conditioned by the reactivity of the three hydroxyl groups of the cellulose unit formula and it appears not unlikely that if some or all of these were acetylated or otherwise "protected" the cellulose complex would be unable to rearrange itself in the direction of alkali solubility, and loss of structural character and the deterioration due to beetling or to micro-organisms might not take place. A suggestion on these lines was put forward by C. F. Cross eight years ago and two cotton fabrics were acetylated by his method approximately to the stage of a mono- and a di-acetate respectively, the structural form of the cotton being retained. These pieces were immersed in sea water at the same time as those detailed in Table I. No growths were visible at any time, and when the fabrics were removed after 16 weeks they appeared completely

<sup>1</sup> These numbers though very much higher than usually found for a purified cellulose were done by a standard method and are comparable

unaffected. The process can be carried out by such a method as that of Eng. Pat. 142,615 of Oct. 1919. One part of fused zinc chloride is dissolved in a mixture of acetic anhydride 5 or 6, glacial acetic acid 4 or 5 parts. The cotton is treated several times with double its weight of this mixture at temperatures below 50°. It is very difficult, however, to acetylate an open fabric such as muslin without largely sealing up the openings and the process is not of much use for marine biological purposes.

*Cellulose Acetate Silk, a resistant Textile Material.*

Attention was therefore directed to the "Acetate Silk" now being manufactured on a large scale by British Cellulose and Chemical Manufacturing Ltd. from the soluble cellulose tri-acetate. Yarns of every grade of fineness are now available and by the courtesy of the Sales Department a supply was sent for experiment.

An acetate silk twist yarn with a breaking load of 3.2 kilos was immersed in sea water under the same conditions as those of Exps. P and Q. At the end of five weeks no growths had appeared, the fibres seemed quite unchanged and the breaking load was still 3.0 kilos. Even after four months no appreciable change had taken place.

It will be seen therefore that a material is at length available which has a satisfactory resistance to sea water. At present fabrics composed wholly of acetate silk are not being manufactured, mixed fabrics only being produced. The yarn is available in any quantity, and it is recommended that this be knitted into suitable meshed fabrics for marine biological use.

SUMMARY.

1. Fabrics of cotton and silk are destroyed by immersion in sea water for three weeks, wool lasting somewhat longer.
2. This destructive action has been shown in the case of cellulose to be due to micro-organisms and not to oxygen, light or the salts present.
3. In its nature it resembles the "mechanical" break-down of cotton sometimes observed under the "beetling" process.
4. If cotton is acetylated to the mono-acetate stage so that its structural qualities are preserved, the resulting material is very resistant to sea water.
5. Cellulose acetate silk now manufactured on a large scale has proved able to withstand the action of sea water for months.

I desire to express my thanks to Dr E. J. Allen, F.R.S. of the Marine Biological Station, Plymouth, for his valuable cooperation throughout this investigation, for the expenses of which I am indebted to a grant from the Dixon Fund of London University.

REFERENCES.

- Cross and Bevan (1918). *J. Soc. Dyers Col.* **34**, 215.  
 Fleming and Thaysen (1920). *Biochem. J.* **14**, 25.  
 Hess and Wittelsbach (1920). *Zeitsch. Elektrochem.* **26**, 232.

## LXVII. A NOTE ON THE DIFFERENTIATION OF THE YELLOW PLANT PIGMENTS FROM THE FAT-SOLUBLE VITAMINE.

BY MARJORY STEPHENSON, *Beit Memorial Research Fellow.*

*From the Biochemical Laboratory, Cambridge.\**

*(Received October 12th, 1920.)*

THE identification of the yellow plant pigments—carotene and xanthophyll—with certain lipochromes of the animal body has been established by Palmer and his co-workers and has formed the substance of an interesting series of investigations.

Palmer and Eckles [1914, 1] first identified the yellow colouring matter of cow's milk with carotene accompanied by insignificant traces of xanthophyll. They showed that cows fed on a carotene-free diet yielded, after some weeks, a completely colourless milk, and further that, on the restoration of green food to the diet, the carotene was immediately diverted to the milk fat. The same writers also showed that carotene—with traces of xanthophyll—forms the yellow colouring matter of the body fat, corpus luteum and skin secretions of the cow and made the further observation that the same pigments form the colouring matter of the serum albumin from which they can be separated by drying with plaster of Paris, and treating the dried mass with alcohol, followed by extraction with light petroleum [Palmer and Eckles, 1914, 2, 3].

On extending these observations to other animals Palmer could discover no trace of carotene in the blood serum of swine on grass feed, traces only were found in the blood serum of the sheep and none could with certainty be isolated from ewe's milk. Goat's milk showed possible faint traces of carotene. Horse serum, on the contrary, showed a content of lipochromes comparable with that of the cow [Palmer, 1916].

Examination of the lipochrome content of the fowl [Palmer, 1915] brought to light interesting differences between the chemical habits of birds and mammals in this respect. It appears that, whilst in mammals the predominant plant pigment stored in the body is carotene, yet in the fowl the pigment stored in the egg-yolk body fat and blood serum is principally xanthophyll. Another striking point of difference is the manner of the association of this pigment in the serum albumin. In the case of the mammal the carotene could not be separated from the blood serum by merely shaking with light petroleum,

in which this pigment is soluble; a preliminary breakdown of the albumin-carotene complex by treatment of the dried mass with alcohol was necessary. In the case of the xanthophyll-albumin complex of the bird mere shaking with light petroleum sufficed to remove the pigment.

The distribution of these yellow plant pigments in animal tissues, and particularly their concentration in the milk of the mammal and the egg yolk of the fowl, has naturally led to considerable speculation as to their function and has tempted several workers to seek for their association or identity with the fat-soluble vitamine. The absence of these pigments from the body fat of the pig—a fat which has been shown to be low in vitamine content—also formed a finger-post pointing in the same direction.

Drummond [1919] fed crude carotene in the proportion of 0.003 % of the total diet and obtained evidence of traces of the fat-soluble vitamine. When, however, pure recrystallised carotene was substituted for the crude product a negative result was obtained. Zilva [1920] found that an alcoholic extract of carrot—which must have contained small quantities of the pigment—carried the fat-soluble factor and when fed in quantities corresponding to 25 g. of fresh carrot per rat per day sufficed to restore normal growth.

Palmer and Kempster [1919] working on the growth and fecundity of fowls obtained interesting results negating the importance of yellow plant pigments in the animal. These workers experimented first with a ration which they stated to be “carotinoid free”; as however they mention that this ration contained bone meal *ad lib.* it would have been more satisfactory had they furnished evidence that this was fat-free, since from freshly ground beef bones it is possible to extract a bright yellow fat which in view of Palmer's earlier work probably contains carotene. In the course of their investigations, however, they resort to a special carotinoid-free diet in which bone meal was replaced by ground limestone. Hens on this ration laid eggs which were almost but not quite pigmentless; the eggs showed normal fertility, but were very slightly below the normal in percentage hatched. Considerable trouble was taken to prove the nature of the traces of pigment in these eggs. Although this was not actually identified, it was shown that it was neither xanthophyll nor carotene. These experiments showed fairly conclusively that the absence of yellow plant pigments does not affect prejudicially the health or fecundity of hens, and as a consequence renders it highly improbable that the fat-soluble vitamine can be identified with these pigments.

The experiments described in the present paper were begun in June, 1919; while they were in progress, work bearing on the same subject has been published both by Palmer and Kempster [1919] and by Zilva [1920]. The experiments about to be recorded are therefore chiefly of interest as independent corroboration of their results. As, however, our knowledge of the fat-soluble vitamine is at present mainly derived from feeding experiments for which the widest possible statistical evidence is desirable, it has been decided to publish them.

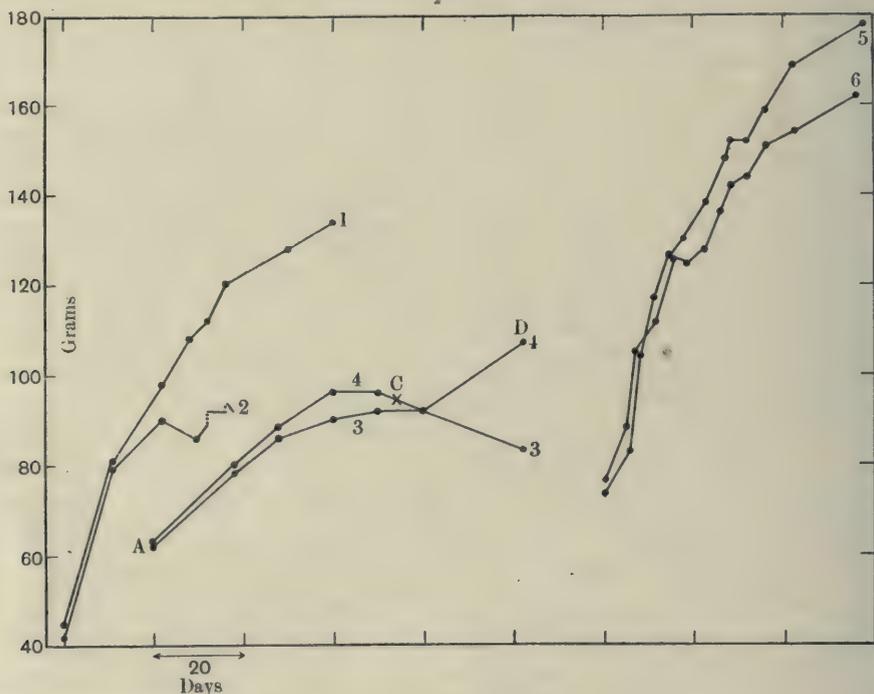
EXPERIMENT 1. *The effect of an alcohol-light-petroleum extract of dried*

*carrot on the growth of young rats.* In order to determine whether carotene is the substance responsible for the growth-promoting properties of butter fat a preliminary trial was made with a crude extract of dried carrot containing the pigment. The fresh carrots were passed through a mincing machine, dried by means of an electric fan between 30° and 35° and the dried substance reduced to powder in a mill. The powder was placed in screw-stoppered bottling jars and covered with 98 % alcohol for 24 hours. The alcohol was then filtered off on a Buchner funnel and the residue covered with light petroleum (B.P. 40°–60°) for 24 hours, again filtered and the light petroleum extraction repeated three times. The yellow alcoholic filtrate was reduced in vacuo to a syrupy residue and the deep red petroleum extracts from the combined washings were concentrated until the volume of the solution was about 0.1 of the original combined extracts. The alcoholic and light petroleum extracts were then mixed, and a portion of the extract corresponding to 250 g. of fresh carrot was evaporated to a syrup on a water-bath and mixed with 250 g. of a palm kernel oil, known by repeated previous experiments to be deficient in the fat-soluble vitamine. Ten young rats (43–52 g. initial weight) were put on the usual synthetic diet employed in this laboratory, consisting of a mixture of starch, sugar, and purified caseinogen to which was added McCollum's salt mixture [McCollum, Simmonds and Pitz, 1916]. The water-soluble vitamine was supplied by a fat-free alcoholic extract of yeast and the anti-scurvy factor by 0.5 cc. lemon juice per rat per day. The fat given was palm kernel oil mixed with the crude extract of carrot as above described. By this means, extract of carrot from 2–3 g. fresh carrot per rat per day was consumed. The growth of these rats is shown on Curve 1. Ten similar rats serving as a control were given the same synthetic diet with palm kernel oil alone. The growth of these rats is shown on Curve 2. After the 36th day the diet of the control set was changed with a view to the study of keratomalacia. No symptoms of eye disease were shown by the rats receiving the extract of carrot, whilst six out of the ten control animals had the disease unmistakably. This, together with the striking dissimilarity in the growth curve, is strong evidence for the presence of the growth promoting factor in the extract of carrot.

EXPERIMENT 2. *The effect of pure carotene on the growth of young rats.* The previous experiment having shown the presence of the fat-soluble vitamine in a crude alcohol-light-petroleum extract of dried carrot, a second experiment was undertaken to determine whether the substance responsible for the activity of the extract was carotene. This was prepared as follows:

5000 g. of fresh carrot were dried as described in Exp. 1, ground and extracted with light petroleum (without preliminary treatment with alcohol) at room temperature; the deep red extract (about eight litres) was concentrated on an electric heater to about 600 cc. and then in vacuo to about 5 cc. The residue was taken up with chloroform (10–20 cc.) and precipitated with methyl alcohol; the crude carotene rapidly crystallised out and was filtered

off under slight pressure. Yield from first crystallisation 0.1678 g. or 0.0033 % of the fresh carrot. The crude product was recrystallised from chloroform and methyl alcohol four times—that is until the m.p. was constant at 172–173° (uncorrected). The final yield was 0.0675 g., *i.e.* 0.0013 % of the fresh carrot, 0.004 g. of the recrystallised carotene corresponding to about 100 g. of fresh carrot was dissolved in 100 g. of palm kernel oil, which thus contained the same concentration of carotene as the palm kernel oil and crude extract of carrot fed in the preceding experiment. Five young rats were fed in the usual way on the standard synthetic diet with palm kernel oil and pure carotene as their source of fat (see Curve 3); five other rats, to serve as a control, were fed



Fat supplied to rats in

- Curve 1 (10 rats). Palm kernel oil + crude extract of carrot.
- Curve 2 (10 rats). Palm kernel oil.
- Curve 3 (5 rats). Palm kernel oil + recrystallized carotene.
- Curve 4 (5 rats). A—C. Palm kernel oil.  
C—D. Palm kernel oil + crude extract of carrot.
- Curve 5 (8 rats). Butter fat decolorised by charcoal.
- Curve 6 (8 rats). Untreated butter fat.

with palm kernel oil alone as their fat supply (see Curve 4 A—C). It is seen from these curves that the growth of the rats receiving carotene showed no superiority over that of the control animals and three out of the five suffered from eye disease. The period represented by Curves 3 and 4 covers 82 days. On the 82nd day one rat was killed and the diet of the remaining four

was changed by the substitution of crude extract of carrot for pure carotene; all symptoms of eye disease cleared up in the three rats suffering from keratomalacia. Three of the four, however, failed to survive and died, two on the 14th and one on the 46th day after the change of diet. From this experiment it is clear that the carotene is not the substance responsible for the growth-promoting properties of the crude light petroleum extract of carrot. (On the 54th day of experiment (Curve 4 C) crude extract of carrot was given to the control animals and resulted in the improved growth (Curve 4 C-D).)

EXPERIMENT 3. *The removal of the pigment from butter fat and the effect on its growth-promoting properties.* The decolorisation of butter was carried out as follows: 300 g. of filtered butter fat were dissolved in one litre of light petroleum and about 4 g. of fine birch charcoal added: the mixture was placed in a Winchester quart bottle and shaken for two hours on a shaking machine. At the end of that time the light petroleum extract was filtered free from charcoal and the solution found to be only very slightly tinted; a second treatment with charcoal and subsequent filtration resulted in the complete decolorisation of the butter fat. The light petroleum was then distilled off in vacuo final traces being removed at 60°. The fat thus treated was perfectly white and resembled lard in appearance; it was not ascertained whether this effect was due to the adsorption of the carotene by the charcoal or to oxidation on its surface. It may also be mentioned that only one out of three samples of birch charcoal (all of which bore the same description on the label) had the effect described. An explanation of this anomaly is probably to be found in the work of Philip, Dunnill and Workman [1920] on the activation of wood charcoal by heat.

Two sets of eight young rats were put on experimental diet, the fat in the one case being the decolorised butter fat (see Curve 5), that in the other case being the same sample of butter fat untreated (see Curve 6). One rat on the decolorised butter died on the 14th day of experiment without previous loss of weight and obviously from accidental causes. Perfect growth was maintained in every other individual of both sets for eight weeks after which the experiment was discontinued.

This experiment shows clearly that the removal (or destruction) of the colouring matter of butter does not affect its vitamine content.

#### SUMMARY.

From these three experiments we may conclude:

1. That a crude alcohol-light-petroleum extract of dried carrot when added to a fat not containing vitamine confers upon it the growth-promoting property and the power of protecting the animal from, or curing it of, keratomalacia.

2. That the substance or substances in the above extract responsible for these properties is not carotene.

3. That the colouring matter of butter fat may be completely removed or destroyed by filtration through charcoal without in the least affecting the vitamine content of the butter.

I wish to take this opportunity of thanking Professor Hopkins for the benefit I have derived from his wide experience in feeding experiments.

The expenses of this Research were in part defrayed by the Planters Margarine Co. and the Maypole Co. to whom my thanks are due.

#### REFERENCES.

- Drummond (1919). *Biochem. J.* **13**, 81.  
McCollum, Simmonds and Pitz (1916). *J. Biol. Chem.* **27**, 33.  
Palmer (1915). *J. Biol. Chem.* **23**, 261.  
—— (1916). *J. Biol. Chem.* **27**, 27.  
Palmer and Eckles (1914, 1). *J. Biol. Chem.* **17**, 191.  
—— (1914, 2 and 3). *J. Biol. Chem.* **17**, 211, 223.  
Palmer and Kempster (1919). *J. Biol. Chem.* **39**, 298.  
Philip, Dunnill and Workman (1920). *J. Chem. Soc.* **117**, 362  
Zilva (1920). *Biochem. J.* **13**, 81.

## LXVIII. NOTE ON THE VITAMINE CONTENT OF MILK.

BY FREDERICK GOWLAND HOPKINS.

*From the Biochemical Laboratory, Cambridge.*

*(Received November 8th, 1920.)*

THE observations described in this note will, it is to be feared, have less interest for others than for myself.

Since, in the early part of 1912, I published a paper describing the startlingly favourable effects of adding minute amounts of milk to synthetic dietaries, incapable, by themselves, of maintaining the growth and health of rats [1912], the conception of Accessory Food Substances or Vitamines has become widely based upon a large mass of experimental evidence. My early experiments have now no more than historic interest.

Since, however, Osborne and Mendel [1920], during their studies of the water-soluble vitamine, have found themselves unable to maintain the growth of rats, when using milk as a source of vitamine in quantities so small as those used by me, I have been naturally anxious to clear up what seemed to be a discrepancy in experience.

I may be allowed the privilege of saying here that the experiments which I described in 1912 followed upon a long experience of the effects of adding tissue extracts, and especially fractionated yeast preparations, to purified diets. Looking back to one's experience in these years during which startling successes were mingled with puzzling failures—failures which led to delay in publication—I realise that the absence of all knowledge concerning the factor associated with fats was the cause of any experimental contradictions. Had I possessed the scientific vision which afterwards led McCollum and Osborne and Mendel to recognise the existence of this, I should have reached full conviction as to the reality of my results long before I did. In the synthetic diets employed by me, the protein and the carbohydrates were purified to the uttermost, but I used little or no discrimination with regard to the fats.

With milk, as an addendum to the purified foodstuffs, I got consistent results in all cases. In preparing at that time a paper for publication I naturally wished to bring out the characteristic quality of the "Accessory Growth Substances" (which in spite of all experimental difficulties had, by 1910-11, become very real to me) of exerting their influence when present in very small amount. I wished in particular to emphasise the fact that their effects had nothing to do with energy supply; hence I used the milk addendum in quantities made as small as possible.

The published experiments, in which quantities of milk as small as 2 cc. per animal per day were employed, were in no sense selected. They were few in number, but gave consistent results and I had no feeling at the time that they would offer any difficulties in repetition.

Some months before they published the paper referred to above, Professors Osborne and Mendel, with great courtesy, communicated the results privately to me.

Even earlier than this I had, as a result of some remarks previously published by Drummond, and by Osborne and Mendel themselves, made, on a small scale, an effort to repeat my original experiments. I became impressed by the apparent influence of seasons on the results.

On the receipt of Osborne and Mendel's private communication, I made a few experiments in the winter of 1919. At this time the results were frankly disappointing. When the animals receiving the 2 cc. of milk in addition to the synthetic diet were compared with others which had the latter alone, the favourable effect upon health and upon the survival periods of the animals was unmistakable. Growth, however, was very slow, and the death rate of the rats was higher than that of animals normally fed.

In April of this year fresh experiments were begun and continued during the summer. The results now became such as to confirm entirely my earlier experiments. Out of 20 animals, each receiving daily 2 cc. of fresh cows' milk as an addition to a highly purified synthetic diet, not one failed to grow almost normally throughout the period of experiment. The observations were not extended beyond 60 days, as they were meant only for comparison with those described in my original paper, and not to determine the amount of milk necessary for continued growth. The technique was exactly that originally used by me. In each experiment control animals were fed upon the synthetic diet alone and the contrast between them and a corresponding set receiving 2 cc. of milk was just as marked as in my original experiments. There has been no selection of results. All the observations made from April onwards are here described.

It may be that such experiments, in which it must be supposed that the supply of vitamins (at any rate of the water-soluble substance) is minimal, are well calculated to bring out the minor variations in the quality of the milk, as it may depend, for instance, upon variations in the summer and winter feeding of cows.

Osborne and Mendel, however, were unable to show that this factor influenced their results. In connection with the experiments just described, I myself endeavoured to get information on the point. Goats were fed upon dietaries corresponding roughly to the winter and summer feed, respectively, of milch cows. Three goats received a mixed cereal diet, together with a small ration of hay and mangold. Three other animals had the same diet, but in addition took grass and green vegetables freely. Two of the animals in each set produced young and their milk was supplied to rats in quantities of 5 cc.

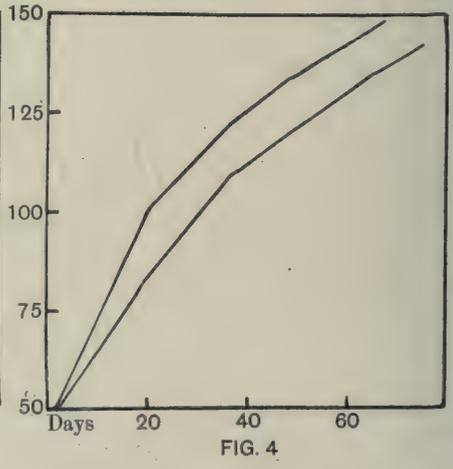
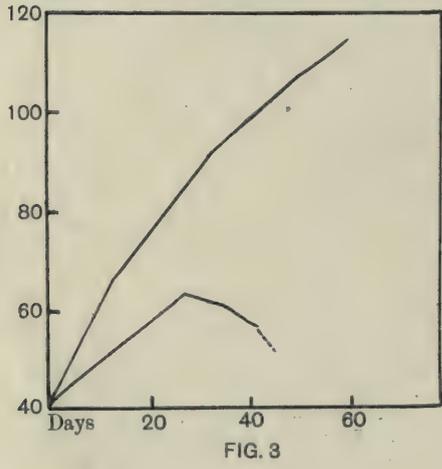
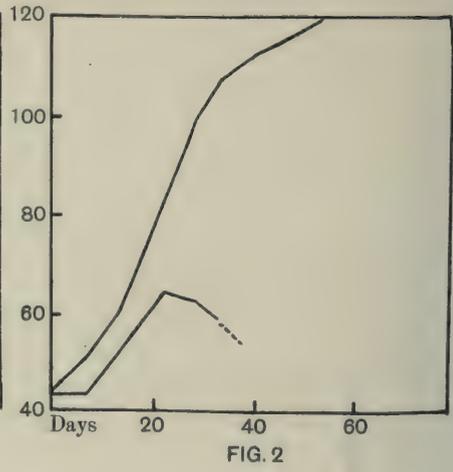
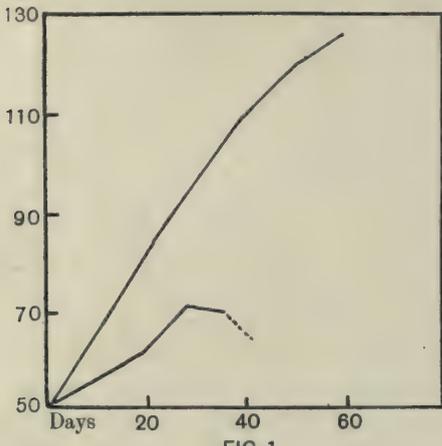
per rat per day. Growth was nearly normal in each case, though, doubtless as an accidental circumstance, the rats upon the "summer" dietary grew rather less well than the others. Analysis of the milk in each case showed that the total solids and fat content was, throughout, almost the same in both. The goats deprived of green stuff produced, however, considerably less milk than the others, a circumstance which would seem to make such comparisons very difficult. It may be when there is deficiency of some essential constituent in the food of a lactating animal that adjustment may occur by a limitation of the total quantity of the milk rather than by a falling off in its quality. The incomplete observation just mentioned, and the experience of Osborne and Mendel, suggest, at any rate, that the apparent seasonal variation in the results of my experiments was not likely to be due to differences in the milk. That there is a seasonal factor in the growth energy of rats is I think sure, but it is doubtful if it could account for the large difference in the experimental results now described. I am endeavouring to obtain further light on the matter. The purpose of this note is merely to point out that given the right conditions my original observations can be repeated.

*Experiment 1*, begun April 9, 1920. Eight rats were placed upon the synthetic diet. Four received this alone, and four were given 2 cc. of milk each before receiving their daily feed; great care being taken that the milk was completely consumed. The latter grew vigorously during the 60 days of the experiment, the former grew slowly for about 25 days, when their growth ceased and on the 35th day three out of the four died. The individual animals showed a strikingly uniform behaviour in each set. This experiment was supervised by Mrs E. C. Bulley (curves of average growth in Fig. 1).

*Experiment 2*, begun April, 1920. Of ten rats, five received the synthetic diet alone, and five had the addition of 2 cc. of milk, fed, as before, in advance of the ration. The latter grew normally for 35 days, then somewhat more slowly, but still vigorously, up to the 60th day, when the experiment was stopped. The former grew slowly for rather more than 20 days, after which their weight rapidly fell off, one dying on the 30th day, and the others soon after. For this experiment I have to thank Miss Killby (average curves of growth in Fig. 2).

*Experiment 3*, begun May 5, 1920. Of 18 rats, eight were upon the synthetic diet alone, and ten received 2 cc. of milk. This experiment, carried out by myself, almost exactly reproduced the results of Experiment 1. The growth curves of individual rats receiving the milk were nearly superimposable, so uniform was their behaviour (average curves in Fig. 3).

*Experiment 4*. This was the experiment referred to above in which rats were fed upon goats' milk. In Fig. 4 the upper curve shows the average growth of five animals receiving 5 cc. of milk from the "winter" fed goats, while the lower curve refers to five rats receiving the milk of goats supplied with abundant green stuff.



REFERENCES.

Hopkins (1912). *J. Physiol.* **44**, 425.  
Osborne and Mendel (1920). *Bioch. J.* **41**, 515.

# LXIX. THE EFFECTS OF HEAT AND AERATION UPON THE FAT-SOLUBLE VITAMINE.

BY FREDERICK GOWLAND HOPKINS.

*From the Biochemical Laboratory, Cambridge.*

*(Received October 15th, 1920.)*

A KNOWLEDGE of the conditions which affect the stability of vitamins is not only of immediate practical importance in connexion with the commercial and domestic treatment of foods, but is, clearly, not without value in setting certain limitations to hypotheses which may be framed as to the nature of these substances, and also in giving guidance when attempts are made to isolate them.

There is at the moment less certain knowledge of this kind in the case of the "fat-soluble A" than in those of the two other recognised vitamins. Thus Steenbock, Boutwell and Kent [1918] came to the conclusion that the substance is readily destroyed by heat, and, later, Drummond [1919] from the results of his earlier experiments came to the same conclusion. Osborne and Mendel [1920], on the other hand, have recently confirmed earlier results of their own which indicated that it is resistant to heat. The investigation to be described in the present paper—to the results of which public reference has already been made [Hopkins, 1920]—confirms Osborne and Mendel's work by showing that the vitamin is relatively resistant to heat. It has demonstrated, on the other hand, that this nutritive factor is rapidly destroyed by exposure to atmospheric oxygen at temperatures ranging from 15° to 120°.

## EXPERIMENTAL.

While the main purpose of this paper is to describe experiments which bear upon the relative importance of temperature and aeration as factors concerned in the destruction of the vitamin A in butter, it will be useful to refer first very briefly to an investigation undertaken with a somewhat different aim.

### I. *Is there any Destruction of Vitamin during the Commercial Purification of Vegetable Fats?*

When vegetable fats are prepared for use in foods such as margarine they are of course first purified. It is a point not without importance to decide whether the processes applied have or have not destroyed vitamin which may have been present, to some extent at least, in the original crude fats.

Some two years ago I was asked by the firms mentioned at the end of this paper to investigate this question. The fats employed were palm kernel oil and ground nut oil, each respectively in the crude condition and purified for use. A large number of rats was used in testing the point, but it is not proposed to describe the results in detail. The practical issue was that in the case of neither fat could any difference in vitamine content be found between the crude and purified material. The average growth curves of the animals fed respectively upon the crude and pure fat were almost super-imposable, and the average curves of those upon the palm kernel oil were exactly similar to those upon ground nut oil. The results indeed may be grouped together as a whole to yield statistics regarding the behaviour of the rat-stocks in my laboratory when on a diet free from—or greatly deficient in—the fat-soluble vitamine. Except for the fats the diet was identical with that employed in all the experiments mentioned in this paper. In the case of 60 of the animals used, coming from the same or strictly comparable stocks, the average date upon which growth ceased was the 25th day, and in respect of this datum individual animals were closely grouped round the mean. The date of death varied of course much more widely. On the average it was the 56th day. 7 % were dead on or about the 30th day; 12 % died between the 40th and the 50th, 54 % between the 50th and 60th, and 27 % outlived the 60th day. These data, since they were obtained from rat-stocks which were essentially the same as those used for the experiments described in the next section, are of value for comparison.

## II. *The Effect of Heat and Aeration on the Vitamine Content of Butter.*

In all the experiments carried out in this connexion the diet comprised highly purified caseinogen, potato starch, cane sugar, butter. The mineral supply was Osborne and Mendel's salt mixture with the addition of 0.10 % potassium iodide and a few milligrams per cent. of sodium fluoride. The butter was always first filtered and, however subsequently treated, formed always 15 % of the food.

The number of animals employed in the whole investigation was large. The growth curves if given in every case would occupy much space, so that certain of the observations must be dismissed with a verbal description. Most of the experiments were made comparative; balanced sets of animals being fed side by side and the one significant factor alone varied in their respective diets. Many authors seem content to compare growth obtained upon a given experimental dietary with the accepted "curve of normal growth" and not with a curve obtained at the time of the experiment from comparable animals on a normal dietary. It is the experience of my laboratory that the curve of normal growth varies both with the season and with the stock. Osborne and Mendel have, I think, commented upon the influence of season on their experimental results. With my stock we seem always to obtain the clearest and most satisfactory results in respect of growth curves during the periods from the

beginning of April to the end of September. This is not a matter of temperature as the laboratory is always uniformly warmed. In the present investigation the comparison has frequently been, not between animals upon the experimental dietary and others upon normal food, but between one set upon fat treated in a given fashion, and another set upon the fat treated differently. Such sets have always been fed side by side.

There is doubtless even greater rigor of proof in the technique used by some others which consists in attempting to restore animals which are presumed to have failed owing to the absence of this or that constituent from their diet by a subsequent supply of that constituent. This method we have occasionally used in the present investigation as in Exp. 6. There are some advantages in the comparative method however.

*Experiments 1 and 2.* Early in 1919 eight rats averaging 57 g. in weight were placed upon a dietary in which the fat was butter previously heated in a steam autoclave for four hours at 120°. Eight similar rats had the same food but with the butter heated at 120° in an oil bath and simultaneously aerated for 12 hours with a brisk stream of air. The fat entirely lost its yellow colour. In the former set, three of the animals exhibited somewhat slow growth from the first, and died before the 60th day, but the remaining five grew vigorously and remained in good health for 160 days when the experiment was stopped. In the case of the second set all the animals failed to grow after the 21st day, five deaths had occurred by the 50th day, while the survivors afterwards developed keratomalacia and were killed. At about the same time eight other rats received butter heated for 12 hours to 80°, in such a way that a considerable surface of the fat was exposed to air. Growth was slow from the first; four deaths occurred about the 60th day, and a fifth occurred shortly afterwards. The three survivors lived to the 140th day when the experiment was stopped; but they had long ceased to grow, and were in poor condition.

These preliminary experiments were sufficient to offer a strong suggestion that aeration is an important factor in the destruction of the vitamine, but the comparison was faulty in the case of the first two sets of animals, since the aerated fat was heated thrice as long as the unaerated.

*Experiment 3.* In this case the comparison was rigorous, but the experiment was begun when but few young rats happened to be available for the purpose, so there were only four animals in each of the sets compared. The butter was in the one case autoclaved at 120° for four hours, and in the other heated to the same temperature and period but at the same time thoroughly aerated. The first set grew vigorously and remained in perfect health; the set on the aerated fat ceased to grow about the 25th day, and all died about the 50th day.

*Experiment 4.* This was a crucial experiment. Two carefully balanced sets of rats were taken, with ten animals in each set. One set received the standard diet with butter previously heated to 120° in the autoclave for four hours; the other set had the same diet with the butter heated at the same temperature

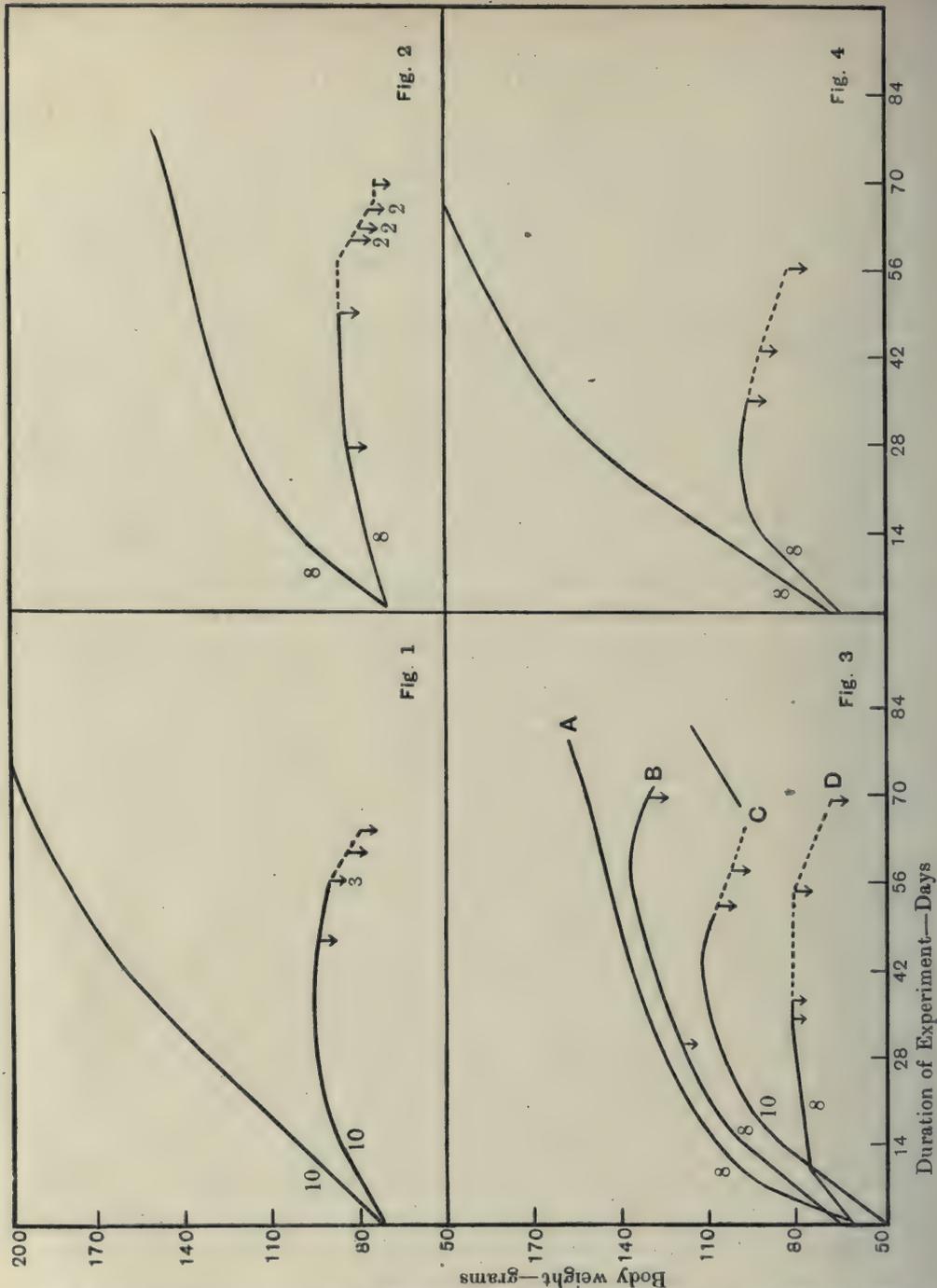


Fig. 1. (Experiment 4.) Upper curve shows average growth of 10 rats on a dietary containing butter heated out of contact with air for 4 hours at 120°. Lower curve shows growth of 10 comparable animals depending upon butter heated for the same time and at the same temperature but simultaneously aerated.

Fig. 2. (Experiment 5.) Curves as in Fig. 1, but the animals were fed with butter respectively autoclaved and aerated for 12 hours at 120°.

Fig. 3. (Experiments 6, 7.) Curve A, animals fed with butter aerated for 1 hour at 120°; B, aeration 2 hours; C, aeration 4 hours; D, aeration 16 hours. The unbroken line continuing Curve C shows the growth of surviving rats when put upon fresh butter.

Fig. 4. (Experiment 8.) Lower curve: animals depending on butter exposed in thin layers to air at ordinary temperatures. Upper curve: animals on butter preserved out of contact with air.

The arrows indicate deaths. When a death appreciably affected the average weight of the group the continuity of the curve is maintained by calculating the weight changes of the survivors on a percentage basis. Change in the curve from a continuous to a dotted line

and for the same time but with simultaneous aeration. The aeration as in other experiments was carried out in a flask immersed in an oil-bath. By means of a filter pump a stream of air was drawn through the melted fat at the rate of four or five bubbles per second.

The average growth curves obtained in this experiment are shown in Fig. 1, and in Fig. 5 are shown the curves of individual growth. In the latter the curve of each animal in one set is reproduced side by side with that of its representative in the other set, each being of the same sex and so far as possible of the same initial weight. (Two extra rats were in the set receiving aerated fat, for which, when the experiment was started, no exact duplicates could be found. These appear in the curves of individual growth, but they did not contribute to the average curve.)

None of the animals upon the autoclaved butter displayed keratomalacia during the course of the experiment. Of those receiving the aerated butter, three showed symptoms of this disorder on the 40th day, three on the 42nd, three on the 53rd, and one on the 64th day.

*Experiment 5.* The conditions in this experiment were exactly those of No. 4, but the butter was respectively autoclaved and aerated for 12 hours instead of 4 ( $120^{\circ}$ ). Curves of average growth in Fig. 2. To save expense of reproduction individual growth curves are omitted in the case of this and subsequent experiments. The degree of variation seen in Exp. 4 (Fig. 5) is quite typical of other experiments. There was no keratomalacia in the case of the animals receiving the fat heated out of contact with air. Of those on the aerated butter all developed keratomalacia between the 40th and 43rd day.

*Experiment 6.* Rats belonging to the same stock were divided into three groups with eight animals in each, the groups being balanced in respect of sex and weight. The diet was the same as in all other experiments, but with the butter aerated at  $120^{\circ}$  for one, two and four hours respectively. A fourth set of animals comparable with the others in respect of sex and weight but from a different stock, and fed at an earlier period, took the diet with butter aerated at the above temperature for 16 hours. The resultant growth curves are shown in Fig. 3.

There was no keratomalacia among the animals receiving fat aerated for one or for two hours; but, in marked contrast with the animals of Exp. 4, there was also none when the butter had received four hours' aeration. The rats concerned came from a stock quite distinct from that employed in Exps. 4 and 5. In the case of the group upon the fat aerated for 16 hours eye trouble developed in several of the animals, but no accurate record of its incidence was kept in this case.

*Experiment 7.* Filtered butter was kept at  $80^{\circ}$  and aerated with a brisk stream of air for 12 hours. Eight rats received the usual dietary with this aerated butter as its fat constituent.

The average growth curve corresponded so exactly with that of the animals

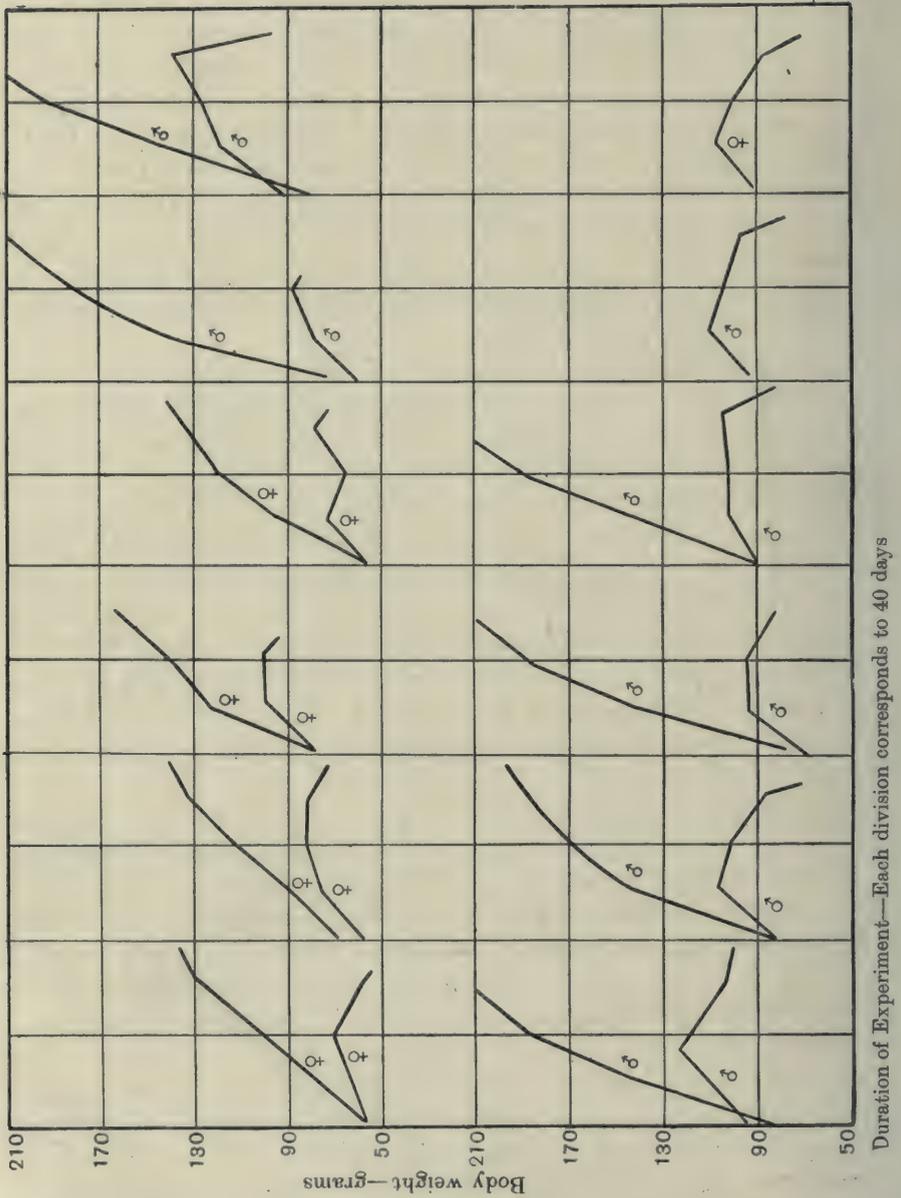


Fig. 5. Growth diagrams of individual rats in Experiment 4.

The upper line in each case shows the changes in body weight of an animal from the group receiving butter heated but not aerated. The lower line refers to an animal corresponding to the first in sex and initial weight, but from the group which depended upon aerated butter. Two rats in the second group were not represented in the first.

In every case the end of the lower curve marks the death of the rat.

which in Exp. 6 received butter aerated at 120° for four hours (Fig. 3, Curve C) that it need not be reproduced. Keratomalacia occurred in four cases between the 40th and 60th days.

*Experiment 8.* Filtered butter was exposed in thin layers at ordinary room temperatures, varying between 15° and 25° (during May, June and July) for periods of about a week. It had by this time lost all, or nearly all, its pigment. The fat so treated was added to the standard diet in the same proportion as in all other experiments, and the animals receiving it were compared with another set upon butter kept *en masse* for corresponding periods without exposure to air or light. The average growth curves (from eight rats in each set) are shown in Fig. 4. The animals were of the stock used for Exp. 6, different therefore from those of Exps. 4 and 5. It is noteworthy that though growth and nutrition failed on the exposed fat keratomalacia developed in one rat only and not before the 65th day.

#### DISCUSSION OF RESULTS.

It will be seen that the experiments described show in the clearest way that while the fat-soluble A factor is relatively resistant to the effects of heat alone, it is easily destroyed by aeration, presumably because it is a substance prone to oxidation.

It was on these points, and on these alone, that information was sought when the experiments were first planned, and for such a purpose the comparative method used seemed to be the best. The animals, in balanced sets, were fed side by side, aeration or non-aeration of their fat supply being the only difference in the treatment of the animals in the sets so compared. The difference in their behaviour was always unequivocal and most striking.

My experiments, however, give no quantitative information as to the *rate* of the destruction of the vitamine, whether by heat alone or with combined aeration at a given temperature. For this extended experiments and a different technique are called for.

The observations indicate that four hours' exposure to 120° does not, in the absence of air, sufficiently reduce the vitamine content of butter to make the heated product any less efficient than normal butter for maintaining rats, at any rate when the fat forms 15 % of their food (Exp. 4). Twelve hours' exposure to the same temperature seems undoubtedly to involve some destruction, but in the absence of air the vitamine is far from being completely destroyed (Exp. 5). On the other hand, aeration for four hours at 120° destroys the greater part of the vitamine, and exposure for 12 hours to the same condition involves what would seem to be almost complete destruction (Exps. 4, 5, 6). At the same temperature, one hour's aeration renders butter definitely less efficient for maintenance than unheated butter, and an increase in the destruction after two hours could be demonstrated (Exp. 6).

Since heating alone for four hours at 120° produced no effect which could

be demonstrated by the method of feeding adopted (*i.e.* with 15 % of fat in the dietary) and exposure to this temperature for even 12 hours produced relatively little, I have not troubled to test the effect of lower temperatures without aeration. Proof that fractions of the vitamines have been destroyed calls of course for experiments in which the total fat administered is reduced to the critical minimum.

With simultaneous aeration destruction of the vitamines proceeds with considerable rapidity at 80° (Exps. 2 and 7).

Very noteworthy are the results of full exposure to air at ordinary room temperatures. Butter exposed in thin layers at 15°–25° for periods of about a week was found to have lost its power of maintaining the growth or health of rats (Exp. 8). It is possible that in this experiment insufficient attention was paid to the accessory influence of light.

Aeration as is well known bleaches butter, but it now seems fully proved that the carotinoid pigments are not related to the fat-soluble A substance.

Though the hypothesis seems to me *prima facie* unlikely it may occur to some that combined heating and aeration might produce toxic products, or deleterious changes in the fat itself. In this connexion it is not without interest to point out that the general behaviour of the rats depending upon butter aerated at 120° for four hours or longer was very similar to that of the animals referred to earlier in this paper which depended on vegetable fats. In the latter growth continued on the average for 25 days and the average (from 60 rats) duration of life was 56 days. In the former the average period which elapsed before growth ceased was 23 days, and the average (from 42 rats) duration of life was 54 days. Aerated fat has frequently been added to the food of animals upon ordinary diet without deleterious effects.

With regard to the effect of the aeration upon the butter fats themselves, I may point out that Miss M. Stephenson has made determination of the iodine value of the fatty acids of butter before and after heating for four hours in a stream of air at 120°. The figures were identical (*e.g.* 39.0 before and 39.0 after).

Keratomalacia was for some reason more frequent in the animals upon aerated butter as just described than in those upon vegetable fats in the earlier investigation mentioned above. The latter formed part of the material used by Miss Stephenson and Miss Clark for their recently published [1920] study of the disorder. They found that only 28 % of the animals were affected. Taking all my animals upon butter aerated at 120° for four hours or longer one finds that just over 60 % showed symptoms of eye trouble. But although the complete absence of keratomalacia from the rats given heated, but un-aerated, butter and its frequency upon the aerated fat is of interest as giving further proof that the loss of nutritive value from the latter is actually due to destruction of the fat-soluble A, I do not attach statistical importance to the above figure. The influence of stock, and perhaps of the nutrition preceding experiment, may affect the incidence of keratomalacia. In Exp. 4 (fat aerated

four hours), and in Exp. 5 (aeration 12 hours), all the animals ultimately developed the condition. In Exp. 6, on the other hand, the animals upon butter aerated for four hours all remained free from it. The last were from a wholly different stock.

#### SUMMARY.

Experiments carried out upon a large number of rats have shown that the fat-soluble A substance in butter, while displaying marked resistance to heat alone at temperatures up to 120°, is readily destroyed by simultaneous aeration of the fat, presumably because it is a substance prone to oxidation by atmospheric oxygen.

The expenses of this research were defrayed from funds supplied by the Planters Margarine Co. and the Maypole Co.

To Mrs E. C. Bulley I am indebted for valuable help in the general supervision of the experiments.

#### REFERENCES.

- Drummond (1919). *Biochem. J.* **13**, 81.  
Hopkins (1920). *Brit. Med. J.* **ii**, 147.  
Osborne and Mendel (1920). *J. Biol. Chem.* **41**, 549.  
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **35**, 517.  
Stephenson and Clark (1920). *Biochem. J.* **14**, 542.

## LXX. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY FACTOR (VITAMIN A). VI: EFFECT OF HEAT AND OXYGEN ON THE NUTRITIVE VALUE OF BUTTER.

BY JACK CECIL DRUMMOND AND KATHARINE HOPE COWARD.

*From the Institute of Physiology, University College, London.*

*(Received October 18th, 1920.)*

A NUMBER of somewhat conflicting statements regarding the influence of heat on the vitamin A are to be found in recent papers. Originally this substance was considered to be comparatively stable to high temperatures, for Osborne and Mendel [1915] had found that butter fat lost little or none of its growth-promoting properties when subjected to the action of live steam for two and one-half hours.

Later, however, experiments were recorded by Steenbock, Boutwell and Kent [1918], the results of which cast some doubt on the supposed stability of the vitamin. They found, for example, that butter aerated for 12 hours at a temperature of 100° completely lost its power to maintain growth. Naturally their first tendency was to ascribe the destruction to oxidation, but when they found that shaking melted butter fat with carbonated water for several hours caused inactivation of the vitamin just as readily as when the shaking was carried out in the presence of air, they were led to conclude that the heat alone was responsible.

A series of experiments on the effect of heat on the vitamin present in certain oils was carried out by Drummond [1919], who obtained results which appeared to support the conclusions of Steenbock and his collaborators. In these experiments reliance was placed upon observations of the iodine value in order to ascertain whether the destruction of the vitamin is a process of oxidation. The results indicated that oxidation was not the cause of destruction of this dietary factor at high temperatures. Early in 1920 a paper appeared by Osborne and Mendel [1920], in which they produced further evidence in favour of their view that the vitamin present in butter is stable to relatively high temperatures. They not only confirmed their earlier result on the treatment of butter fat with steam, but also found that a sample of butter fat heated to 96° for 15 hours retained its growth-promoting activity.

In view of their results it was obvious that the question required re-investigating, and accordingly we carried out a new series of experiments in order to seek the cause of the discrepancies.

The result of this work has been to establish that destruction of the growth factor A does take place with ease at high temperature, provided facilities for oxidation are present.

While these experiments were in progress we learnt from Professor Hopkins that he had also arrived at a similar conclusion [1920, 1, 2], and further confirmation is given by the experiments of Zilva [1920]. It is particularly interesting to note that Hopkins has observed that destruction of the vitamin at high temperatures may occur without an appreciable change in the iodine value of the accompanying oil.

#### EXPERIMENTAL.

Throughout these experiments we employed the technique described in a previous paper [Drummond and Coward, 1920]. The various samples of butter were tested by administering them to young rats whose growth had been completely inhibited by withholding the factor A from their diet. The daily ration of butter was always given separately to the animals before they received the day's ration of the basal diet. In order to be able to detect any destruction of the vitamin, the amount of the daily supplement was fixed at 0.2 g., a quantity which, in the case of the sample of butter used in these tests, was found to give slow but steady growth.

##### *Effect of Exposure of Butter to High Temperatures.*

I. *Experiments at 100°.* A sample of the butter was subjected to the action of a current of live steam for six hours, an exposure longer by three and a half hours than that employed by Osborne and Mendel. Experiments on rats showed that this treatment had caused practically no loss of the growth-promoting factor A, an observation which is in agreement with that of the American workers. This treatment would of course effect the removal of the air in the vessel by the current of steam (Curves 1-3, Fig. 1).

II. *Experiments at 96°.* Quantities of butter were heated to 96° for 15 hours, as was done by Osborne and Mendel [1920]. One sample was placed in a large shallow dish in order to expose a considerable surface to the air, whilst another was enclosed during the heating in an air-free vessel. As may be seen from Fig. 1 destruction of the growth-promoting properties took place in the sample exposed to air (Curves 4-6) but not in that protected from oxidation (Curves 7-9).

Further experiments in which two samples of butter were heated to 96° for only three hours with and without air, respectively, gave similar results (Curves 10-12 and 13-15, Fig. 1).

III. *Experiments at 50°.* In order to re-investigate the effect of heating at comparatively low temperatures, another series of butter samples was heated for six hours at 50°. The results as a whole support the conclusion that oxidative processes are responsible for destruction of the vitamin A. As will

be seen from the Curves 16-18, Fig. 2, one animal (16) fed on the fat heated out of contact with air grew as if no deterioration in the nutritive value of the butter had occurred, but two others (17, 18) showed the opposite result. On the other hand, the animals fed on the exposed fat showed that this had been inactivated (Curves 19-21, Fig. 2).

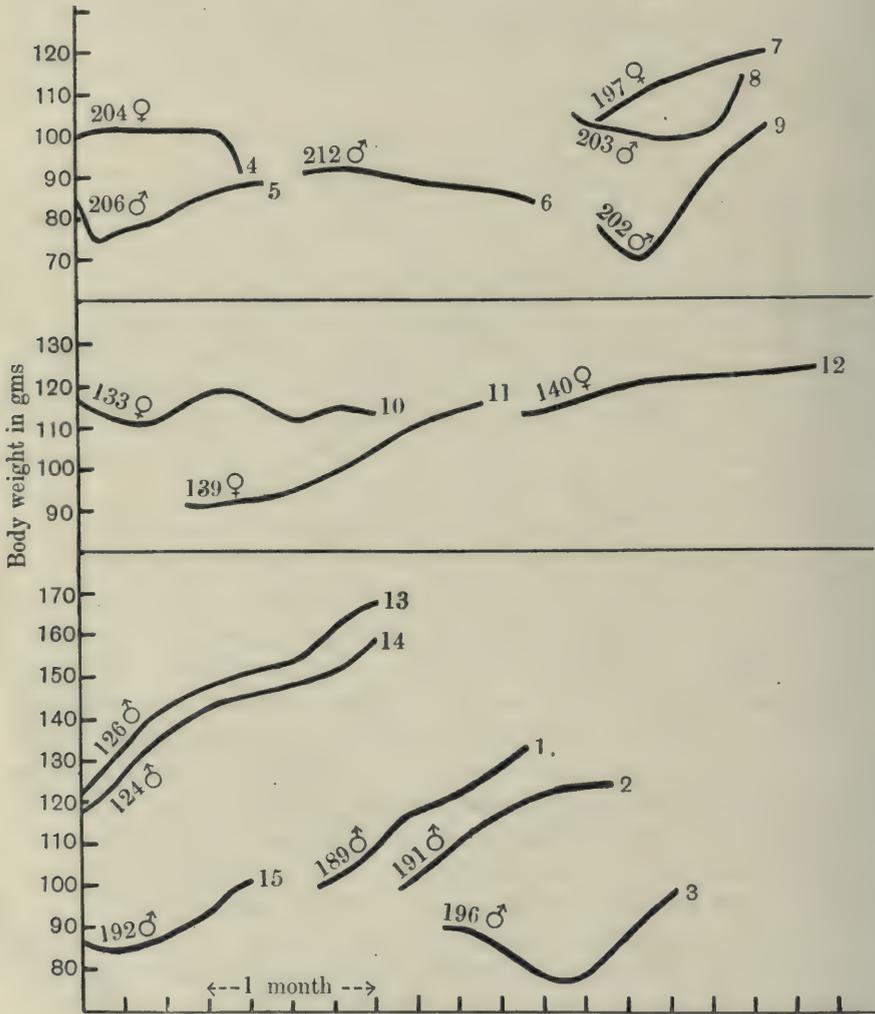


Fig. 1

N.B. In all the curves presented in this paper the preliminary period when the rats were fed on the basal diet alone is omitted.

A third sample of butter which was exposed to air in a vessel permitting a much smaller surface to come in contact with the atmosphere was only slightly inactivated (Curves 22-24, Fig. 2).

IV. *Experiments at 37°.* The most striking observation recorded in the earlier investigation [Drummond, 1919] was the instability of the vitamin A

at temperatures as low as 37°. It seemed, however, advisable to repeat the experiments employing the more satisfactory method of testing fractions which we now use. Four samples of the butter were heated at a temperature of 37° for a period of three weeks. Of these, two were exposed to air in thin layers in shallow glass dishes, whilst the other two were protected from air in closely stoppered vessels.

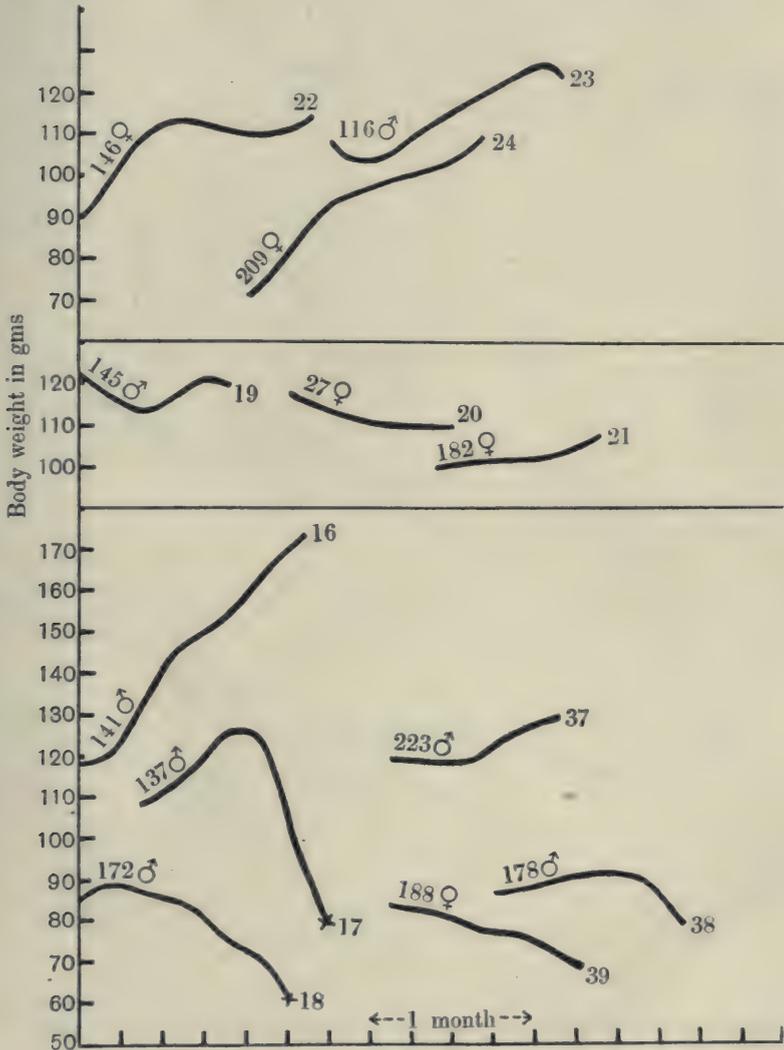


Fig. 2

One of each group was kept in a light corner of the laboratory, whilst the other two were placed in a darkened chamber. By this means it was hoped to ascertain the influence of light and air, since it has already been shown by Zilva [1919] that the vitamin A is destroyed on exposure to ultra-violet rays, a process which he suggested might be due to the action of the ozone

produced by the rays. Zilva has now obtained evidence that the cause of inactivation is oxidation by ozone [Zilva, 1920]. The samples exposed to air in this laboratory both became bleached, that kept in the light almost completely, and they were found at the end of three weeks to have lost their power of restoring growth in rats (Curves 25-27 and 32-34, Fig. 3). No appreciable impairment of the nutritive value of the samples protected from air was detected (Curves 28-30, and 31, 35, 36, Fig. 3).

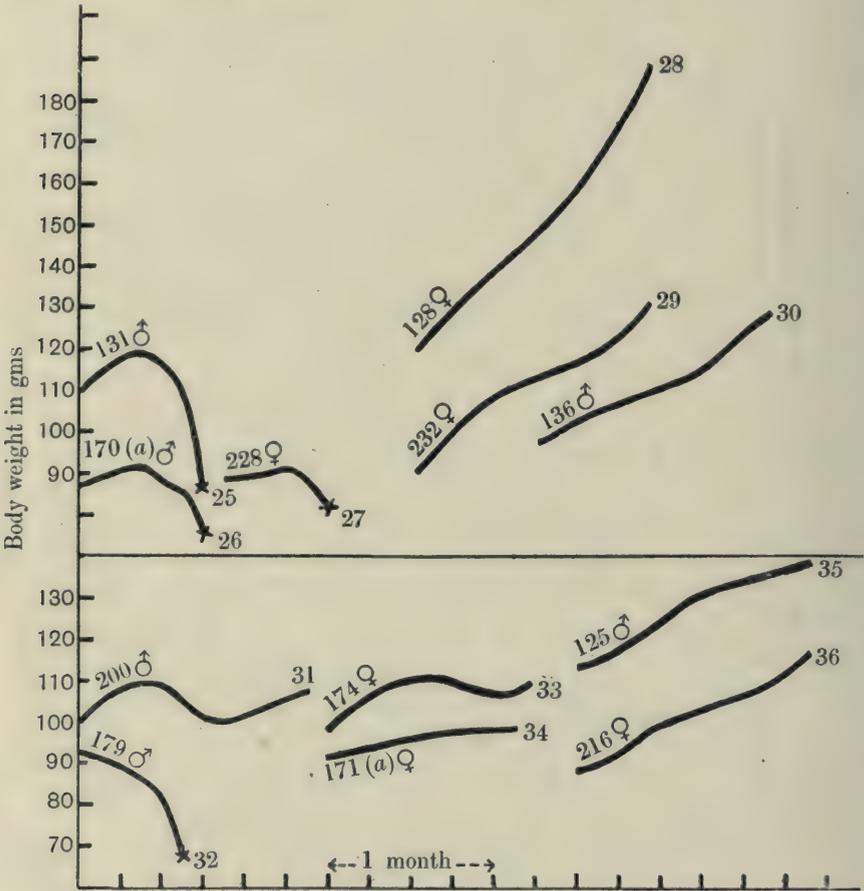


Fig. 3

In connection with these experiments we also tested a sample of butter fat, which Dr O. Rosenheim of King's College, London, very kindly placed at our disposal. This sample had during a previous investigation in 1917 been found to possess considerable growth-promoting power. It had been then dissolved in alcohol, and the solution allowed to stand for many months in the laboratory. When he sent it to us all the alcohol had evaporated, and there remained behind a perfectly white fat resembling a soft tallow. Tests on rats showed that the vitamin had been destroyed (Curves 37-39, Fig. 2).

## SUMMARY.

1. Destruction of the vitamin present in butter fat occurs on heating in the presence of air. It is therefore probable that the loss is due to changes of an oxidative nature.

2. The destruction takes place rapidly at high temperatures, but provided the exposure to air is extensive, considerable loss of nutritive value may take place at temperatures as low as 37°.

The authors wish to express their indebtedness to the Medical Research Council for a grant out of which the expenses of this research were defrayed.

## REFERENCES.

- Drummond (1919). *Biochem. J.* **13**, 81.  
Drummond and Coward (1920). *Biochem. J.* **14**, 661  
Hopkins (1920, 1). *Brit. Med. J.* July 31st, 147.  
—— (1920, 2). *Biochem. J.* **14**, 725.  
Osborne and Mendel (1915). *J. Biol. Chem.* **20**, 379.  
—— (1920). *J. Biol. Chem.* **41**, 549.  
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **35**, 517  
Zilva (1919). *Biochem. J.* **13**, 164.  
—— (1920). *Biochem. J.* **14**, 740.

# LXXI. THE ACTION OF OZONE ON THE FAT-SOLUBLE FACTOR IN FATS.

## PRELIMINARY NOTE.

BY SYLVESTER SOLOMON ZILVA.

*From the Biochemical Department, Lister Institute.*

*(Received October 18th, 1920.)*

In a previous communication [Zilva, 1919] it was shown that when butter was spread in a thin layer on a plate and exposed to ultra-violet rays for six to eight hours, the fat-soluble factor in it was inactivated. It was then pointed out that as ozone was produced by the mercury quartz lamp from the atmospheric oxygen the butter was at the same time exposed to the action of this gas, and that further investigation would be required in order to ascertain whether the inactivation was due to the action of the rays or to that of the ozone. It was therefore decided to establish the influence of ultra-violet rays on the fat-soluble factor in the absence of oxygen and the action of ozone on it in the dark, as well as to determine the iodine value of the fat under different conditions of exposure. As the results were of great interest, the inquiry into the action of ozone was extended to the antineuritic and antiscorbutic factors. The object of this note is to give a brief summary of the results obtained by studying the action of ozone on the fat-soluble factor only. The experimental data are reserved for another communication in which the influence of ozone on all the accessory factors will be described and discussed in detail.

For technical convenience active fats which are transparent and liquid at room temperature, like whale oil and cod liver oil, were chosen for this investigation. The latter oil being very active was found to be the more suitable and most of the experiments were therefore carried out with it. On exposure for six to eight hours in shallow layers in Petri dishes to the action of the ultra-violet rays in an atmosphere containing ozone these oils were entirely inactivated.

Unlike the butter, however, the cod liver oil was not bleached but assumed a slightly darker colour, which suggests that the nature at least of some of the colouring matter associated with it is different from that of butter.

In order to study the effect of ultra-violet rays on the fat-soluble factor in cod liver oil in the absence of oxygen the oil was placed in a thin layer a few millimetres thick between two tubes, one of which fitted loosely into the

other. The outer tube was made of quartz, so that the ultra-violet rays could reach the oil without being previously absorbed. The air above the layer of oil was displaced by carbon dioxide gas, and the tube was revolved by a water motor during the exposure. On testing the exposed oil on rats receiving a diet deficient in the fat-soluble factor it was found that an exposure even of 16 hours' duration did not inactivate the oil, nor was there any evidence that the activity of the oil was impaired to any great extent by such an exposure.

The next set of experiments was carried out with cod liver oil exposed to ozone in the dark. This was done by introducing some of the oil into a dark-stained glass bottle through which a current of ozone was passed. By rolling the bottle at short intervals the oil was thoroughly exposed. About ten hours of this treatment almost solidified the oil at the concentration used. After six hours' exposure the oil was much more viscous than before treatment, and high doses of this modified oil, which was originally extremely active, failed to promote growth in rats deficient in the fat-soluble factor.

It is evident then that ozone inactivates the fat-soluble factor in active oils and fats. This is in agreement with the recent observations of Hopkins [1920, 1, 2] and Drummond and Coward [1920] that the fat-soluble factor in fats on being exposed to atmospheric oxygen becomes inactivated. The action of ozone is of course much more drastic and therefore more rapid.

I wish to express my indebtedness to Dr J. S. Edkins for having kindly permitted me to use his ozone generator.

A part of the expenses of this research was defrayed from a grant made by the Medical Research Council to whom my thanks are due.

#### REFERENCES.

- Drummond and Coward (1920). *Biochem. J.* **14**, 734.  
Hopkins (1920, 1). *Brit. Med. J.* **ii**, 147.  
— (1920, 2). *Biochem. J.* **14**, 725.  
Zilva (1919). *Biochem. J.* **13**, 164.

## LXXII. THE NUTRITIVE VALUE OF LARD.

BY JACK CECIL DRUMMOND, JOHN GOLDING, SYLVESTER SOLOMON ZILVA AND KATHARINE HOPE COWARD:

*From the Institute of Physiology, University College, London, the Research Institute in Dairying, Reading, and the Biochemical Department, Lister Institute, London.*

(Received October 18th, 1920.)

With Plate XII.

DURING the last few years a considerable amount of attention has been devoted to the study of the distribution of the so-called fat-soluble accessory factor, or vitamin A, in naturally occurring oils and fats. The results of these investigations tend to show that the oils and fats derived from the animal kingdom are, as a rule, decidedly richer sources of this essential dietary constituent than those prepared from vegetable sources. Most authorities, however, regard lard as an exception, having found it practically devoid of vitamin A. Quite early in the study of the growth-promoting vitamins McCollum and Davis [1913] observed that butter fat is of much higher nutritive value for growth than lard.

A similar conclusion was reached by Osborne and Mendel [1913], who suggested that the difference might be ascribed to the fact that lard is a fat derived from storage depôts, whereas butter fat is a product of the synthetic processes of the mammary gland. Many later workers have confirmed these experimental results, and lard has usually been regarded as of little or no value as a source of the fat-soluble A factor.

When one considers the enormous quantities of lard which are prepared for edible purposes at the present time, the importance of ascertaining why lard is thus deficient will be realised. So far as we are aware, no systematic attempt to solve this problem has been made; and only one reference bearing on this question has been encountered in the literature.

Osborne and Mendel [1915] refer to one experiment which they carried out in order to ascertain whether the inefficiency of lard is due to the technical processes to which the fat of pigs is subjected in preparation for the market. Pig fat, direct from the slaughter-house, was finely divided and filtered through filter paper at a temperature just above its melting-point. The filtered product, which they termed "Laboratory Lard," was found to be as inadequate for growth as the commercial products. This result led the authors to conclude that the inferior nutritive value of lard is not due to the heating

which the fat may have received in the course of preparation. This experiment must, however, be regarded as of little value, since apparently no test of the growth-promoting power of the unheated pig fat was made.

In view of the very great importance of the question of the food value of lard, we decided to subject the matter to an experimental study, particularly since it had been observed by Drummond and Coward [1920, 1] that some specimens of raw pig fat contain appreciable amounts of the vitamin A.

Since it is now experimentally proven that the mammalian organism does not possess the power to synthesise the vitamin A, and that it is dependent on its diet for supplies of this essential factor, we concluded that the investigation must proceed along two lines, first, a study of the influence of the diet of the pigs on storage of the vitamin in the fat depôts, and secondly, an investigation of the influence of the technical processes of lard manufacture on the vitamin when present in the pig fat.

The first series of experiments was carried out at the farm attached to University College, Reading, and we desire to thank Prof. Pennington, the Director of the farm, for granting us facilities for the work. The rat feeding tests were carried out at University College, London.

## I. INFLUENCE OF DIET OF PIGS ON THE VITAMIN CONTENT OF PIG FAT.

### *Experimental.*

A litter of Berkshire pigs farrowed in the early part of 1920 was selected for this experiment. With the exception of one pig, which was a weakling, all the animals were strong and healthy. They were permitted to remain with the sow until weaned at the age of eight weeks, when they were given a ration of toppings (wheat pollards) and whey.

At the age of nine and a half weeks they were divided into five groups. Groups I-IV, which were to be placed on controlled diets, each contained a hog and a sow, whilst Group V, which were the real controls, contained two hogs.

Groups I and II were kept in large, well-designed, stone-floored experimental styes, since it was intended to give them a diet deficient in vitamin A. Groups III and IV were kept in a form of moveable pen improvised by one of us, which permitted the animals to have access to a new plot of green pasturage every day. Group V were reared in the usual manner employed on this farm, and not only received a mixed diet, but also had free run of a small grass paddock.

Group I were given a seriously deficient diet, namely one of toppings and a "synthetic whey." This latter constituent was prepared from caseinogen, lactose, olive oil and salt mixture so as to represent the composition of the whey used in the other experiments, as determined by frequent analysis.

Experiments on rats indicated that this diet was almost devoid of all three

vitamins A, B and C. It was not, therefore, considered likely that the pigs would thrive at all on the food mixture. To our astonishment both animals showed excellent increments of weight for a considerable period of time.

Fig. 1 shows the growth of the hog of this series. It will be seen that the initial growth is considerable in spite of the deficient nature of the ration. Ultimately the animals in this group showed retardation of growth, and lost their healthy appearance. We do not, however, intend to discuss this side of the experiments at this point since we have made a number of other observations on the growth of pigs on deficient diets which will form the subject of a later communication.

Group II were fed on a food mixture of similar composition to that employed for Group I, but the whey was the natural product. Tests of the whey made on rats indicated that it contained insignificant traces of the factor A.

Group III received a basal diet identical with that given to Group II, namely whey and toppings, but in addition were allowed to have an unlimited supply of fresh green food, an addition which they welcomed and of which they made good use.

Group IV received no whey, and were confined to a diet of toppings and green foods. Group V received a well-balanced and varied farm diet, including ample grass.

Growth curves of one animal (hog) from each group are given in Figs. 1-5, but, as we have remarked above, we do not intend to discuss this side of the investigation here. Records of food intake were made daily in the case of Groups I-IV, and frequent analyses of the toppings and whey were carried out to control the intake of nutrients. Attached to the growth curves are diagrams indicating the average daily food consumptions at various periods of the experiments.

At the end of nearly three months on the experimental diets, the condition of the hogs on the deficient diets 1 and 2 and the size of the control animals led us to conclude that a suitable point had been reached for testing the body fats for storage of the factor A. In view of the fact that our material was limited, we decided to slaughter the hogs of each group only, retaining the sows for the purpose of other observations which we desired to make. Accordingly this plan was carried out.

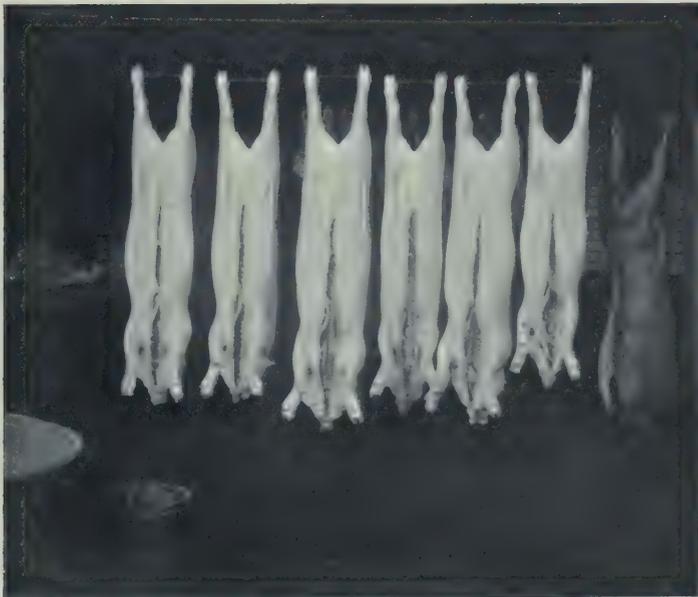
The hogs were sent to the butcher 24 hours before being slaughtered. A post-mortem examination did not reveal any noticeable abnormality in the organs of the animals. After the carcasses had been "dressed" in the usual manner, samples of back fat and perinephritic fat (fleck or leaf) were removed. Photographs of the dressed carcasses are given which show the relative sizes of the animals from each group (Plate XII). The small size of the hog fed on the deficient diet given to Group I is obvious, as are the well-developed bodies of the two animals in the control Groups III and V, especially in the latter.

The fats were tested for the presence of the factor A by observing the

744<sup>a</sup>



1. Side view.



2. Front view.

Photographs of the slaughtered pigs.

From left to right

Group	Sex	Diet	Weight lbs
II	♂	Toppings, whey	122
IV	„	Toppings, grass	103
V	„	Full diet with grass	150
V	„	„ „	—
III	„	Toppings, whey and grass	120
I	„	Toppings, synthetic whey	84



influence of daily supplements of known weight on the growth of rats, whose growth had been brought to a standstill by a deficiency of that substance.

Small cubes of the fat weighing approximately 1.5 g. were administered to each rat before the daily ration of the basal diet devoid of the factor A

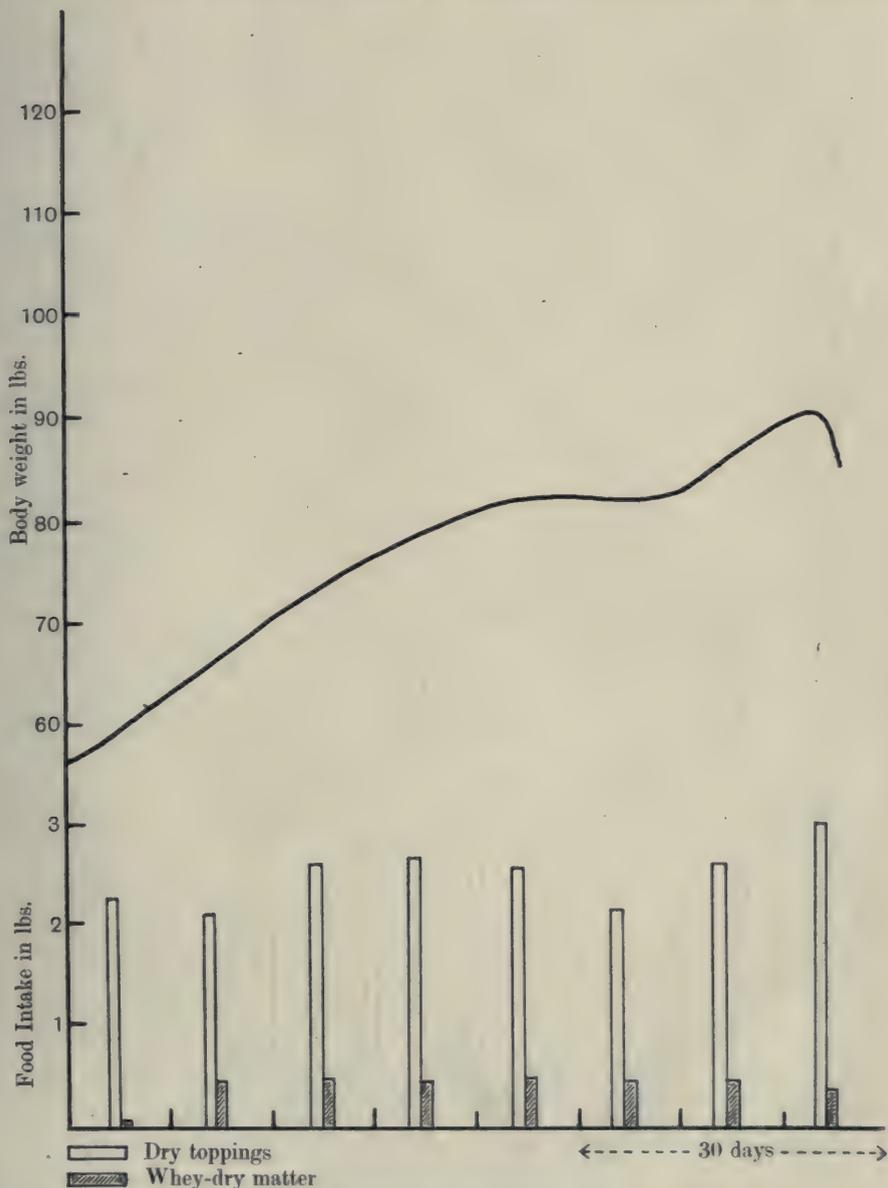


Fig. 1. Growth curve and food intake of hog from Group I.

was given. Practically without exception the whole of the supplements were consumed. The results of these feeding tests are in our opinion quite striking. The body fats derived from pigs in Groups I and II fed on diets deficient in

the vitamin A were found to be of no value as sources of that essential substance for the rat (Curves 1-11, Fig. 6). On the other hand, there is marked evidence that the body fats of the grass-fed animals, particularly those

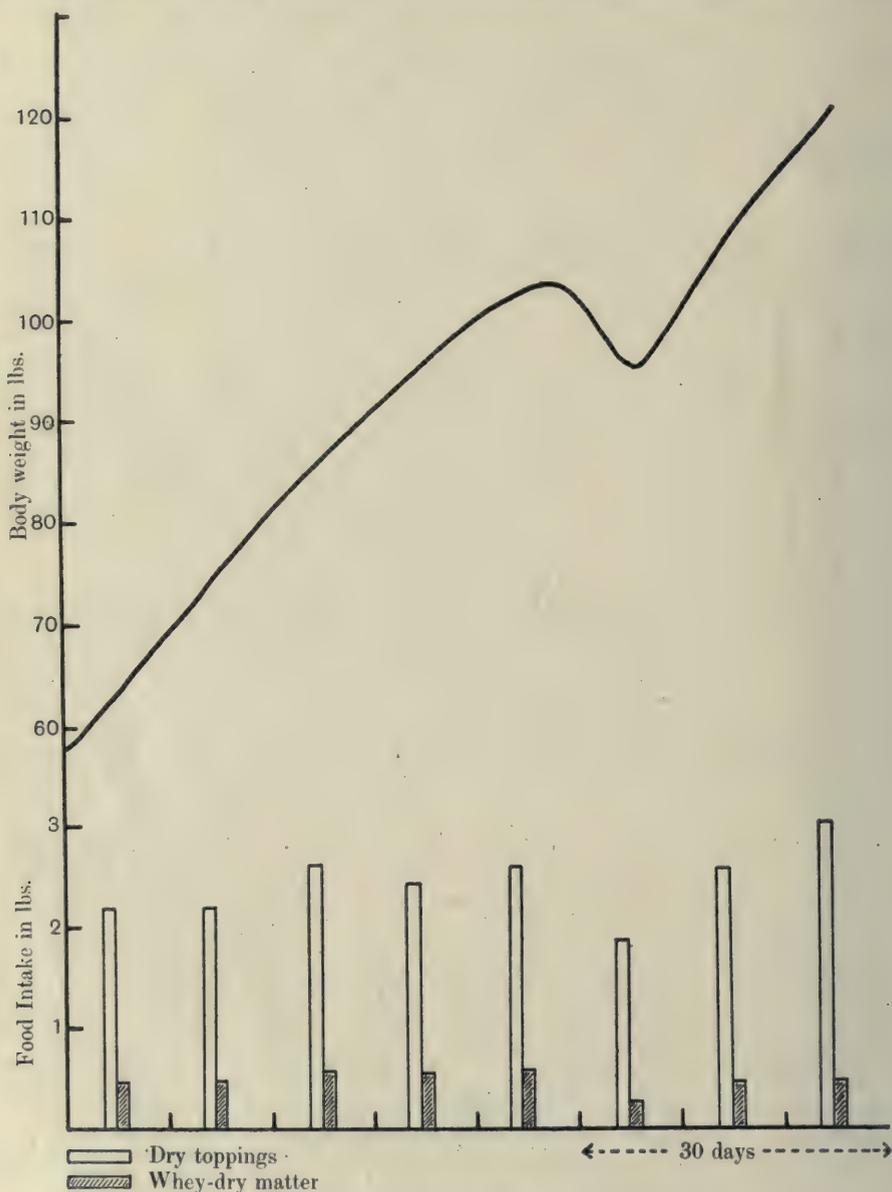


Fig. 2. Growth curve and food intake of hog from Group II.

receiving a mixed farm ration in Group V contained appreciable amounts of the important growth-promoting accessory substance (Curves 12-28, Fig. 6).

The fats from the abdominal cavity and from the subcutaneous deposits were tested separately in each case.

These experiments demonstrate that storage of the vitamin A will occur in the body fat of the pig, provided that the animal receives a diet containing considerable amounts of that substance. This finding is of importance since

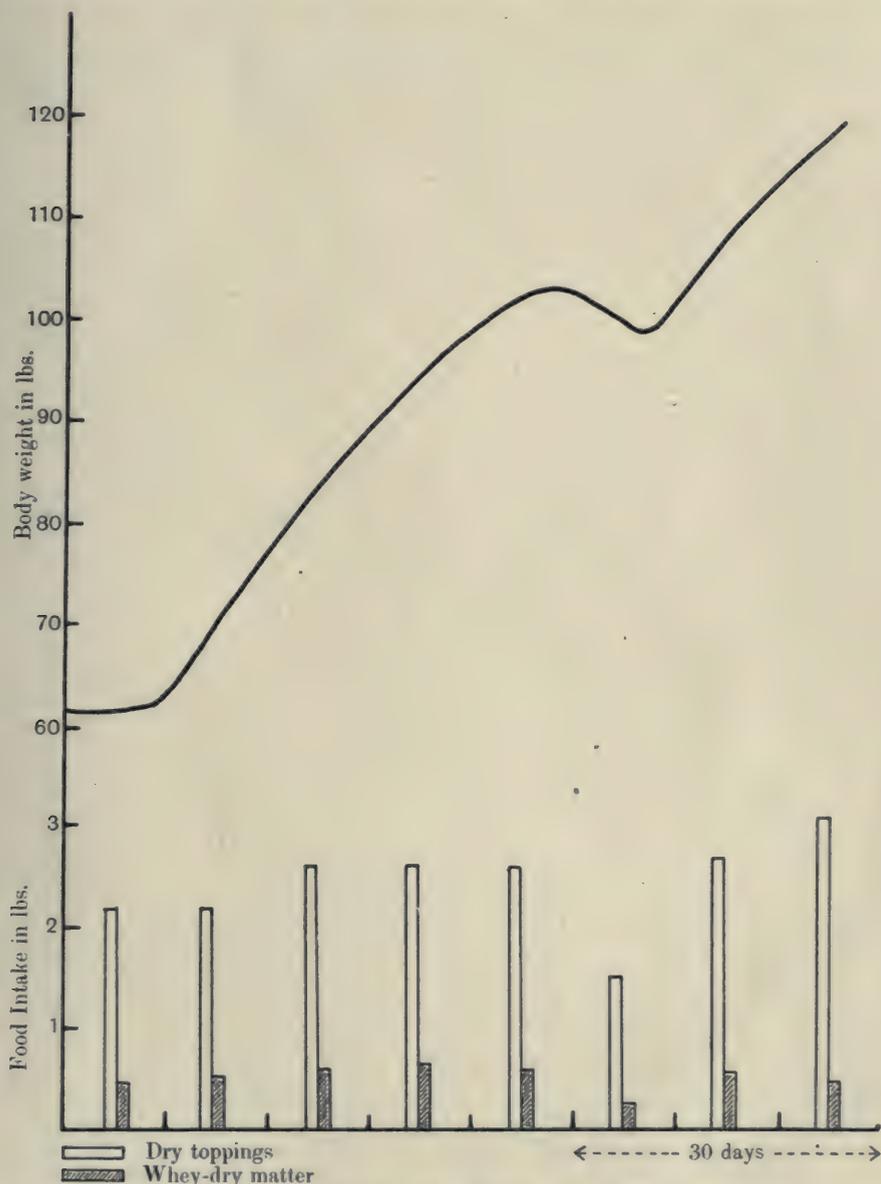


Fig. 3. Growth curve and food intake of hog from Group III.

it shows that the pig is not an exception to the rule that storage of the fat-soluble vitamin A in the fat depôts occurs in animals under suitable conditions.

It does not appear that pig fat is, weight for weight, as rich in vitamin A as is the body fat of other animals fed on a similar type of diet. This may,

however, be due to the fact that the mass of adipose tissue is so much greater in the former species, and that the concentration of vitamin per unit weight of fat would tend to be smaller than in a species such as the cow, where fat deposition is less marked.

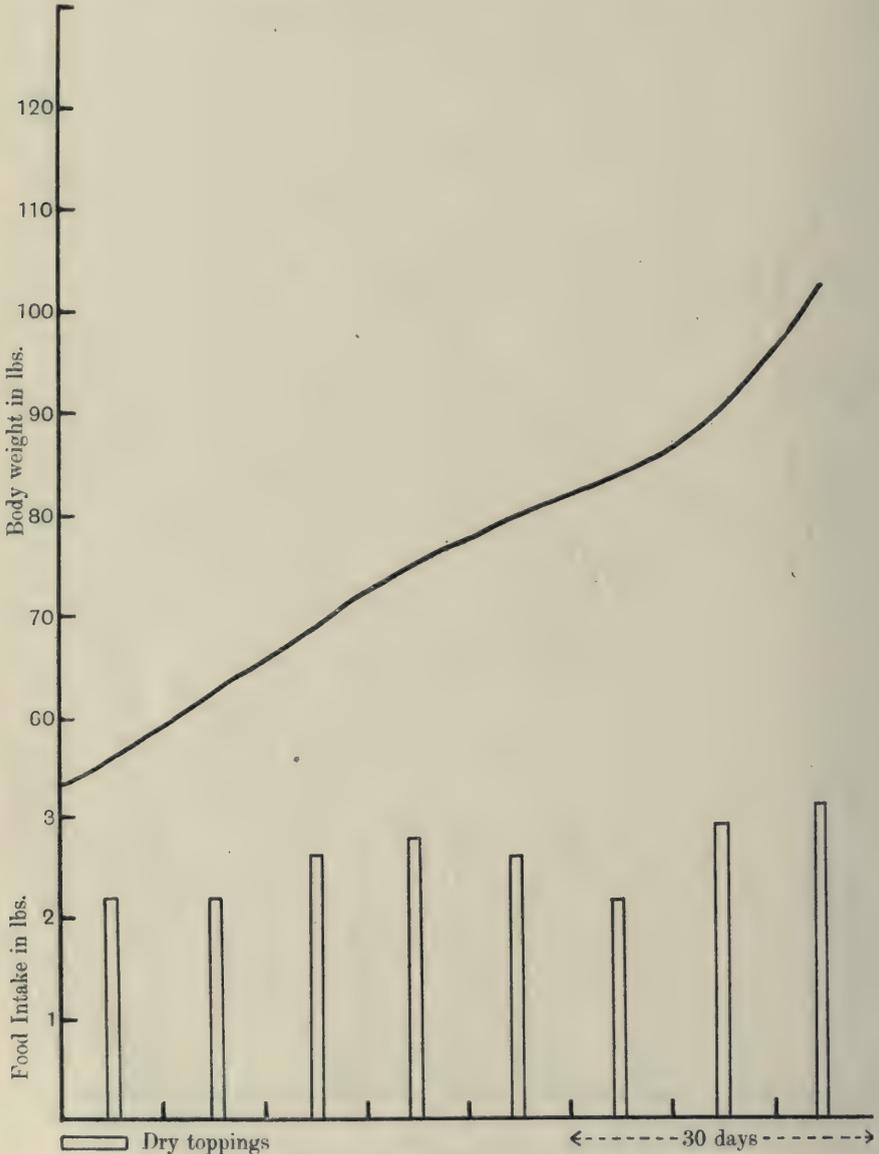


Fig. 4. Growth curve and food intake of hog from Group IV.

## II. INFLUENCE OF THE PROCESSES EMPLOYED IN THE MANUFACTURE OF LARD ON THE VITAMIN PRESENT IN PIG FAT.

The enormous quantities of pig fat that are converted into lard for human consumption every year may be judged from the fact that in 1912 the total

weight of lard exported from the United States was over 500 million lbs. A considerable proportion of this production is utilised for margarine manufacture, an industry which has grown to many times the size it was in 1912. It is therefore of very great importance to ascertain the influence of the method of preparation employed in lard manufacture on the nutritive value of the fat. Having found by the feeding experiments described above that

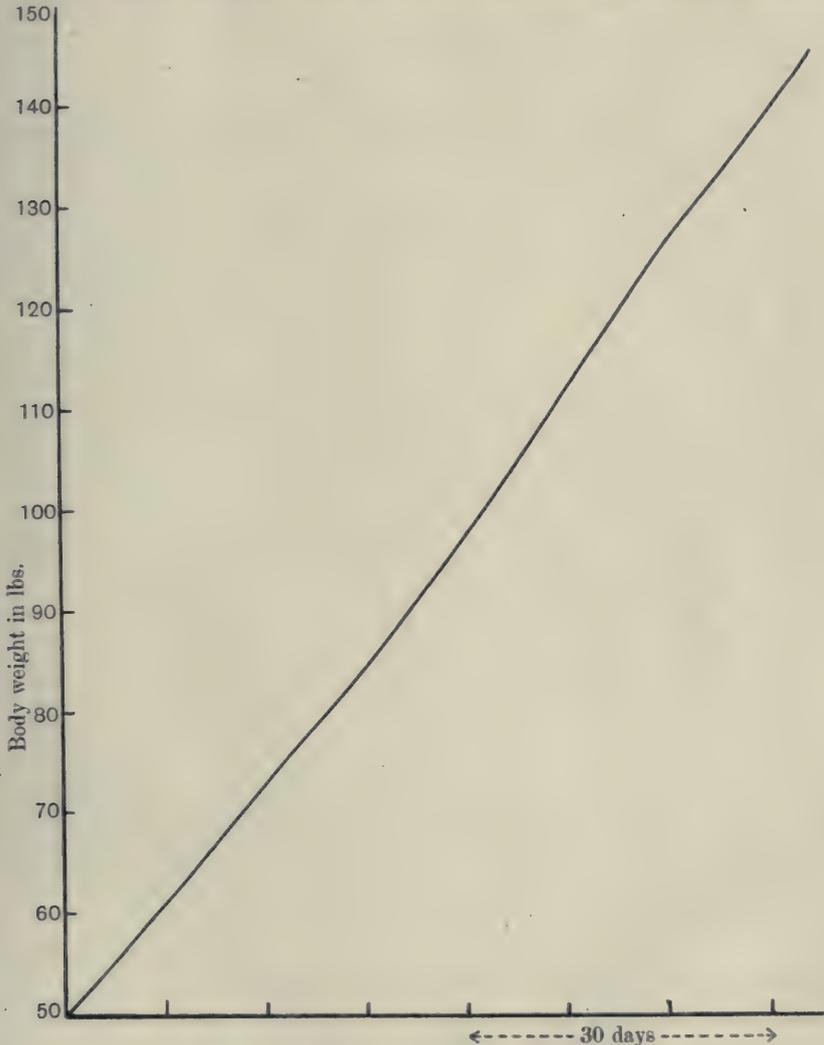


Fig. 5. Growth curve of hog from Group V.

pig fat may contain appreciable quantities of vitamin A when the pigs have been fed on a diet rich in that factor, we proceeded to investigate how the accessory factor is affected by the processes of lard manufacture and refinement. Lard manufacture in this country is not carried out on anything approaching the enormous scale that may be seen in America, and there are

distinct differences between the methods employed there and in this country. The oldest and simplest method of lard preparation still survives in the country parts of England, and we had the opportunity of seeing one preparation by this method. It is only employed by local pig butchers or farmers who handle small numbers of swine, and the amount prepared in this manner is negligible when one considers the total production of lard; the product is largely used for consumption in the immediate locality. This process consists



Fig. 6. Growth curves of rats whose diets were supplemented by a daily ration of 1.5 g. fat from the slaughtered pigs.

Rats 1-3	Group I (back fat);	Rats 14-16	Group III (abdominal fat);
„ 4-5	Group I (abdominal fat);	„ 17-19	Group IV (back fat);
„ 6-8	Group II (back fat);	„ 20-22	Group IV (abdominal fat)
„ 9-11	Group II (abdominal fat);	„ 23-25	Group V (back fat);
„ 12-13	Group III (back fat);	„ 26-28	Group V (abdominal fat).

The preliminary period of inhibited growth on the basal ration is omitted.

in heating the pig fat in an open pan over a fire, stirring meanwhile. The melted fat is from time to time skimmed from the surface, reheated to drive off moisture, strained, and poured into bladders. Lard prepared in this manner usually has a brownish tint due to admixture with products derived from the charred tissue. In view of the very small amount of lard which is prepared by this simple farmhouse method, we decided that it was unnecessary to investigate its food value.

For permission to view a large modern lard manufactory in this country we are indebted to Mr R. J. Harris, of the firm of Harris and Co., Bacon Curers, Calne, Wiltshire. To him, and to Mr O. Jones, the chief chemist at

that factory, we wish to express our appreciation of the very kind manner in which they assisted us in our investigation, by placing much information and numerous samples at our disposal. The method employed in this factory is essentially as follows:

The pigs which are drawn from a large area of the surrounding country are slaughtered and the carcasses dressed in the usual manner. When cold, the abdominal fat or fleck, which has been stripped and hung up beside the carcass, is minced in a large mechanical mill. The fatty pulp passes into a steam-jacketed pan provided with stirrers, where it is heated to a temperature of about 82°. The fat which separates from the tissues runs off into a second steam-heated pan, where heating at a temperature just above boiling-point (102°) is maintained with stirring for about 10 or 15 minutes to drive off any moisture. The hot dehydrated fat is then clarified either by passing it through a filter press or by allowing it to stand in a settling tank. The lard is now ready to be converted into a suitable solid form and packed for the market. The details of these latter processes have no bearing on the subject of this paper and are therefore omitted.

Very little pig fat other than the perinephritic deposits is used for lard making in this factory, and consequently the product is one of very high standard. The dimensions of the lard industry in the United States have necessitated the adoption of a system of classifying lards. Many of the preparations are made from inferior sources of fat.

The Rules of the Chicago Board of Trade define the following brands of edible lard. (a) Neutral Lard No. 1, which is a high quality lard prepared only from the abdominal leaf. (b) Neutral Lard No. 2, a similar product prepared from the back fat. These two types are carefully prepared and since they have not been "cooked," they do not keep well. Their chief use is in the manufacture of high class margarines. Only a small amount of lard is prepared by this method in this country. (c) Leaf Lard. This is essentially a product which has been prepared by a process similar to the one we have described in detail above. (d) Choice Lard or Kettle-rendered Lard, and (e) Prime Steamed Lard are products of much lower standard. The latter is indeed usually prepared from the trimmings of the carcasses and not from the true fat deposits. They are frequently rendered by the use of high pressure steam, and since they are unpalatable and discoloured in the crude state, they are usually treated by "blowing," or some such process involving oxidation, in order to remove colouring substances and a somewhat unpleasant odour and taste.

We began our investigation by examining an average sample of the pig fat such as is used at Calne for lard manufacture. Curves 4-6, Fig. 7 show that this sample contained an appreciable amount of the fat-soluble vitamin. This confirms the observations by Drummond and Coward [1920, 1] that pig fat may contain the factor A. We also tested a sample of lard which had been made from the same batch of pig fat. This proved to be inactive (Curves 1-3,

Fig. 7) as did several other samples of lard made there on other occasions. Apparently the processes of manufacture were responsible in some manner for the removal or destruction of the vitamin. Naturally the inactivation of the vitamin present in the pig fat will depend on the amount present, which will presumably depend to some extent on the age of the pigs when slaughtered, and to a greater extent on the diet upon which they had been reared.

It was impossible to obtain precise information regarding the diet which had been given to the pigs from which the samples of fat ultimately made into lard were taken. We were informed, however, that it is general in the areas from which the majority of the pigs are drawn to supplement the usual type of diet of toppings, barley meal, whey, etc. with kitchen refuse and green foods. Grass feeding of swine is not carried out to any large extent in this country.

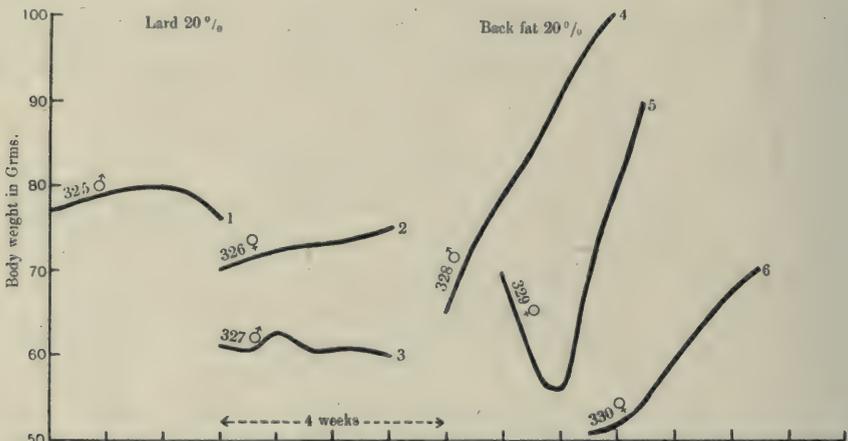


Fig. 7. Growth curves of rats whose diets contained 20% lard (1-3) and back fat (4-6) respectively.

The preliminary period of inhibited growth on the basal ration is omitted.

The cause of the loss of vitamin during lard manufacture is in our opinion largely due to oxidation, since it has recently been shown that the factor is rapidly inactivated at high temperatures by air or oxygen or by ozone in the cold [Hopkins, 1920, 1, 2; Drummond and Coward, 1920, 2; Zilva, 1920].

The concentration of vitamin A in pig fat, even in grass-fed animals, appears to be considerably lower than in fat derived from grass-fed cattle, and much lower than that usually found in butter, so that when it is remembered that butter fat exposed over a large surface to air at temperatures about 100° may be inactivated in a time as short as one or two hours, it can be understood how the pig fat loses its accompanying vitamin during the heating and stirring used in its conversion into lard.

Recently a paper has appeared in which Daniels and Loughlin [1920] claim to have examined samples of lard which showed considerable growth-promoting power. Not only good growth, but reproduction and satisfactory

rearing of the young were accomplished by their rats when their diet contained 28 % of a commercial preparation of lard. It is possible that the sample with which they worked was one prepared from a pig fat of very rich vitamin content, and that appreciable amounts of that factor had escaped destruction.

Since this communication was completed a sample of lard prepared at Calne from some very active back fat has been examined by us and has been found to possess some activity although not nearly as pronounced as that observed by Daniels and Loughlin. This suggests that some of the fat-soluble vitamin may remain in the lard after treatment in certain cases.

#### SUMMARY.

1. The pig is able to store up supplies of vitamin A in the body fat when fed upon a diet containing ample supplies of that factor, as for example when grass-fed.

2. When the diet of the pig is deficient in vitamin A, as for example when the diet consists almost entirely of toppings and whey, no appreciable amounts of that dietary factor can be detected in the body fat.

3. The processes employed in the manufacture of lard on a large scale in this country cause a very marked destruction of the vitamin present in the pig fat.

4. The low nutritive value of lard is therefore believed to be due to two causes. First, the diet usually given to fattening pigs in this country is seldom rich in vitamin A, so that the average sample of pig fat contains little or none of that substance; secondly, the processes of lard manufacture undoubtedly cause the destruction of much of the vitamin present in the original pig fat, probably owing to the exposure of the fat to oxygen at high temperature.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

#### REFERENCES.

- Daniels and Loughlin (1920). *J. Biol. Chem.* **42**, 359.  
Drummond and Coward (1920, 1). *Biochem. J.* **14**, 668.  
——— (1920, 2). *Biochem. J.* **14**, 734.  
Hopkins (1920, 1). *Brit. Med. J.* **ii**, 147.  
——— (1920, 2). *Biochem. J.* **14**, 725.  
McCullum and Davis (1913). *J. Biol. Chem.* **15**, 167.  
Osborne and Mendel (1913). *J. Biol. Chem.* **15**, 311.  
——— (1915). *J. Biol. Chem.* **20**, 379.  
Zilva (1920). *Biochem. J.* **14**, 740.

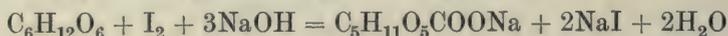
## LXXIII. THE IODIMETRIC ESTIMATION OF SUGARS.

BY JULIAN LEVETT BAKER AND  
HENRY FRANCIS EVERARD HULTON.

(Received October 18th, 1920.)

IN the course of an enquiry on the iodimetric estimation of sugars, Hilda M. Judd [1920] examined the methods of Romijn [1897], Colin and Lièvin [1918], and Willstätter and Schüdel [1918]. The principle involved in these processes is the oxidation of the sugar to the corresponding monobasic acid by means of iodine in alkaline solution. In the first two methods it was found by Miss Judd that the sugar was not oxidised quantitatively; the third method, however, was adopted by her as it afforded good results. We also found it accurate and rapid.

Briefly the method consists in adding 20 cc. of *N*/10 iodine and 30 cc. of *N*/10 soda to 0.1 g. of the sugar dissolved in 10 cc. of water. The mixture is left at the air temperature for 15 to 20 minutes, then acidified with *N*/1  $\text{H}_2\text{SO}_4$  and the excess of iodine titrated with *N*/20 thiosulphate using starch solution as indicator. The reaction in its simplest form may be represented in the case of a hexose as proceeding according to the equation



from which it will be found that 1 g. of the anhydrous hexose reacts with 1.411 g. of iodine, or 1 g. of anhydrous biose, such as lactose or maltose, with 0.743 g. of iodine. How closely this theoretical iodine gram-equivalent is reached in practice will be seen from the figures given in Table I.

In practice an excess of iodine and alkali is employed beyond that required by the equation; for 0.1 g. of sugar, 20 cc. of *N*/10 iodine and 30 cc. of *N*/10 NaOH are taken, although theoretically the necessary iodine for the reaction is present in 11 cc. of the decinormal solution when the sugar is a hexose or in 6 cc. for a biose sugar. Attention may be drawn to the following points.

(1) Our experiments show that it is essential that the reacting solutions should be mixed in the order of sugar, iodine, alkali. If the iodine and alkali are mixed prior to the addition of the sugar a lower iodine value is obtained, due probably to the loss of oxygen from the sodium hypoiodite.

(2) The time required for the oxidation mentioned by Willstätter and Schüdel and adopted by Miss Judd is 15 to 20 minutes; we find, however, that 3 to 5 minutes suffice for the completion of the reaction.

Working under the conditions described we obtained the following results:

Table I.

Sugar	Gram-equivalent		H. M. Judd's results
	Found	Calculated	
Dextrose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> ) ...	1.41	1.411	1.315
Levulose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> ) ...	0.10	—	0.103
Galactose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> ) ...	1.37	1.411	1.418
Sucrose (C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> ) ...	0.02	—	0.01
Maltose (C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> ) ...	0.746	0.743	0.7456
Lactose (C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> ) ...	0.762	0.743	1.502
Raffinose (C <sub>18</sub> H <sub>32</sub> O <sub>16</sub> ·5H <sub>2</sub> O)	0.05	—	—
Soluble starch ... ..	0.06	—	—

The anhydrous dextrose used had a specific rotatory power of  $[\alpha]_D + 53.21^\circ$  after crystallisation from alcohol. The galactose had  $[\alpha]_D + 80^\circ$  and levulose recrystallised from alcohol had  $[\alpha]_D - 88.4^\circ$  and a reducing power with Fehling's solution, calculated as levulose, of 99%. The maltose and sucrose we employed were chemically pure; the former sugar as used for the determination contained 1 mol. of water which has been allowed for in the iodine equivalent recorded in the table. It will be noted that the gram-equivalent of iodine found by us for lactose differs very markedly from the value recorded by Miss Judd. We worked with four separate specimens of carefully purified lactose hydrate and in over a dozen determinations found figures similar to those recorded in the table. We believe, therefore, we are justified in concluding that lactose behaves with iodine in the same way as maltose and gives practically a quantitative yield of the monobasic acid. Consequently, there is no reason to suppose, as suggested by Miss Judd [1920], that lactose is hydrolysed and that both the glucose and galactose molecules are oxidised.

The citation by Miss Judd of Lobry de Bruyn and van Ekenstein's results [1896] in this connection would only be justified if the complete hydrolysis of lactose under the influence of cold alkali were effected in a few minutes, a transformation which was never obtained by these chemists. To settle the point we added alkali to solutions of lactose, maltose and levulose for a period of 20 minutes before the addition of iodine which was then allowed to act for the usual time. Under these conditions we should have obtained even more evidence of the Lobry de Bruyn transformation had it occurred, in the form of a higher gram-equivalent of iodine, but the values obtained were identical with those found when working under normal conditions.

In the course of this work we attempted to ascertain something of the nature of the mechanism of the iodimetric oxidation of carbohydrates and to this end made a series of determinations of the rate at which oxidation took place. Fifty per cent. of the total iodine we found was used up in the first five seconds and the reaction is in all cases complete within five minutes in the cases of dextrose, maltose and lactose. On investigating the values thus obtained by the customary formulae we found that the reaction is not mono-, di- or tri-molecular.

The fact that the time necessary for the reaction as employed by Willstätter and Schüdel [1918] can be reduced from 20 to 5 minutes considerably enhances the usefulness of the method and makes it as convenient as the volumetric Fehling reduction, while an accuracy of 1 % in the case of maltose and lactose and 0.5 % with dextrose and galactose is obtainable.

We applied the method to the analysis of the products of the action of diastase on starch and found that iodine does not differentiate between free maltose and that supposed to exist as "maltodextrin" in a state of combination with dextrin. When precipitated malt diastase was allowed to act on soluble starch for three hours at 50°, the conversion products had the constants  $[\alpha]_D + 156.5^\circ$  and  $R$ , by Fehling's solution, 85.8 % of maltose. A mean of six iodimetric oxidations showed 85.4 % of maltose. From this it is evident that dextrin is unattacked by hypoiodite and that the whole of the maltose present both free and combined is quantitatively oxidised. When starch conversion products are fractionated with alcohol the iodine values agree with the apparent maltose content as found by Fehling's solution. Soluble starch and  $\alpha$ -amylodextrin [J. L. Baker, 1902]—the dextrin obtained from starch when hydrolysed with barley diastase—are unacted on by an alkaline solution of iodine.

In the results recorded above solutions of pure sugars were employed. It should, however, be borne in mind that proteins are oxidised by iodine, thus egg albumin and Witte peptone have iodine gram-equivalents of 0.53 and 0.58 respectively. If any intention be entertained of employing the method for the analysis of solutions other than those of the pure sugars, the possible disturbing effects of protein and impurities will have to be considered.

*Note added November 15th.*

Since writing the above paper our attention has been drawn to a communication by N. Bland and L. L. Lloyd [1914]. These authors have examined (*inter alia*) the behaviour of the sugars towards neutral sodium hypoiodite and find that the oxidation of maltose, dextrose and lactose proceeds quantitatively to the corresponding monobasic acids in five minutes at ordinary temperature; sucrose and levulose were unaffected. The neutral hypoiodite used was obtained by mixing equal volumes of  $N/10$  iodine and  $N/10$  caustic soda.

REFERENCES.

- Baker (1902). *J. Chem. Soc.* **77**, 1177.  
Bland and Lloyd (1914). *J. Soc. Chem. Ind.* **33**, 948.  
de Bruyn and van Ekenstein (1896). *Rec. Trav. Chim.* **15**, 95.  
Colin and Lièvin (1918). *Bull. Soc. Chim.* **47**, 402.  
Judd (1920). *Biochem. J.* **14**, 255.  
Romijn (1897). *Zeitsch. anal. Chem.* **36**, 18, 349.  
Willstätter and Schüdel (1918). *Ber.* **51**, 780.

## LXXIV. THE "AMMONIA COEFFICIENT" OF PREGNANCY.

BY WINIFRED CLARA CULLIS AND EVELYN E. HEWER.

*From the Physiological Laboratory, London (Royal Free Hospital)  
School of Medicine for Women.*

*(Received September 21st, 1920.)*

A STUDY of the nitrogen distribution in the urine during pregnancy has led to the view that the "ammonia coefficient<sup>1</sup>," although it shows a tendency to rise above the normal, should not differ from it very markedly. Indeed it has been claimed by some obstetricians that a value of 15 means a serious departure from a normal pregnancy, and that a value of over 30 is a sign that the pregnancy should be terminated.

The following case is given as a contribution to the study of nitrogen metabolism in pregnancy because it showed a high ammonia coefficient and yet had a successful termination. It is to be regretted that other observations on respiratory values and blood reactions were not made at the same time, as they would have added considerably to the value of the observations. Further, no attempt was in this case made to differentiate between the ammonia and the amino-acids, the "ammonia" being determined by the Folin method. Nevertheless, the figures obtained from the urinary analysis are sufficiently striking to be worth recording<sup>2</sup>.

The subject, Mrs W., was a primipara aged 22, who was admitted to the Royal Free Hospital for prolonged vomiting. She was found to be pregnant, retroversion was corrected, and then as the vomiting continued in spite of dieting she was transferred to the Marlborough Maternity Hospital, Endsleigh Street, under charge of Lady Barrett, with a view to induction. Lady Barrett's resident medical officer was Dr Dorothy Chick, to whose keenness and interest we are indebted for the collection of the specimens over the long period before parturition, during which we were enabled to make the analyses at the school laboratories. This was only one of the cases in which Dr Chick's interest in the scientific aspect of her work was manifested, but it was the last before her untimely death in the influenza epidemic of 1919. In publishing these

<sup>1</sup> By the "ammonia coefficient" is meant the ratio between the ammonia and the nitrogen excreted in the urine, taking the nitrogen as 100. The usual way of determining is to estimate the percentage of ammonia and of nitrogen in a 24-hours' specimen of urine: this will give an average coefficient of from 4 to 8, most often about 5 or 6.

<sup>2</sup> All the analyses in this investigation were done by one of us (E.E.H.).

results it is only fitting to record once again our keen sense of the loss that all her colleagues sustained in the death of this particularly able young medical woman, with her scientific attitude towards the problems of work, an attitude strengthened and made valuable by her previous training at Cambridge.

The first specimen of urine in which the ammonia coefficient was determined gave the extraordinarily high value of 70. For other reasons immediate induction was not considered essential, and therefore in view of the urinary analysis we suggested that possibly the high value was in part due to an acidosis of starvation, and particularly to protein deficiency. That the high coefficient could not be explained only by fasting is shown by the fact that in fasting the ratio may rise up to 35, but probably not higher. However, as a trial, the patient was put on to a diet containing much more protein, and almost at once she began to improve.

The volume of urine passed increased (see accompanying charts), and the ammonia coefficient dropped rapidly (although still high). Intermittent vomiting continued for some time, but the patient looked and felt much better. Two months after admission to the Maternity Hospital she was discharged, with instructions to report at out-patients, and to bring fortnightly twenty-four-hourly samples of urine.

Two months later she was readmitted for vomiting, and a trace of albumin in the urine, but was discharged a week later much improved.

Six weeks later she was readmitted for confinement. The baby was born slightly jaundiced, and weighed  $5\frac{3}{4}$  lb. ♀. The patient was discharged doing well, and feeding the baby.

In view of the interest of the case, she was instructed to report periodically, and to bring samples of urine with her. From these we have been able to draw up a chart showing her urinary excretion when not pregnant; the analyses give a normal ammonia coefficient, a very high urea average, and a small volume of urine.

At the present time she is again pregnant, and the urine is being systematically analysed: up till the present nothing abnormal has been detected.

A study of the accompanying tables and charts will show the way in which the ammonia coefficient varied with the condition of the patient. It is particularly unfortunate that no urinary analyses were made just before and after confinement: this was due to the serious outbreak of influenza at the Hospital, during which Dr Chick herself died.

The following points appear from the charts:

1. The patient has no inherent abnormality of metabolism, the coefficient being of the average physiological value, *i.e.* from 4 to 8.
2. The high ammonia percentage was not due to concentration of the urine.
3. There was a very definite retention of nitrogen.
4. There was a definite parallelism between the daily total  $\text{NH}_3$  and daily

Table I. *Urinary Analysis (Mrs W., Non-pregnant condition)*

Date	Quantity oz.	S.G.	Titration acidity %	NH <sub>3</sub> %	N %	Urea %	NH <sub>3</sub> Coeff.	Total NH <sub>3</sub>	Total N	Total Urea
Mar. 16-17	—	1023	71.1	0.1	1.6	2.75	6.2	—	—	—
Sept. 7-8	—	1030	58	0.15	1.81	3.2	8.3	—	—	—
Sept. 21-22	30	1030	55.1	0.12	1.78	3.85	6.7	1.02	15.1	32.8
Sept. 29-30	—	1030	53.8	0.12	1.73	3.8	6.9	—	—	—
Nov. 21-22	36	1021	24.8	0.074	1.26	2.2	5.9	0.76	12.8	22.5
Dec. 2-3	22	1027	12	0.064	1.13	1.92	5.6	0.4	7.1	11.98

In this table and in Table II

Titration acidity Expressed as number of cc. of N/10 NaOH required for 100 cc. urine using phenolphthalein as indicator.

Urea ... Expressed as grams. Estimated by hypobromite method.

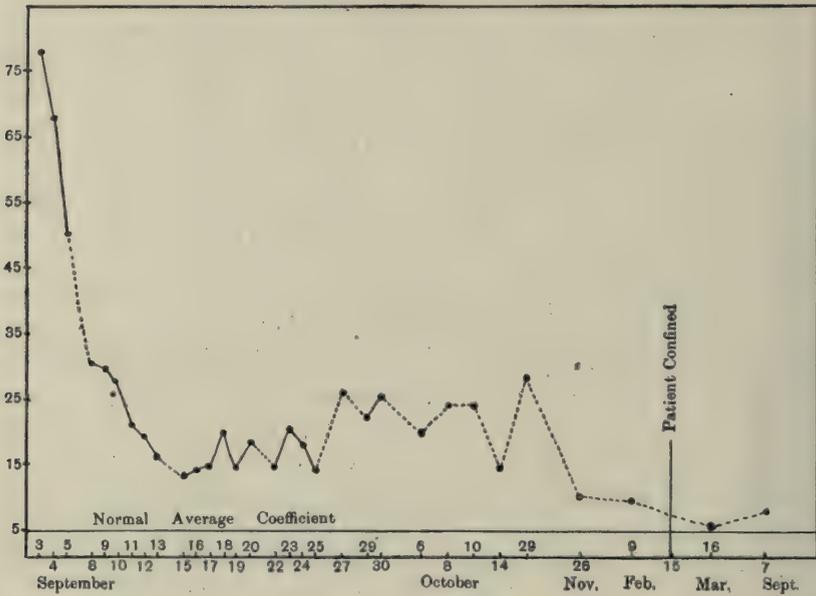
Nitrogen ... Expressed as grams. Estimated by Kjeldahl method.

Ammonia ... Expressed as grams. Estimated by Folin's formaldehyde method.

Table II. *Urinary Analysis (Mrs W., Pregnant condition)*

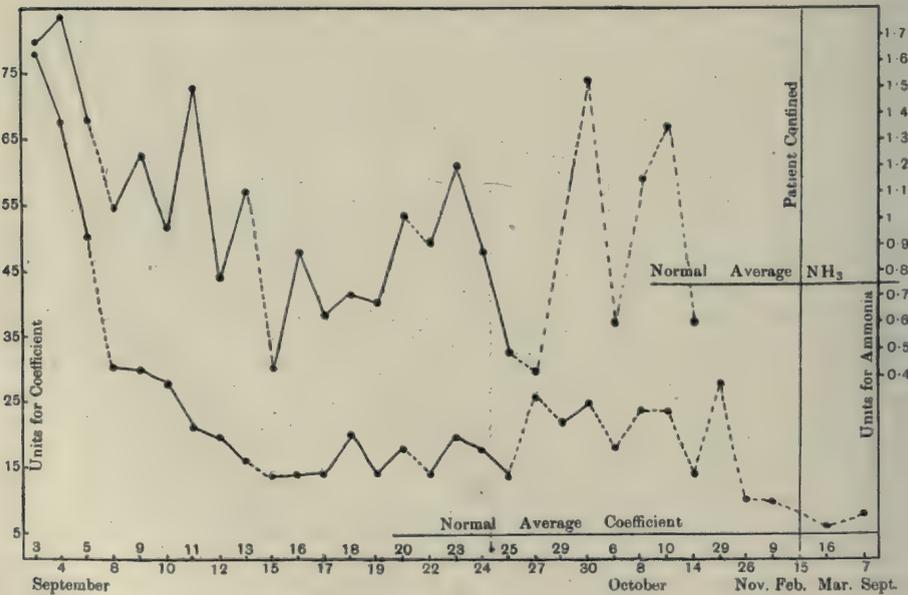
Date	Quantity oz.	S.G.	Titration acidity %	NH <sub>3</sub> %	N %	Urea %	NH <sub>3</sub> Coeff.	Total NH <sub>3</sub>	Total N	Total Urea
Sept. 3-4	8½	1022	33.3	0.67	0.86	—	77.9	1.66	2.1	—
4-5	9	1017	48	0.7	1.03	—	67.9	1.79	2.6	—
5-6	9	1019	78.5	0.54	1.08	—	50	1.38	2.8	—
8-9	16	1016	30	0.23	0.76	—	30.25	1.04	3.45	—
9-10	20	1015	24.7	0.22	0.74	—	29.7	1.25	4.2	—
10-11	20	1016	21.4	0.17	0.61	—	27.8	0.96	3.46	—
11-12	44	1012	17.6	0.12	0.57	0.9	21	1.5	7.1	11.23
12-13	23	1012	26.6	0.12	0.62	1.1	19.35	0.78	4.04	7.2
13-14	30	1016	28.1	0.13	0.77	1.2	16.8	1.11	6.55	10.2
15-16	30	1005	12.85	0.05	0.38	0.5	13.2	0.43	3.2	4.3
16-17	45	1009	30.9	0.07	0.5	0.8	14	0.89	6.4	10.3
17-18	25	1012	34.3	0.09	0.62	0.8	14.5	0.64	4.4	5.7
18-19	25	1010	50	0.1	0.5	0.8	20	0.71	3.5	5.7
19-20	30	1007	22.85	0.08	0.54	0.8	14.8	0.68	4.6	6.8
20-21	36	1011	37.6	0.1	0.54	0.75	18.5	1.02	5.5	7.7
22-23	40	1008	17.1	0.08	0.54	0.7	14.8	0.91	6.12	7.9
23-24	39	1010	41.9	0.11	0.54	0.65	20.3	1.22	5.97	7.2
24-25	28	1011	34.2	0.11	0.61	0.8	18	0.87	4.84	6.3
25-26	29	1008	9.1	0.06	0.43	0.52	13.95	0.49	3.54	4.3
27-28	7	1018	49.5	0.21	0.82	1.45	25.6	0.42	1.63	2.9
29-30	+	1009	13.8	0.09	0.4	0.55	22.5	—	—	—
Oct. -1	27	1015	61.4	0.2	0.8	1.3	25	1.53	6.13	10
6-7	15	1016	37.1	0.14	0.73	1.2	19.18	0.6	3.1	5.1
8-9	27	1012	52.8	0.15	0.63	1	23.8	1.15	4.8	7.7
10-11	25	1014	59	0.19	0.8	1.37	23.75	1.35	5.7	9.7
14-15	30	1013	2	0.07	0.5	0.62	14	0.6	4.25	5.3
29-30	—	—	9.5	0.23	0.8	0.8	28.7	—	—	—
Nov. 26-27	—	—	17.5	0.07	0.66	0.85	10.6	—	—	—
Feb. 9-10	—	1016	18.5	0.07	0.71	1.25	9.8	—	—	—
15	Patient confined									
Mar. 16-17	—	1023	71.1	0.1	1.6	2.75	6.2	—	—	—

Chart 1



Ammonia coefficient  
 i.e.  $\frac{100 \times \text{Percentage of NH}_3}{\text{Percentage of N}}$

Chart 2

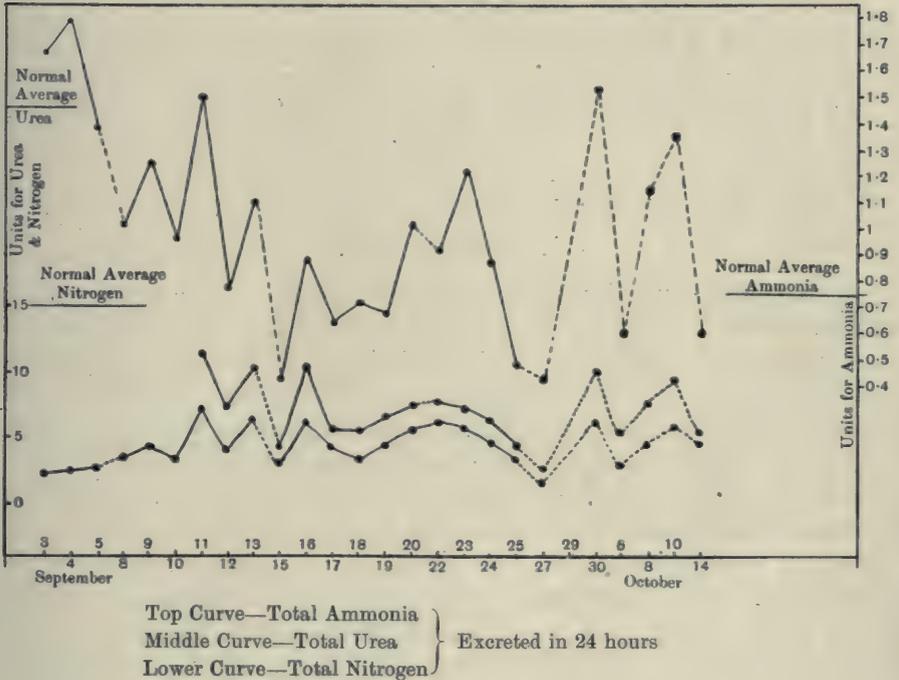


Top Curve—Total NH<sub>3</sub> excreted in 24 hours  
 Lower Curve—Ammonia coefficient

total urea: comparison with a normal chart shows that increased  $\text{NH}_3$  in the usual way means decrease of urea (on a constant diet): in this case, increase of  $\text{NH}_3$  went with increase of urea.

5. There appeared to be no relationship between the vomiting and the coefficient: the latter was much more dependent on the diet. (It is, of course, well known that starvation raises the coefficient.)

Chart 3



6. There was no relationship between the total  $\text{NH}_3$  excreted and the  $\text{NH}_3$  coefficient.

Quite recently another case of abnormally high ammonia coefficient has been met with, in connection with an investigation that is being carried out at the London (R.F.H.) School of Medicine for Women, on the metabolism of pregnancy under the Medical Research Council's Scheme of Child Life Investigation.

We should like to thank Lady Barrett for permission to publish this case.

# LXXV. THE PENETRATION OF ELECTROLYTES INTO GELS. II: THE APPLICATION OF FOURIER'S LINEAR DIFFUSION LAW.

BY GILBERT SMITHSON ADAIR,

*R. J. Smith Student, King's College, Cambridge.*

*From the Food Investigation Board and the Physiological Laboratory,  
Cambridge.*

*(Received October 9th, 1920.)*

## I. INTRODUCTION.

THE ways of studying diffusion can be divided roughly into three classes, two of which, electrical measurements and quantitative analysis, may be described as the standard methods of determining diffusion rates; the third experimental procedure, marked off from the first two by its simplicity, has been named the indicator method. The characteristic of simplicity is appreciated on looking at the list of essential apparatus—a rack of test-tubes and a ruler. The tubes are filled half full with a gelatin solution containing some indicator, and after the formation of a solid jelly a solution of the substance under investigation is poured in and a colour change in the indicator demonstrates the forward march of the diffusing substance. When this is a neutral salt the change consists of the formation of a precipitate with the reagent placed in the jelly.

This method has been investigated recently by von Fürth and Bubanović [1918]. Their research on the quantitative aspects of the problem is a notable advance, but their method of calculation of the diffusion coefficient (defined below) merely gives ratios and not absolute values. This is a serious drawback, and Professor Stiles, who is revising the indicator method [1920], urged me to attempt the calculation if there was the slightest hope of success. The winning of new results is beyond the scope of this essay. It is limited to the effort to work out a theoretical formula free from arbitrary constants, and secondly the illustration of the method of using this formula by which new results can be won.

The mathematical expression arrived at is general, therefore it does not matter what substance is used in the experimental testing—accordingly it will be put to the trial with the familiar salt, sodium chloride. Anyone reading this account will see how essential is such a test, though unfortunately it expands the writing to an inordinate length. The theory, from the point of view of the laboratory worker, lacks the charming simplicity of the experimental technique.

This disadvantage is inevitable—in fact, it must be confessed that in the beginning, the prospect of working out the result looked small indeed. The given measurement was doubtless a function of the diffusion coefficient “ $\kappa$ ,” but it was a function also of about a dozen other variables. The complexity of the theory might easily have discounted the advantages of the practice, but a review of these advantages had better follow the account of the method of calculation—a method mainly due to a lucky coincidence.

## II. SUMMARY OF THE THEORETICAL PRINCIPLES OF DIFFUSION.

Leaving more general aspects, we may now proceed to the special problems of the calculation. The laboratory measurements are given in terms of centimetres of salt penetration. These distances depend on a variety of factors. Firstly, we have to consider the laws of diffusion, which are expressed in the form of differential equations, integrable for certain “boundary conditions,” which are the second group of factors to be examined. Essentially they depend on the form of the mass of gelatin and the stirring of the salt solution. Thirdly, there are the phenomena peculiar to the experiment, for example, the temperature, the nature of the diffusing substance and the resistance of the medium.

The theory of the first two of these groups of phenomena belongs to the realm of mathematical physics, and was worked out by Fourier 100 years ago. The last group falls within the province of the experimenter.

The factors of interest to him all affect the diffusion coefficient,  $\kappa$ , so he has to reverse the original procedure, to find what value of  $\kappa$  must be assigned in the formulae to give the observed relation between concentration, time and place. This is easy in the two standard methods, but if an indicator is present, the problem becomes much more complicated, and there is nothing for it but to go back to first principles. The following portion of this section is devoted to the explanation of the terms in the formulae required, and the definition of the conditions under which they hold good. For the explanation of the formulae themselves, it is enough to give a general reference to Fourier's *Théorie Analytique de la Chaleur*, or the modern text-books of the mathematical theory of heat conduction, for example, Carslaw's *Fourier's Series and Integrals*. Even though we need only the simpler formulae it would take far too long and serve no useful purpose to go through the expansion of unity in a sine series—which is needed for formula (I) or Laplace's solution for the flow of heat in an infinite solid, required for formulae (II) and (III).

For our present work they are merely stated in the form most suitable for calculation with ordinary tables.

There is no possibility of avoiding these formulae. Von Fürth and Bubanović's penetration factor can be used for certain purposes, but the overwhelming reasons for calculating “ $\kappa$ ” are stated below.

(1) There is no need to introduce an empirical law expressing the effect of concentration, so that the true effects of concentration are laid before us.

(2) We can test the absolute accuracy of the work by comparison with standard methods.

(3) Thirdly,  $\kappa$  must be known if we are to make any practical calculations on the diffusion of salt in the system.

(4) It is the fundamental constant which is of use to us in the study of the phenomena, in which we are interested—free from the complications due to the “boundary conditions.”

These reasons for making the attempt to calculate  $\kappa$  are quite decisive. The fundamental law, that the rate of diffusion is proportional to the concentration gradient, was published by Fick as a special case of Fourier's linear diffusion law.

$$\frac{\partial u}{\partial t} = \kappa \frac{\partial^2 u}{\partial x^2}$$

$t$  = time in seconds,

$x$  = distance in centimetres,

$u$  = concentration of salt,

$\kappa$  = diffusion coefficient.

The diffusion coefficient is the amount of salt in equivalents which would flow across an area of one square centimetre, if the concentration at one point was one equivalent per litre greater than that at another point, one centimetre distant in the line of flow.

This partial differential equation has, of course, many different solutions, depending on the initial and boundary conditions. We will consider first the example which resembles the type of salt penetration of most practical interest. In this example the surface is kept at constant salt concentration by immersion in a large bath of solution.

To define the conditions more accurately, let us call the direction of penetration the “ $x$ ” axis, and at the surface of the gel let  $x = 0$ . Suppose the test-tube is 14 cm. long, and distances into it are reckoned positive. Let  $u$  = the salt concentration.

Let  $u_0$  be the concentration of salt in the bath of solution. Then we have the following conditions:

$$u = 0, \text{ when } 0 < x < 14 \text{ at } t = 0,$$

$$u = u_0 \text{ at } x = 0,$$

$$du/dx = 0 \text{ at } x = 14.$$

This last condition is represented mathematically by adding another 14 cm. to the length, with the condition that

$$u = u_0 \text{ at } x = 28.$$

Both the differential equation and the boundary conditions are satisfied by the following series of sine and exponentials, where  $l$  is double the length of the test-tube:

$$u = u_0 \left\{ 1 - \frac{4}{\pi} \left( e^{-\frac{\kappa\pi^2 t}{l^2}} \cdot \sin \frac{\pi x}{l} + \frac{1}{3} e^{-\frac{9\kappa\pi^2 t}{l^2}} \cdot \sin \frac{3\pi x}{l} + \frac{1}{5} e^{-\frac{25\kappa\pi^2 t}{l^2}} \cdot \sin \frac{5\pi x}{l} + \dots \right) \right\} \dots \text{(I)}$$

This series converges too slowly to be very helpful in practical work, but with its aid we can define the limits within which a simpler formula will hold good. For this purpose the calculations need not be carried very far and in any case tubes do not agree in length to four places of decimals. Taking Öholm's figure  $\kappa = 1.07$  per day, and assuming diffusion has been taking place for six days, we find that

$$u = 0.0007N \text{ if the calculation includes the fourth term,}$$

$$u = 0.0003N \text{ if five terms are summed.}$$

The amounts are so small that we shall probably not exceed the error in calculations with four figure tables if we assume that no salt has reached the end of the tube in the first two or three days of the experiment.

This conclusion greatly simplifies the problem, since the initial and boundary conditions may be re-stated with the assurance that the length of the tube does not enter into the problem, and for purposes of calculation may be taken as infinite.

$$u = 0, \text{ when } x > 0 \text{ at } t = 0,$$

$$u = u_0 \text{ at } x = 0.$$

The solution for these conditions is

$$u = u_0 \left\{ 1 - \frac{2}{\sqrt{\pi}} \int_0^q e^{-q^2} dq \right\}, \dots\dots\dots(\text{II})$$

where

$$q = \frac{x}{2\sqrt{\kappa t}}.$$

The formula could be stated more briefly if Kramp's table of the integral between  $q$  and infinity were more generally available, but the form given above is used because the probability integral tables are much more likely to be at hand. Given the table, it is the work of a few moments to determine " $\kappa$ " when " $u$ " is known.

The second type of experiment is carried out as follows. The salt solution is put in a tube half filled with gelatin and diffusion is allowed to take place without any circulation of the solution, so that we have a concentration gradient in the liquid itself.

If, as before, we consider only the first few days, and assume  $\kappa$  is constant, we have the following conditions:

$$u = u_0, \quad x < 0,$$

$$u = 0, \quad x > 0.$$

After reversing the sign of  $x$  the solution which satisfies these conditions is

$$u = u_0 \left\{ \frac{1}{2} + \frac{1}{\sqrt{\pi}} \int_0^q e^{-q^2} dq \right\} \dots\dots\dots(\text{III})$$

### III. NEGATIVE SALT CONVENTION.

The formulae stated in the preceding section are well-established in the study of diffusion by the classical methods—they can be used to calculate " $\kappa$ " directly. The indicator method, however, does not give us the direct

relation between  $u$  and  $u_0$  at the point  $x$  and at the time  $t$ . At the demarcation surface, we could get the concentration of chloride ions from the solubility of silver chloride 0.00015 %, but this does not help us much. It is obvious that the more silver nitrate is streaming up, the more slowly will the precipitate advance, because there will be longer to wait for enough sodium chloride to stream down and precipitate all the silver ions in a given space.

Some method of handling the problem must be devised, and the most promising line of attack seemed to have as base the obvious fact that one silver ion removes one chloride ion from the sphere of action, when silver chloride is precipitated. The development of this idea is to assume that we can call the silver nitrate "negative sodium chloride."

That is to say, a gel with 0.01 g. molecules of the silver salt would be called a - 0.01N sodium chloride. Making the usual shift in the scale, this is described for the next calculation as 0, and at the zone of precipitation, where the sodium chloride really is 0, the concentration therefore becomes + 0.01N sodium chloride.

Calling the silver nitrate concentration  $v_0$  and the sodium chloride concentration  $u_0$ , we have at the edge of the precipitation the relation

$$u = \frac{v_0}{u_0 + v_0}.$$

This is a known fraction of  $u_0$ , and therefore we can find  $q = x/2\sqrt{\kappa t}$  from the tables of the probability integral. This formula is only a first approximation, as it neglects the rate of movement of the silver nitrate. Before entering on this secondary complication our policy is to find if the approximation gives us a result which is at all promising, because the next step must obviously depend on whether the percentage error is 10 or 10,000.

#### IV. EXPERIMENTS WITH ORIGINAL TECHNIQUE.

The first data which will be used to test the formula were obtained on July 29th, 1919, by the half test-tube method.

Table I.

Diffusion coefficient of sodium chloride in 1 % agar gel. July 29th, 1919.

NaCl solution poured into half test-tubes of gel.

Concentration of silver nitrate 0.005N.

$\kappa_1$  calculation of " $\kappa$ " from 1st reading, 19 hours.

$\kappa_2$  calculation of " $\kappa$ " mean of later readings.

$q = x/2\sqrt{\kappa t}$  taken from tables, using formula (III).

The values of  $\kappa$  are given in c.g.s. units  $\times 10^9$ .

Salt concentrations	2N	N	0.5N	0.2N	0.1N	0.05N
Corresponding "q"	1.98	1.82	1.65	1.39	1.18	0.94
Temp. 22° $\kappa_1$	1.54	1.55	1.54	1.79	2.0	1.84
22° $\kappa_2$	1.7	1.7	1.7	2.08	2.40	2.46
13.6° $\kappa_1$	1.19	1.18	1.21	1.48	1.65	1.75
13.6° $\kappa_2$	1.36	1.27	1.34	1.67	1.85	1.95
0° $\kappa_1$	0.82	0.86	0.87	0.99	1.16	1.42
0° $\kappa_2$	0.88	0.90	0.89	1.13	1.25	1.31

The error is generally nearer 10 % than 100 % in these figures, but it is quite clear that the indicator method is of no use unless it turns out more consistent results than these. Perhaps the most serious discrepancy is the difference between the  $\kappa$  calculated from the first reading and the mean  $\kappa$ . If this is a real physical fact, it would of course be very interesting, but there are other causes we must examine first.

(1) Is the "negative salt" formula too crude?

(2) Is there an error in the experiment?

No. (1) may be dismissed for the time being, as it could not be held responsible for the difference of  $\kappa_1$  from  $\kappa_2$ . We are left with the second alternative. There are a great many possible sources of error—but some can be ruled out at once, as for instance the suggestion that the dense precipitate obstructs diffusion—such a phenomenon would produce an error in the opposite direction.

The most probable explanation of the discrepancy is that the salt solution was circulating in the test-tubes. The liquid at the gel surface continually loses salt, and, as the heavier solution is above it, mixing is bound to take place. This can be eliminated by a slight modification of the experimental technique.

The original method with the solution above the gel will be called type A. The modified method with tube inverted will be called type B. Type C unlike A and B requires formula (II), and it is theoretically more reliable because the diffusion gradient is in gel throughout, not one-half in gel, the other in water.

#### V. DATA OBTAINED WITH MODIFIED TECHNIQUE.

Some experiments by methods B and C are given in the tables below. They illustrate the theory and are not without value in showing what degree of consistency may be obtained in a first trial, but the numerical results are not very reliable, because the temperature was not controlled.

Table II.

Diffusion of sodium chloride into a 5 % gelatin gel, 0.0201N AgNO<sub>3</sub>. "Depth" in cm. of ppt.  
 $\kappa$  in c.g.s. units  $\times 10^5$ . Temperature 12°.

Time		N NaCl		0.5N NaCl		0.1N NaCl	
hrs.	mins.	Depth	$\kappa$	Depth	$\kappa$	Depth	$\kappa$
1	0	0.55	0.99	0.5	1.11	0.3	1.06
22	36	2.5	0.92	2.2	0.97	1.3	1.14
25	44	2.8	1.01	2.4	—	1.5	1.15
28	53	3.0	1.02	2.5	0.98	1.9	1.18
35	50	3.7	1.25	3.2	1.26	2.0	0.99
48	16	3.8	0.99	3.3	1.02	2.6	1.08
73	15	4.6	0.96	—	—	—	—

The figures given in this table are a great improvement on those of Table I, with the exception of the readings taken at the 35th hour. The remaining determinations show that  $\kappa$  does not really change with time, and therefore

method A is discredited. The constant of diffusion, however, does vary with the concentration, being greater in the more diluted and therefore more highly ionised solution.

$N$ NaCl	Mean $\kappa$	$0.98 \times 10^{-5}$	Mean error 3 %,
$0.5N$ NaCl	Mean $\kappa$	$1.02 \times 10^{-5}$	Mean error $4\frac{1}{2}$ %.
$0.1N$ NaCl	Mean $\kappa$	$1.10 \times 10^{-5}$	Mean error $5\frac{1}{2}$ %.

The mean error on a single determination is large, but on the whole the results prove that the indicator method gives reasonable results at the first trial, a most important advantage where a diffusion coefficient must be measured as an incidental portion of a wider research.

The results in Table II were all obtained by method B, so our next step is to give observations, made with the surface of the gelatin kept at constant salt concentration by exposure to a large bath of stirred salt solution.

Table III.

Penetration of  $N$  NaCl into a gelatin gel, with  $0.0201$  gram-mol. of  $AgNO_3$ . Method "C." Depth in cm. of precipitate.

$\kappa$  = diffusion coefficient in c.g.s. units  $\times 10^5$ .

Temperature  $12^\circ$ . Time, hours and minutes.

Time	1.0	2.49	4.9	6.0	7.30	11	22.36	25.44	28.53
Depth	0.6	0.9	1.2	1.5	1.62	1.95	2.85	3	3.3
$\kappa$	0.92	0.82	0.89	0.96	0.90	0.93	0.93	0.90	0.97
Time	48.16	53.30	73.15	96.20	120.6	197.30	219.0	263.30	
Depth	4.25	4.45	5.23	6	6.7	8.7	9.1	10	
$\kappa$	0.96	0.96	0.96	0.96	0.96	0.98	0.97	0.97	

The results in this table are in accordance with those in Table II, and because the method and formula are different, the "negative salt convention" is placed in a much stronger position than if it depended on one method alone; but the figures also prove that we must qualify the claim, made in the beginning, that all the apparatus needed is a rack of test-tubes and a ruler. The distances measured in the first few hours, when the temperature was constant, are so small that the experimental error with a wooden ruler is a large fraction of the whole, and if the experiment is protracted, the cupboard temperature cannot be relied upon. The night temperature would be below  $12^\circ$ . We must add a thermostat to our list of apparatus.

It is not absolutely essential because the heat reserve in a pan of water prevents much temperature variation, as is shown in Table IV, an experiment carried out to test the effect of varying the silver concentration, and also to get a direct comparison between the old method A and the method C, which was adopted for later experiments.

Table IV.

Penetration of $0.1N$ NaCl into 5 % gelatin, with $0.034$ gram-mol. $AgNO_3$ .			
Time	0	17.3 hours	25.2 hours
Temp.	$12.0^\circ$	$12.3^\circ$	$12.2^\circ$
Method A	$\kappa = 10^{-5} \times$	1.35	1.35
" C	$\kappa = 10^{-5} \times$	1.09	1.04
" A	depth in cm.	1.95	2.35
" C	depth in cm.	2.13	2.5

Method A makes " $\kappa$ " come out much too large, an error we must put down to circulation inside the test-tube. The data for B and C are set forth below, and for comparison a calculation of  $\kappa$  for water is made from Öholm's data [1905] (using his value of the temperature coefficient, in the neighbourhood of 18°, a rise of 2.5 % per degree centigrade).

Table V.

Method B	$N \text{ NaCl } \kappa = 0.98 \times 10^{-5}$	$N/10 \text{ NaCl } \kappa = 1.10 \times 10^{-5}$
Method C	0.96	1.06
Öholm	1.06	1.11

The negative salt convention has been subjected to three tests:

- (i) Variation in the silver concentration.
- (ii) Variation in the sodium chloride concentration.
- (iii) Alteration of the boundary conditions. If it was wildly in error, at least one of these tests should expose it, but if we allow for the nightly fall in temperature, the differences from Öholm's figures are about as large as the experimental error. The difference between the four experimental values of  $\kappa$  is in accordance with expectations.

A 5 % gelatin offers some resistance to diffusion, therefore "B" should give bigger values than "C," and the relatively faster movement of the ions causes the more highly dissociated 0.1*N* salt to diffuse more quickly than normal sodium chloride.

## VI. DERIVATION OF CORRECTED FORMULA.

The data given above are sufficient to establish the "negative salt" convention as a first approximation, and for diffusion experiments it is a fairly close approximation. To carry it further requires a considerable extension of the work, mathematical and practical.

The temperature control in particular must be very reliable but it is the former branch of the subject which must be considered here.

There are two difficulties in particular which were omitted in the preliminary treatment. The first of these is ionisation. As a general rule the undissociated molecule diffuses more slowly than the ions, so that other things being equal, the more dilute the solution, the larger is the value of  $\kappa$ . This variation in  $\kappa$  is quite big enough to be noticeable in the results calculated above. Each concentration of salt has a particular value of  $\kappa$ . In the gel the concentration varies from say *N* to 0. The  $\kappa$  we calculate will be that of some intermediate concentration, probably about *N*/2, but the indicator method does not give us information on this point, so it has perforce to be neglected.

The second and much the more important factor is the rate of diffusion of the silver nitrate. The original convention was that silver nitrate could be treated as negative sodium chloride. This assumption is arbitrary and it is to be feared it offends common sense.

In the following section, an attempt is made to treat the indicator method theoretically without this simplifying assumption. The change of  $\kappa$  with concentration will not be introduced into the calculations and the average value between the two limits will be used. This approximation is unavoidable.

The experimental facts to be considered have been stated before, but they must now be defined with greater precision. The silver nitrate is streaming down the tube. Let its concentration be  $v$  and its diffusion coefficient  $\lambda$ , then

$$\frac{\partial v}{\partial t} = \lambda \frac{\partial^2 v}{\partial x^2} \dots\dots\dots(1)$$

Similarly for the chloride

$$\frac{\partial u}{\partial t} = \kappa \frac{\partial^2 u}{\partial x^2} \dots\dots\dots(2)$$

At the position  $a$ , the demarcation line between the precipitate and clear gel, it is assumed that the concentrations  $v$  and  $u$  both equal 0. This is not strictly true. Really the product of their concentration is a constant, and it is possible that  $u$  for instance might be quite large. This possible complication need not be considered here, as at the boundary it is certain that the chloride concentration must be small. If this assumption is to be true, the amount of salt brought to the point "a" must be equalled by the amount of silver nitrate. Therefore at  $a$

$$\lambda \frac{\partial v}{\partial x} + \kappa \frac{\partial u}{\partial x} = 0 \dots\dots\dots(3)$$

In addition to these equations the following boundary conditions must be satisfied:

$$\begin{aligned} v &= v_0 \text{ when } x > 0 \text{ at } t = 0, \\ u &= u_0 \text{ at } x = 0, \\ u &= v = 0 \text{ at } x = a. \end{aligned}$$

Let

$$\phi_a = \frac{2}{\sqrt{\pi}} \int_0^a e^{-\beta^2} d\beta.$$

Then  $\phi\left(\frac{x}{2\sqrt{\lambda t}}\right)$  and  $\phi\left(\frac{x}{2\sqrt{\kappa t}}\right)$  are particular solutions of the differential equations (1) and (2). The combination of these two integrals in a general solution is not immediately obvious.

The only method is to make some trial assumptions, and find out what values must be assigned to the constants if the formula is to satisfy the conditions of the problem. The simplest possible guess we can make is that  $v$  and  $u$  are linear functions of  $\phi\left(\frac{x}{2\sqrt{\lambda t}}\right)$  and  $\phi\left(\frac{x}{2\sqrt{\kappa t}}\right)$ . F. Neumann [Weber-Riemann] has shown that this method can be used successfully in solving a problem with similar equations, the formation of ice on a pool of still water.

Let

$$\begin{aligned} v &= A + B\phi\left(\frac{x}{2\sqrt{\lambda t}}\right), \\ u &= C + D\phi\left(\frac{x}{2\sqrt{\kappa t}}\right), \end{aligned}$$

where  $A, B, C$  and  $D$  are constants determined by the experimental conditions.

The data below enable one to calculate them.

- (i)  $at^{-\frac{1}{2}} = p =$  the penetration factor of von Fürth and Bubanović.
- (ii)  $A + B\phi \frac{p}{2\sqrt{\lambda}} = 0.$
- (iii)  $C + D\phi \frac{p}{2\sqrt{\kappa}} = 0.$   
 $\phi_0 = 0, \phi_\infty = 1.$
- (iv)  $A + B = v_0.$
- (v)  $C = u_0.$
- (vi)  $D = \frac{-u_0}{\phi \frac{p}{2\sqrt{\kappa}}}.$
- (vii)  $B = \frac{v_0}{1 - \phi \frac{p}{2\sqrt{\lambda}}}.$

From formula (III), by differentiating the linear functions, we find

$$-\kappa \left\{ \frac{2D}{\sqrt{\pi}} e^{-\frac{x^2}{4\kappa t}} \right\} \frac{1}{2\sqrt{\kappa t}} = \lambda \left\{ \frac{2B}{\sqrt{\pi}} e^{-\frac{x^2}{4\lambda t}} \right\} \frac{1}{2\sqrt{\lambda t}}$$

at  $x = a,$

$$-\kappa^{\frac{1}{2}} D e^{-\frac{p^2}{4\kappa}} = \lambda^{\frac{1}{2}} B e^{-\frac{p^2}{4\lambda}}.$$

Substituting for  $D$  and  $B,$  we arrive at our final formula :

$$\frac{\kappa^{\frac{1}{2}} u_0 e^{-\frac{p^2}{4\kappa}}}{\frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\kappa}}} e^{-\beta^2} d\beta} = \frac{\lambda^{\frac{1}{2}} v_0 e^{-\frac{p^2}{4\lambda}}}{1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\lambda}}} e^{-\beta^2} d\beta} \dots\dots\dots(IV)$$

$\kappa$  can be calculated from this equation if  $u_0, v_0, \lambda$  and  $p$  are known. When  $\kappa = \lambda$  it simplifies to the old "negative salt" formula

$$\frac{v_0}{v_0 + u_0} = 1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{x}{2\sqrt{\lambda t}}} e^{-\beta^2} d\beta.$$

VII. MODIFICATION OF FORMULA (IV).

In its present form, the new formula is not suitable for computations. The arithmetic with formula (IV) takes 50 times as long as that required for formula (II). If this work had to be gone through for each experiment, the beauty of the indicator method would be gone. Much less formidable would be the difficulties of bad electrodes, and of gravimetric analysis, when compared with the labour in the calculations. For example, the right-hand side of the equation is objectionable when the concentration of silver nitrate is small, because then "p" is large, consequently with five figure tables of the probability integral, the error in calculating the denominator may easily exceed 10 %. On the left-hand side, although this difficulty is avoided, we have a worse trouble, since  $\kappa,$  the unknown, can only be arrived at by the method of trial and error.

In the light of these considerations, the calculation of (IV) appeared to be a fruitless effort, but when the formula was placed before Mr Berry, Fellow of King's College, Cambridge, it was a source of much pleasure to a naturalist to see both of these difficulties immediately cleared away, the former by the use of a series formula, the latter by a rearrangement. Both sides of the equation could be so modified that the whole series of calculations could be carried out once and for all.

The modification necessary is given below.

Let  $\frac{p}{2\sqrt{\lambda}} = y,$

$\frac{p}{2\sqrt{\kappa}} = z,$

$\phi_a = \frac{2}{\sqrt{\pi}} \int_0^a e^{-\beta^2} d\beta,$

then

$$\frac{\phi z}{\frac{p}{2z} u_0 e^{-z^2}} = \frac{1 - \phi y}{\frac{p}{2y} v_0 e^{-y^2}};$$

$$\therefore \frac{v_0}{u_0} = \frac{y e^{y^2} \cdot (1 - \phi y)}{z e^{z^2} \phi z}.$$

The problem of calculating  $\kappa$  is thus reduced to tabulation of these two functions of  $y$  and  $z$ , and when this has been done, the diffusion coefficient can be worked out with no trouble.

The logarithms of the functions are tabulated, to facilitate arithmetical work

$f(y) = f \frac{p}{2\sqrt{\lambda}} = \log_{10} y + \cdot 4343y^2 + \log_{10} (1 - \phi y).$

$f(z) = f \frac{p}{2\sqrt{\kappa}} = \log_{10} z + \cdot 4343z^2 + \log_{10} (\phi z).$

These functions are referred to elsewhere as the  $\lambda$  function and the  $\kappa$  function, because the physical significance of these symbols is an aid in distinguishing them—an association which is lost when  $y$  and  $z$  are used.

The computation of tables with the  $\kappa$  function is not a difficult matter, but with higher values of  $y$ , the probability integral tables are unsuitable for calculating the values of the  $\lambda$  function

$= \log [y e^{y^2} (1 - \phi y)],$

replacing  $1 - \frac{2}{\sqrt{\pi}} \int_0^y e^{-v^2} dy$  by  $\frac{2}{\sqrt{\pi}} \int_y^\infty e^{-v^2} dy$

we can use the series for  $e^{v^2} \int_y^\infty e^{-v^2} dy$

$= \frac{1}{2y} - \frac{1}{2^2 y^3} + \frac{1.3}{2^3 y^5} - \frac{1.3.5}{2^4 y^7} + \dots$

VIII. EXPERIMENTAL VERIFICATION OF NEW FORMULA.

When calculations are made with these tables the gulf is a wide one between the experimental data and the diffusion coefficient, so the bridge requires testing before we can rely upon it. There are two methods of doing this—the first by trying the effect of different concentrations of silver nitrate, the second

by examining substances which diffuse at a rate differing from that of sodium chloride.

The problem will be treated initially by the negative salt method, on account of the lack of reliable data concerning the diffusion of silver nitrate in gelatin. The figures below, copied from Landolt and Börnstein, refer to water.

Table VI.

Conc. AgNO <sub>3</sub>	0.02	0.1	0.9	3.9	0.03	0.433
Temperature	12°	12°	12°	12°	7.2°	7.2°
$\kappa$ (c.g.s.) $10^{-5} \times$	1.2	1.14	1.02	0.62	1.04	0.752

The measurement even for the most dilute solution differs by over 20 % from the coefficient calculated from Nernst's formula [Nernst, p. 273]. It may be remarked when a similar calculation is made for dilute NaCl, the difference is very much smaller—only 2 %.

Table VII.

Diffusion of *N* NaCl into 1 % agar with different concentrations of AgNO<sub>3</sub>. *d* = depths in cm.  $\kappa$  = diffusion coefficient in c.g.s. units  $\times 10^5$ .

		Concentrations of AgNO <sub>3</sub> (normalities)							
Time	Temp.	0.006	0.0154	0.06	0.114	0.006	0.0154	0.06	0.114
hrs. mins.	°C.	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	$\kappa$	$\kappa$	$\kappa$	$\kappa$
16 6	36.4	4.1	3.61	2.78	2.4	1.92	1.91	1.92	1.88
25 3	36.4	5	4.7	3.45	3.02	1.88	2.06	1.90	1.91
39 48	36.4	—	6	4.39	3.8	—	2.13	1.94	1.90

		Concentrations AgNO <sub>3</sub>					
Time	Temp.	0.006	0.0154	0.1143	0.006	0.0154	0.1143
hrs. mins.	°C.	<i>d</i>	<i>d</i>	<i>d</i>	$\kappa$	$\kappa$	$\kappa$
15 23	11.5	3	2.7	1.79	1.1	1.11	1.09
24 50	11.7	3.77	3.42	2.24	1.09	1.12	1.07
39 17	10.8	4.75	—	2.85	1.08	—	1.08

		Concentrations AgNO <sub>3</sub>					
Time	Temp.	0.5	0.1	0.02	0.5	0.1	0.02
hrs. mins.	°C.	<i>d</i>	<i>d</i>	<i>d</i>	$\kappa$	$\kappa$	$\kappa$
0 54	11.9	0.265	0.475	0.6	1.2	1.2	0.99
2 50	—	0.45	0.765	0.99	1.06	0.99	0.88
6 0	—	0.68	1.16	1.6	1.15	1.09	1.09
8 56	—	0.8	1.35	1.95	1.06	0.99	1.09

The examination of this table points to the conclusion that variations in the concentration of silver nitrate have less effect on " $\kappa$ " than the errors of an individual observation, when the experiment is made with sodium chloride.

In other words, we have been lucky enough to find an indicator which fulfils the condition postulated, therefore silver nitrate does behave as negative sodium chloride.

We may infer that the diffusion coefficient of silver nitrate probably lies between 1.0 and  $1.1 \times 10^{-5}$  under these experimental conditions.

The study of bodies with diffusion rates differing from this figure will give more interesting results.

Table VIII. *Hydrogen Chloride.*

Diffusion of a 1.03*N* solution of HCl into 2% agar with different concentrations of AgNO<sub>3</sub>.  
 $d$  = depth in cm.  $\kappa$  = coefficient in c.g.s. units  $\times 10^5$ . Temperature 11.9°.

Time hrs. mins.	Concentrations of AgNO <sub>3</sub>									
	0.5 $d$	0.1 $d$	0.02 $d$	0.004 $d$	0.0008 $d$	0.5 $\kappa$	0.1 $\kappa$	0.02 $\kappa$	0.004 $\kappa$	0.0008 $\kappa$
0 46	0.22	0.6	0.8	0.1	1.2	0.92	2.24	2.06	2.12	2.20
2 40	0.475	0.96	1.4	1.8	2.15	1.21	1.62	1.81	1.97	2.04
5 59	0.74	1.44	2.15	2.7	3.23	1.31	1.64	1.95	2.02	2.12
8 54	0.9	1.8	2.62	3.31	3.95	1.31	1.73	1.95	2.05	2.14
32 49	1.75	3.5	5.1	—	7.7	1.35	1.76	2.0	—	2.20

This table shows that the negative salt convention breaks down when applied to hydrochloric acid. The variation in  $\kappa$  is far greater than can be accounted for by the experimental error, even though the work was carried on for a brief period of time. The result that  $\kappa = 1.35 \times 10^{-5}$  (with 0.5*N* AgNO<sub>3</sub>) is obviously not a natural constant: it corresponds to an "artefact" in histology, produced by crude technique.

The first step to a more exact analysis of the problem is to work out  $\lambda$ , the diffusion coefficient of silver nitrate. This proved to be quite simple. Since the coefficient of sodium chloride is fairly close to that of silver nitrate, the "negative" method will give reasonable results if the sodium chloride is used as indicator in small concentrations. The results are given below.

Table IX.

Diffusion of 0.4*N* AgNO<sub>3</sub> into a 1% agar gel, with 0.01*N* NaCl.  $d$  = depth in cm.  
 $\kappa$  = coefficient  $\times 10^5$ . Temperature 10°.

Time hrs. mins.	Experimental number							
	1 $d$	2 $d$	3 $d$	4 $d$	1 $\kappa$	2 $\kappa$	3 $\kappa$	4 $\kappa$
16 47	2.4	2.45	2.5	2.5	0.935	0.98	1.02	1.02
24 10	2.97	2.98	3.09	3.0	0.995	1.01	1.08	1.02
40 57	3.82	3.89	3.9	—	0.975	1.01	1.01	—

The diffusion of silver nitrate deserves a more extended study, but these figures seem to be consistent enough for us to use the figure  $1.01 \times 10^{-5}$  in further calculations—at any rate for a preliminary survey, which is all that can be attempted in this paper.

The figure is probably a fair enough approximation to illustrate distinctly the differences when calculations are made by the new and old formulae.

Table X. *Hydrogen Chloride.*

Diffusion of a 1.035*N* solution of HCl into agar gels with different concentrations of AgNO<sub>3</sub>.  
 $t$  = time (hours and minutes).  $d$  = depth in cm.

Temp. °C.	Concentrations of AgNO <sub>3</sub>									
	0.5 <i>N</i>		0.1 <i>N</i>		0.02 <i>N</i>		0.004 <i>N</i>		0.0008 <i>N</i>	
	$t$	$d$	$t$	$d$	$t$	$d$	$t$	$d$	$t$	$d$
10.0	16 20	1.29	16 15	2.4	16 7	3.46	16 0	4.3	15 54	5.1
10.3	23 50	1.55	23 45	2.93	24 41	4.2	23 39	5.3	23 37	6.19
9.7	40 29	2.05	40 21	3.83	40 14	5.49	40 7	6.9	40 3	8.05
10.0	48 41	2.265	48 44	4.21	47 48	6.00	48 47	7.6	—	—

The data in this table are probably more reliable than the figures in Table VIII: especially in cases where the depth of penetration was small—though this advantage is offset to some extent by temperature variations. The materials are now complete for carrying out calculations with the aid of our tables of the  $\lambda$  and  $\kappa$  functions.

Table XI.

Illustration of calculations made with the aid of tables of the  $\kappa$  and  $\lambda$  functions where  $\kappa$  is the diffusion coefficient of HCl and  $\lambda = 1.01 \times 10^{-5}$  that of  $\text{AgNO}_3$ .

Concentration of $\text{AgNO}_3$	0.5N	0.1N	0.02N	0.004N	0.0008N
" $p^2$ " = $\frac{x^2}{l}$ in seconds	$2.86 \times 10^{-5}$	$1.0 \times 10^{-4}$	$2.05 \times 10^{-4}$	$3.3 \times 10^{-4}$	$4.51 \times 10^{-4}$
$\log_{10} v_0$ ... ..	1.699	1.0	2.301	3.602	4.903
$\log_{10} u_0$ ... ..	0.014	0.014	0.014	0.014	0.014
$\lambda$ function ... ..	1.602	1.687	1.716	1.7265	1.733
$\kappa$ function ... ..	1.917	0.701	1.429	2.1384	2.844
$\kappa$ by new formula ...	1.38	1.65	1.84	1.96	1.98
$\kappa$ by old formula ...	1.48	1.72	1.87	1.97+	1.99
Error in old formula	7.2 %	4 %	1.6 %	0.7 %	0.5 %

The same method is applied to magnesium chloride below. The scale of the experiments is too small to eliminate chance errors, but in spite of this the essential difference between the two methods of calculation is well brought out.

Table XII.

Diffusion of *N* magnesium chloride into 2 % agar with various concentrations of  $\text{AgNO}_3$ . Temperature 11.9°. Time, 5 hours 55 minutes.  $\lambda = 1.09 (\times 10^{-5})$ .

Concentration of $\text{AgNO}_3$	0.5	0.1	0.02	0.0008
Depth of penetration ...	0.5	1.0	1.4	2.05
$\kappa$ by new formula ...	0.765	0.835	0.857	0.88
$\kappa$ by old formula ...	0.628	0.82	0.845	0.875
Error in old formula ...	11 %	1.8 %	1.5 %	0.6 %

With the data given in this table we may bring to a close our compilation of experimental material, and proceed to discuss the results.

### IX. REVIEW OF THE EXPERIMENTAL DATA.

*Sodium chloride.* The results with this salt are satisfactory when the negative salt convention is used, on account of the lucky coincidence that silver nitrate diffuses as quickly as sodium chloride.

The numerical values of  $\kappa$  at 12°—in the two sets of experiments of Table VII—are  $1.04$  and  $1.09 \times 10^{-5}$ . Öholm's figure at that temperature, obtained by interpolation, works out at  $1.06 \times 10^{-5}$  for a normal solution. No stress can be laid on the accuracy of the agreement—the number of experiments is far too small, but it supports Voigtländer's observations on the smallness of the effect of agar gels in retarding diffusion. A 5 % gelatin appears to have a much more noticeable effect.

*Hydrogen chloride.* The results obtained with this substance are interesting, but the interpretation is doubtful. Table XI shows that the negative salt convention is not reliable, especially when the concentration of silver nitrate is high. Put into words, the table shows that the slower rate of diffusion of silver nitrate helps the hydrogen chloride to advance, and the assumption that the silver nitrate streams up with equal rapidity to meet it cannot give a reliable result. The coefficient appears to be larger than is actually the case, because the weakness of the opposition is ignored.

The difficulty is overcome by the new formula, but it only serves to emphasise the second trouble—the coefficient is far too small when there is a high concentration of silver nitrate in the gel. It is necessary to exercise caution in working out the explanation of this difficulty because we have no right to assume that the silver chloride precipitate has no influence on the rate of diffusion—the precipitate from a strong solution of silver nitrate in the presence of acid may quite easily differ from that formed in a neutral medium. In the absence of direct evidence, this danger can only be mentioned as a possibility—it is not likely that it will prove to be the real explanation. The root of the difficulty is that hydrogen chloride cannot be considered as a simple body. If the compound  $\text{HCl} \cdot \text{AgNO}_3$  was formed, the formula would give accurate results at all concentrations. In reality, the hydrogen ions are not eliminated—therefore the diffusion of fresh hydrogen chloride is retarded. In other words, the “effective concentration” of acid is reduced. Below are given the strengths of HCl which, but for this complication, would give the observed penetration.

Normality of $\text{AgNO}_3$	0.5	0.1	0.02	0.004	0.0008
Strength of HCl ...	0.63	0.71	0.82	0.98	1.035

As a matter of fact, this difficulty is rather academic, because the silver nitrate is put in as an indicator—in small concentration—therefore the complications which turn up when its concentration is high are of no practical importance. Nevertheless, it demands our consideration, when deciding what concentration of silver nitrate is the highest we can use. The strength is best kept above 0.0008N to ensure the distinctness of the demarcation surface.

*Magnesium chloride.* The experiments on this substance are on too small a scale to justify any confidence in the absolute numerical values—but as chance errors will affect both results equally, they serve to illustrate the case where the diffusing substance travels more slowly than the silver nitrate—in complete contrast to hydrochloric acid.

In Table XII it will be observed that the negative convention makes the diffusion coefficient of magnesium chloride appear too small, because the “opposition” of the silver is thereby arbitrarily reduced, but the material is not sufficient for further developments of the theory.

CONCLUSION.

The number of definitions required foils any attempt to give a brief account of certain sections, but rather than repeat lengthy explanations, an illustration is given to show the simplicity of the calculation required in practice.

The indicator technique differs from the standard methods in securing rapidity of execution at the expense of theoretical simplicity. This sacrifice has been of sufficient importance to cause the neglect of the indicator method in the past. The diffusion of two salts resulting in the formation of a zone of precipitate is a problem too full of complications to admit of a rigorous treatment. This disadvantage need not concern us practically, for such a treatment would lead to long calculations consuming more time than that gained by the rapidity of the experimental work. Consequently it is necessary to make some approximations. These are described in sections III and VI, but the following terms are re-defined: "u" = conc. NaCl, "u<sub>0</sub>" = conc. NaCl in bath, "κ" = diff. coeff. of NaCl, "v<sub>0</sub>" = conc. AgNO<sub>3</sub> in jelly, "λ" = diff. coeff. of AgNO<sub>3</sub>, p = penetration "x" divided by square root of time "t."

The first approximation, called the negative salt convention, leads to the formula

$$\frac{v_0}{u_0 + v_0} = u = 1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{x}{2\sqrt{\kappa t}}} e^{-\beta^2} d\beta. \dots\dots\dots(\text{II})$$

The second approximation, which allows for the different velocity of silver nitrate, leads to the formula

$$\frac{\kappa^{\frac{1}{2}} u_0 e^{-\frac{p^2}{4\kappa}}}{\frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\kappa}}} e^{-\beta^2} d\beta} = \frac{\lambda^{\frac{1}{2}} v_0 e^{-\frac{p^2}{4\lambda}}}{1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\lambda}}} e^{-\beta^2} d\beta} \dots\dots\dots(\text{IV})$$

The calculation of κ with these formulae is a matter of two or three minutes' work at most, but this can be best shown by an illustration, that having once looked up the factor in the table, κ can be worked out in 45 seconds, using formula (II). Given a jelly with 0.005N silver nitrate, then κ in c.g.s. units is equal to the square of the penetration in cm. divided by the product of the time in hours and 10,000 times the factor—given below the corresponding strength of the salt solution.

Concentration	5N	N	0.5N	0.2N	0.1N
Factor	7.8	5.68	4.78	3.65	2.83

The negative salt convention introduces a serious error if the velocity of the diffusing salt is very different from that of silver nitrate unless the concentration of the latter is very small. The method of working out κ, illustrated in Table XI though it takes a few minutes longer, still has the advantage of checking the first result even though the indicator has been diluted enough to conceal the error.

To work out a formula by neglecting secondary phenomena is one thing, but the proof that these can be overlooked is another. The judgment of the

experimental test is a delicate matter for the experimenter, but it is advisable to draw attention to one table, No. XI. The errors in the indicator method are obviously due to the complications caused by silver nitrate and these increase as the strength of the latter is increased; 100 times the normal amount of nitrate gives a difference of 35 %, four times gives 7 %, but when we get down to a 0.005*N* silver nitrate, further dilution is practically without effect.

This shows that we can deliberately increase the magnitude of the "errors of method" but to do this unusual concentrations of silver nitrate are required.

A balanced account of the indicator method would require a description of the advantages comparable in length with the discussion of the difficulties, but the space taken by these leaves little room for the other side of the question. But because there are other diffusion methods, it seems necessary to give some reasons for departing from them.

(1) The measurement of length is direct, it can be made with great accuracy, and the same tube can be measured again at different times. The presence of gelatin or agar-agar, so useful to avoid mechanical mixing, apart from its intrinsic interest, complicates the technique of the standard methods.

(2) The ease with which a large number of experiments can be performed makes the method particularly suitable for an introductory survey even if it gives only a two figure accuracy—and the rough preliminary experiments described above could be greatly improved upon. Specially interesting results could be verified by other methods—but much time would be saved in finding where accurate measurements were most needed.

(3) The fact that no special apparatus is needed enables a worker to determine several diffusion coefficients at a few hours notice, a useful point when these figures are required for another research—for example the comparison with simple diffusion of the rate of absorption of nutrient salts by living tissues.

For educational purposes there is a further advantage due to the simplicity of the apparatus, combined with the fact that the readings can be taken whenever it is convenient.

Data can be obtained to illustrate the methods of handling numerical results in a branch of science which generally demands long experiments before a table or graph can be constructed. The temptation to continue the list of applications must be suppressed, for after all, the special qualities of the method are matters of laboratory technique in which experience is superior to description, but I cannot conclude this account without acknowledging my indebtedness to Professor Stiles, but for whom the work would never have been attempted, and Mr Berry, Fellow of King's College, Cambridge, for his help in dealing with formula (IV).

APPENDIX.

Table of values of the "λ" function.

$$f \frac{p}{2\sqrt{\lambda}} = \log_{10} \frac{p}{2\sqrt{\lambda}} \cdot e^{\frac{p^2}{4\lambda}} \left( 1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\lambda}}} e^{-\beta^2} d\beta \right).$$

For values of  $\frac{p}{2\sqrt{\lambda}}$

$\frac{p}{2\sqrt{\lambda}}$	$f \frac{p}{2\sqrt{\lambda}}$	$\frac{p}{2\sqrt{\lambda}}$	$f \frac{p}{2\sqrt{\lambda}}$	$\frac{p}{2\sqrt{\lambda}}$	$f \frac{p}{2\sqrt{\lambda}}$
0.4	1.429	1.8	1.699	3.4	1.734
0.5	1.488	1.9	1.703	3.6	1.736
0.6	1.532	2.0	1.709	3.8	1.737
0.7	1.566	2.1	1.712	4.0	1.739
0.8	1.593	2.2	1.715	4.5	1.741
0.9	1.614	2.3	1.718	5.0	1.743
1.0	1.631	2.4	1.720	5.5	1.744
1.1	1.646	2.5	1.722	6.0	1.745
1.2	1.657	2.6	1.723	6.5	1.746
1.3	1.668	2.7	1.725	7.0	1.747
1.4	1.676	2.8	1.726	8.0	1.748
1.5	1.683	2.9	1.727	9.0	1.749
1.6	1.689	3.0	1.729	10.0	1.750
1.7	1.695	3.2	1.732	∞	1.751

Table of values of the "κ" function.

$$f \frac{p}{2\sqrt{\kappa}} = \log_{10} \frac{p}{2\sqrt{\kappa}} \cdot e^{\frac{p^2}{4\kappa}} \left( \frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\kappa}}} e^{-\beta^2} d\beta \right).$$

For values of  $\frac{p}{2\sqrt{\kappa}}$

$\frac{p}{2\sqrt{\kappa}}$	$f \frac{p}{2\sqrt{\kappa}}$	$\frac{p}{2\sqrt{\kappa}}$	$f \frac{p}{2\sqrt{\kappa}}$	$\frac{p}{2\sqrt{\kappa}}$	$f \frac{p}{2\sqrt{\kappa}}$
0.4	1.3034	1.4	0.956	2.4	2.88
0.5	1.5240	1.5	1.138	2.5	3.112
0.6	1.7154	1.6	1.305	2.6	3.36
0.7	1.8890	1.7	1.48	2.7	3.60
0.8	0.0516	1.8	1.66	2.8	3.85
0.9	0.2074	1.9	1.845	2.9	4.115
1.0	0.3600	2.0	2.036	3.0	4.38
1.1	0.5115	2.1	2.238	3.5	5.864
1.2	0.6637	2.2	2.442	4.0	7.551
1.3	0.8182	2.3	2.659		

REFERENCES.

von Fürth and Bubanović (1918). *Biochem. Zeitsch.* 90, 265; 92, 139.  
 Nernst. *Theoretical Chemistry*, 273.  
 Öholm (1905). *Zeitsch. physiol. Chem.* 50, 309.  
 Stiles (1920). *Biochem. J.* 14, 58.  
 Weber-Riemann. *Part. Diff. Gleich.* 2, 117.



## INDEX

- Aceto-acetic acid, passage of, into urine (Widmark) 364
- Acetone, elimination of, through the lungs (Widmark) 379
- Acetone: *n*-butyl alcohol fermentation, products of (Reilly, Hickinbottom, Henley and Thaysen) 229
- Acetone, passage of, into urine (Widmark) 364
- Acids, organic, rapid volumetric method for estimation of (Foreman) 451
- ADAIR, G. S. The penetration of electrolytes into gels. II: The application of Fourier's linear diffusion law 762
- ADAM, N. K. A modification of the Barcroft and Winterstein microrespirometers 679
- ADKINS, D. M. Digestibility of germinated beans 637
- Aldehydes, effect of, on fermentation of glucose by yeast preparations (Harden and Henley) 642
- Alkaline tide in Singapore (Campbell) 603
- Amino-acid excretion in Singapore (Campbell) 603
- Amino-acids, rapid volumetric method for estimation of (Foreman) 451
- Ammonia coefficient of pregnancy (Cullis and Hewer) 757
- Ammonia excretion in Singapore (Campbell) 603
- Amylase, presence of, in milk and cheese (Sato) 120
- ANDRESEN, K. G., *see* KROGH, A.
- ANNETT, H. E. Factors influencing alkaloidal content and yield of latex in the opium poppy (*Papaver somniferum*) 618
- Anthocyanins, butyl alcohol as a solvent for (Rosenheim) 73
- Anthocyanins, of young leaves of the grape vine (Rosenheim) 178
- Antitoxin, diphtheria, heat inactivation of (Homer) 565
- Antitoxins, association of, with proteins of immunised horse serum (Homer) 42
- Anti-scorbutic factor, of vegetable and fruit juices, effect of heat on (Delf) 211
- Anti-scorbutic properties of concentrated fruit juices (Harden and Robison) 171
- Anti-scorbutic requirements of the monkey (Harden and Zilva) 131
- BAKER, J. L. and HULTON, H. F. E. The iodimetric estimation of sugars 754
- BARRATT, J. O. W. The action of thrombin upon fibrinogen 189
- Bases, organic, rapid volumetric method for estimation of (Foreman) 451
- Beans, germinated, digestibility of (Adkins) 637
- Bile, inhibitor for hepatic esterase in (Wihart) 406
- BRADFORD, S. C. Adsorptive stratification in gels, III 29
- BRADFORD, S. C. Adsorptive stratification in gels, IV 474
- BRADFORD, S. C. On the theory of gels. II: The crystallisation of gelatin 91
- BROWN, A. J. Obituary notice of 1
- BURNS, D. A note on the effect of purgation on the creatinine content of urine 94
- Butter, effect of heat and oxygen on the nutritive value of (Drummond and Coward) 734
- Butyl alcohol as a solvent for anthocyanins (Rosenheim) 73
- Cabbage, fat-soluble factor of, extraction of, by solvents (Zilva) 494
- CAMPBELL, J. A. Ammonia excretion, amino-acid excretion and the alkaline tide in Singapore 603
- Carbohydrate and fat, relative values of, as sources of muscular energy (Krogh and Lindhard) 290
- Carrots, fat-soluble factor of, extraction of, by solvents (Zilva) 494
- Caseinogen, molecular weight of, determination by Barger's method (Yamakami) 522
- Cellulose, fermentation of, in paunch of ox (Krogh and Schmit-Jensen) 686
- Cheese, presence of amylase in (Sato) 120
- CHICK, H. and HUME, E. M. The production in monkeys of symptoms closely resembling those of pellagra, by prolonged feeding on a diet of low protein content 135
- CLARK, A. B., *see* STEPHENSON, M.
- Cooking, of eggs, formation of ferrous sulphide during (Tinkler and Soar) 114
- Cooking of green vegetables, methods employed for (Masters and Garbutt) 75
- Cotton, action of sea water on (Dorée) 709
- Cotton, deterioration of, on wet storage (Fleming and Thaysen) 25
- COWARD, K. H. and DRUMMOND, J. C. Researches on the fat-soluble accessory substance. IV: Nuts as a source of vitamin A 665
- COWARD, K. H., *see also* DRUMMOND, J. C.
- Creatinine content of urine, effect of purgation on (Burns) 94
- CULLIS, W. C. and HEWER, E. E. The "ammonia coefficient" of pregnancy 757
- Cuorin (MacLean and Griffiths) 615
- DELFF, E. M. Effect of heat on the anti-scorbutic accessory factor of vegetable and fruit juices 211
- Digestibility of germinated beans (Adkins) 637

- DORÉE, C. The action of sea water on cotton and other textile fabrics 709
- DRUMMOND, J. C. The nomenclature of the so-called accessory food factors (vitamins) 660
- DRUMMOND, J. C. and COWARD, K. H. Researches on the fat-soluble accessory substance. III: Technique for carrying out feeding tests for vitamin A (fat-soluble A) 661
- DRUMMOND, J. C. and COWARD, K. H. Researches on the fat-soluble accessory substance. V: The nutritive value of animal and vegetable oils and fats considered in relation to their colour 668
- DRUMMOND, J. C. and COWARD, K. H. Researches on the fat-soluble accessory factor (vitamin A). VI: Effect of heat and oxygen on the nutritive value of butter 734
- DRUMMOND, J. C., GOLDING, J., ZILVA, S. S. and COWARD, K. H. The nutritive value of lard 742
- DRUMMOND, J. C., *see also* COWARD, K. H.
- Eggs, formation of ferrous sulphide during cooking of (Tinkler and Soar) 114
- Electrolytes, penetration of, into gels (Stiles) 58
- Electrolytes, penetration of, into gels, mathematical discussion of (Adair) 762
- ELMHIRST, R. and SHARPE, J. S. On the colours of two sea anemones, *Actinia equina* and *Anemonia sulcata* 48
- Enzymes of direct oxidising systems in plants (Onslow) 535
- Enzymes, oxidising, of fruits (Onslow) 541
- Esterase, hepatic, inhibitor for, in the bile (Wishart) 406
- Faeces, guanidine content of, in idiopathic tetany (Sharpe) 46
- Fat and carbohydrate, relative values of, as sources of muscular energy (Krogh and Lindhard) 290
- Fats, nutritive value of, in relation to colour (Drummond and Coward) 668
- Fat soluble factor, action of ozone on, in fats (Zilva) 740
- Fat-soluble factor of cabbage and carrot, extraction of, by solvents (Zilva) 494
- Fat-soluble factor of fats and oils, relation of, to colour (Drummond and Coward) 668
- Fat-soluble factor, technique of feeding tests for (Drummond and Coward) 661
- Fat-soluble factor, *see also* Vitamin A, and Vitamine
- FEARON, W. R. A study of some biochemical tests. No. 2. The Adamkiewicz protein reaction. The mechanism of the Hopkins-Cole test for tryptophan. A new colour test for glyoxylic acid 548
- Fermentation of cellulose in paunch of ox (Krogh and Schmit-Jensen) 686
- Fermentation, of glucose by yeast preparations, effect of aldehydes, etc. on (Harden and Henley) 642
- Ferrous sulphide, formation of, in eggs during cooking (Tinkler and Soar) 114
- Fibrinogen, action of thrombin upon (Barratt) 189
- FLEMING, N. and THAYSEN, A. C. On the deterioration of cotton on wet storage 25
- Flour, bread-making properties of, effect of various substances on (Masters and Maughan) 586
- FOREMAN, F. W. Rapid volumetric methods for the estimation of amino-acids, organic acids and bases 451
- FREEAR, K. and VENN, E. C. V. The acidity of ropy milk 422
- Frogs, dietetic experiments with (Harden and Zilva) 263
- Fruit juices, concentrated, anti-scorbutic properties of (Harden and Robison) 171
- Fruit juices, effect of heat on anti-scorbutic factor of (Delf) 211
- Fruits, oxidising enzymes of (Onslow) 541
- GARBUTT, P., *see* MASTERS, H.
- Gas analysis apparatus, accurate form of (Krogh) 267
- Gas analysis, application of Krogh's micro-methods of (Schmit-Jensen) 4
- Gas meters, calibration and use of (Krogh) 282
- Gelatin, contracting clot in gel of (Lloyd) 584
- Gelatin, crystallisation of (Bradford) 91
- Gelatin, swelling of, in acid and alkali (Lloyd) 147
- Gels, adsorptive stratification in (Bradford) 29, 474
- Gels, agar-agar, containing silver nitrate, penetration of sodium chloride into (Stiles) 58
- Gels, penetration of electrolytes into (Stiles) 58
- Gels, penetration of electrolytes into, mathematical discussion of (Adair) 762
- Gels, theory of (Bradford) 91
- Glycine, neutral salt addition compounds of (King and Palmer) 574
- Glyoxylic acid, new colour test for (Fearon) 548
- Goat, collection of uncontaminated samples of urine of (Peters) 697
- Goat, normal metabolism of (Peters) 697
- GOLDING, J., *see* DRUMMOND, J. C.
- Grape vine, anthocyanins of young leaves of (Rosenheim) 178
- GRIFFITHS, W. J., *see* MACLEAN, H.
- Growth-promoting substances, plant, occurrence of, in manurial composts, and properties of (Mockeridge) 432
- Guanidine content of faeces in idiopathic tetany (Sharpe) 46
- HARDEN, A. and HENLEY, F. R. The effect of pyruvates, aldehydes and methylene blue on the fermentation of glucose by yeast juice and zymine in presence of phosphate 642
- HARDEN, A. and ROBISON, R. The anti-scorbutic properties of concentrated fruit juices. Part III 171
- HARDEN, A. and ZILVA, S. S. Dietetic experiments with frogs 263
- HARDEN, A. and ZILVA, S. S. The anti-scorbutic requirements of the monkey 131
- HATSCHEK, E. A series of abnormal Liesegang stratifications 418
- Heat, effect of, on anti-scorbutic factor of vegetable and fruit juices (Delf) 211
- Heat inactivation of diphtheria antitoxin (Homer) 565

- HENLEY, F. R., *see also* HARDEN, A. and REILLY, J.
- HEWER, E. E., *see* CULLIS, W. C.
- HEWITT, J. A. and PRYDE, J. The metabolism of carbohydrates. Part I. Stereochemical changes undergone by equilibrated solutions of reducing sugars in the alimentary canal and in the peritoneal cavity 395
- HICKINBOTTOM, W. J., *see* REILLY, J.
- HOMER, A. On the association of antitoxins with the proteins of immunised horse serum 42
- HOMER, A. The heat inactivation of diphtheria antitoxin 565
- HOPKINS, F. G. Note on the vitamine content of milk 721
- HOPKINS, F. G. The effects of heat and aeration upon the fat-soluble vitamine 725
- HULTON, H. F. E., *see* BAKER, J. L.
- HUME, E. M., *see* CHICK, H.
- Iron, inorganic, distribution of, in plant and animal tissues (Jones) 654
- JONES, H. W. The distribution of inorganic iron in plant and animal tissues 654
- JUDD, H. M. The iodometric estimation of sugars 255
- Keratomalacia, in rats (Stephenson and Clark) 502
- KING, H. and PALMER, A. D. Glycine and its neutral salt addition compounds 574
- KROGH, A. A gas analysis apparatus accurate to 0.001 % mainly designed for respiratory exchange work 267
- KROGH, A. The calibration, accuracy and use of gas meters 282
- KROGH, A. and LINDHARD, J. (with the collaboration of LILJESTRAND, G. and ANDRESEN, K. G.). The relative value of fat and carbohydrate as sources of muscular energy. With appendices on the correlation between standard metabolism and the respiratory quotient during rest and work 290
- KROGH, A. and SCHMIT-JENSEN, H. O. The fermentation of cellulose in the paunch of the ox and its significance in metabolism experiments 686
- Lard, nutritive value of (Drummond, Golding, Zilva and Coward) 742
- LILJESTRAND, G., *see* KROGH, A.
- LINDHARD, J., *see* KROGH, A.
- LLOYD, D. J. Note on the production of a contracting clot in a gel of gelatin at the iso-electric point 584
- LLOYD, D. J. On the swelling of gelatin in hydrochloric acid and caustic soda 147
- MACLEAN, H. and GRIFFITHS, W. J. Curon 615
- MACLEAN, I. SMEDLEY and THOMAS, E. M. The nature of yeast-fat 483
- Manurial composts, occurrence of plant growth-promoting substances in (Mockeridge) 432
- MARTIN, C. J. The preparation of Sørensen's pure phosphate solutions when the pure salts are not available 98
- MASTERS, H. and GARBUTT, P. An investigation of the methods employed for cooking vegetables, with special reference to the losses incurred. Part II: Green vegetables 75
- MASTERS, H. and MAUGHAN, M. An experimental study of the effect of certain organic and inorganic substances on the bread-making properties of yeast and on the fermentation of yeast 586
- MAUGHAN, M., *see* MASTERS, H.
- Metabolism experiments, significance of fermentation of cellulose in paunch of ox for (Krogh and Schmit-Jensen) 686
- Metabolism in Singapore (Campbell) 603
- Metabolism, normal, of the billy goat (Peters) 697
- Metabolism, standard, and respiratory quotient, correlation between (Krogh and Lindhard) 290
- Methylene blue, effect of, on fermentation of glucose by yeast preparations (Harden and Henley) 642
- Microrespirometer, modified form of (Adam) 679
- Milk, presence of amylase in (Sato) 120
- Milk, rosy, acidity of (Freear and Venn) 422
- Milk, vitamine content of (Hopkins) 721
- MOCKERIDGE, F. A. The occurrence and nature of the plant growth-promoting substances in various organic manurial composts 432
- Molecular weight, application to caseinogen of Barger's method for determining (Yamakami) 522
- Molecular weight, Barger's microscopical method of determining (Yamakami) 103
- Monkey, anti-scorbutic requirements of the (Harden and Zilva) 131
- Monkeys, pellagra in (Chick and Hume) 135
- Muscular energy, relative value of fat and carbohydrate as sources of (Krogh and Lindhard) 290
- NIERENSTEIN, M. Note on the oxidation of quinine with hydrogen peroxide 572
- Nuts, vitamin A of (Coward and Drummond) 665
- Obituary notice, A. J. Brown 1
- Oils, animal and vegetable, nutritive value of, in relation to colour (Drummond and Coward) 668
- ONSLow, M. WHELDALe. Oxidising enzymes. II: The nature of the enzymes associated with certain direct oxidising systems in plants 535
- ONSLow, M. WHELDALe. Oxidising enzymes. III: The oxidising enzymes of some common fruits 541
- Opium poppy, factors influencing alkaloidal content and yield of latex of (Annett) 618
- Oxidising enzymes of fruits (Onslow) 541
- Oxidising enzymes of plants, nature of (Onslow) 535
- Ozone, action of, on vitamin A in fats (Zilva) 740
- PALMER, A. D., *see* KING, H.
- Pellagra, symptoms of, in monkeys (Chick and Hume) 135
- PETERS, R. A. A method for obtaining uncontaminated specimens of urine from the billy goat with some notes upon the normal metabolism of this animal 697

- Phosphate, fermentation of glucose in presence of, by yeast preparations, effect of aldehydes, etc. on (Harden and Henley) 642
- Pigments, the yellow plant, differentiation of, from fat-soluble vitamine (Stephenson) 715
- Pigs, "scurvy" in (Plimmer) 570
- PLIMMER, R. H. A. Note on "scurvy" in pigs 570
- Pregnancy, "ammonia coefficient" of (Cullis and Hewer) 757
- Protein, Adamkiewicz test for (Fearon) 548
- PRYDE, J., *see* HEWITT, J. A.
- Pyruvates, effect of, on fermentation of glucose by yeast preparations (Harden and Henley) 642
- Quinine, oxidation of, with hydrogen peroxide (Nierenstein) 572
- Rats, keratomalacia in (Stephenson and Clark) 502
- Reaction, influence of, on colour changes in tyrosine solutions (Venn) 99
- REILLY, J., HICKINBOTTOM, W. J., HENLEY, F. R. and THAYSEN, A. C. The products of the "acetone: *n*-butyl alcohol" fermentation of carbohydrate material with special reference to some of the intermediate substances produced 229
- Respiratory quotient and standard metabolism, correlation between (Krogh and Lindhard) 290
- ROBINSON, R., *see* HARDEN, A.
- ROSENHEIM, O. Note on the use of butyl alcohol as a solvent for anthocyanins 73
- ROSENHEIM, O. Observations on anthocyanins. I: The anthocyanins of the young leaves of the grape vine 178
- SATO, M. On the presence of amylase in milk and cheese 120
- SCHMIT-JENSEN, H. O. Estimation of carbon dioxide, oxygen and combustible gases by Krogh's method of micro-analysis 4
- SCHMIT-JENSEN, H. O., *see also* KROGH, A.
- "Scurvy" in pigs (Plimmer) 570
- Sea anemones, colours of (Elmhirst and Sharpe) 48
- Sea water, action of, on cotton and other textile fabrics (Dorée) 709
- Serum, immunised horse, association of anti-toxins with (Homer) 42
- SHARPE, J. S. The guanidine content of faeces in idiopathic tetany 46
- SHARPE, J. S., *see also* ELMHIRST, R.
- Singapore, alkaline tide in (Campbell) 603
- Singapore, excretion of ammonia and amino-acids in (Campbell) 603
- SOAB, M. C., *see* TINKLER, C. K.
- Sørensen's phosphate solutions, preparation of (Martin) 98
- STEPHENSON, M. A note on the differentiation of the yellow plant pigments from the fat-soluble vitamine 715
- STEPHENSON, M. and CLARK, A. B. A contribution to the study of keratomalacia among rats 502
- STILES, W. The penetration of electrolytes into gels. I: The penetration of sodium chloride into gels of agar-agar containing silver nitrate 58
- Stratification, adsorptive in gels (Bradford) 29, 474
- Stratifications, Liesegang, series of abnormal (Hatschek) 418
- Sugars, iodimetric estimation of (Baker and Hulton) 754; (Judd) 255
- Sugars, reducing, stereochemical changes undergone by, in alimentary canal and peritoneal cavity (Hewitt and Pryde) 395
- Tetany, idiopathic, guanidine content of faeces in (Sharpe) 46
- Textile fabrics, action of sea water on (Dorée) 709
- THAYSEN, A. C., *see also* FLEMING, N. and REILLY, J.
- THOMAS, E. M., *see* MACLEAN, I. SMEDLEY
- Thrombin, action of, upon fibrinogen (Barratt) 189
- TINKLER, C. K. and SOAB, M. C. The formation of ferrous sulphide in eggs during cooking 114
- TODD, A. H. A note on Braunstein's modification of the Mörner-Sjöqvist process for the estimation of urea 252
- Tryptophan, mechanism of Hopkins-Cole test for (Fearon) 548
- Tyrosine, influence of reaction on colour changes of (Venn) 99
- Urea, estimation of, note on (Todd) 252
- Urine, creatinine content of, effect of purgation on (Burns) 94
- Urine, of billy goat, method of collecting uncontaminated samples of (Peters) 697
- Vegetable juices, effect of heat on anti-scorbutic factor of (Delf) 211
- Vegetables, green, methods of cooking (Masters and Garbutt) 75
- VENN, E. C. V. The influence of reaction on colour changes in tyrosine solutions 99
- VENN, E. C. V., *see also* FREEAR, K.
- Vitamin A, effect of heat and oxygen on, in butter (Drummond and Coward) 734
- Vitamin A, of nuts (Coward and Drummond) 665
- Vitamin A, technique of feeding tests for (Drummond and Coward) 661
- Vitamin A, *see also* Fat-soluble factor and Vitamine
- Vitamine content of milk (Hopkins) 721
- Vitamine, the fat-soluble, differentiation of, from the yellow plant pigments (Stephenson) 715
- Vitamine, the fat-soluble, effect of heat and aeration on (Hopkins) 725
- Vitamine, the fat-soluble, effect of heat and oxygen on, in butter (Drummond and Coward) 734
- Vitamine, the fat-soluble, *see also* Fat-soluble factor and Vitamin A
- Vitamins, nomenclature of (Drummond) 660
- WIDMARK, E. M. P. Studies in the acetone concentration in blood, urine and alveolar air. II: The passage of acetone and acetoacetic acid into the urine 364

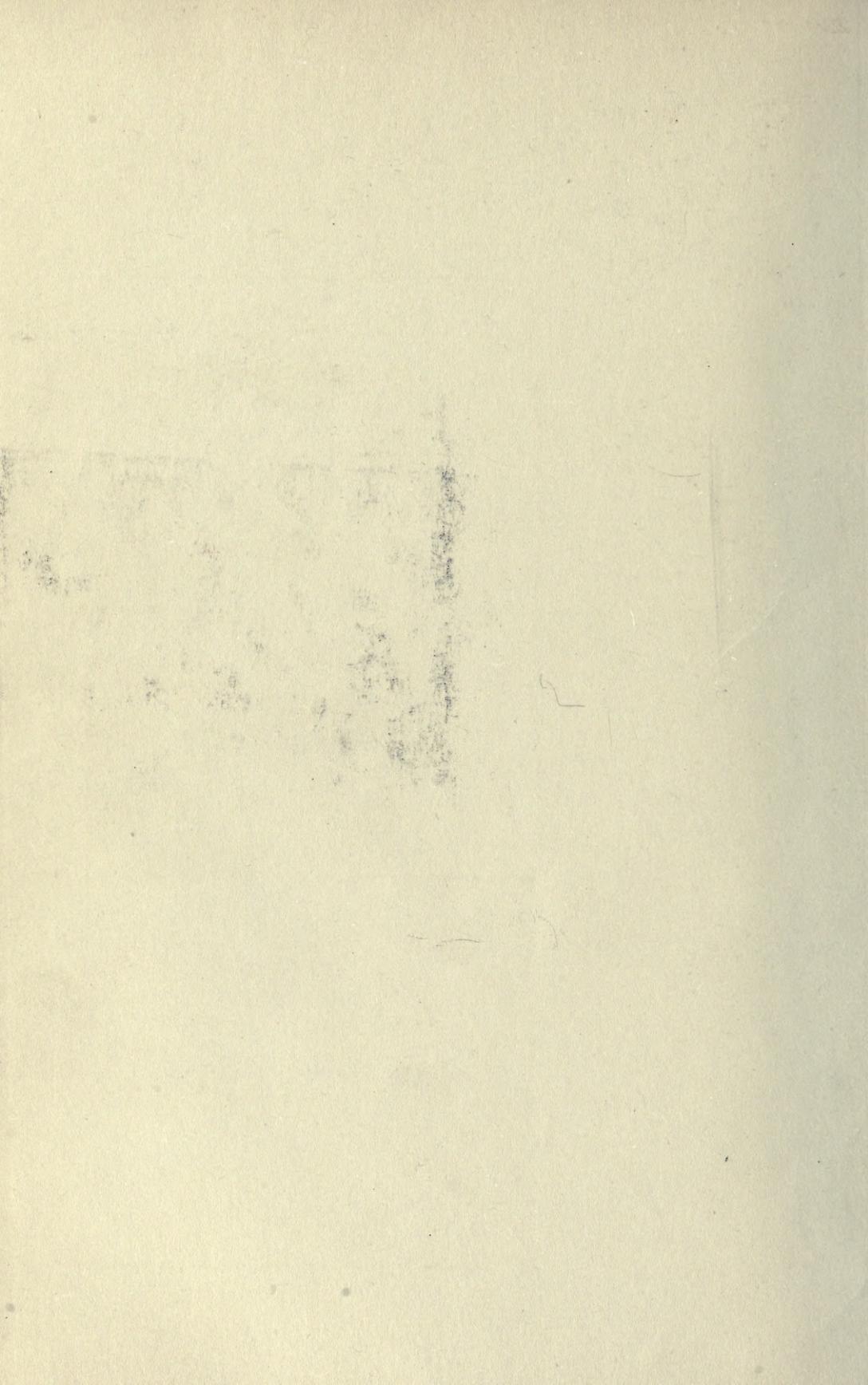
- WIDMARK, E. M. P. Studies in the acetone concentration in blood, urine and alveolar air. III: The elimination of acetone through the lungs 379
- WISHAET, G. M. The existence in the bile of an inhibitor for hepatic esterase, and its nature 406
- YAMAKAMI, K. Barger's microscopical method of determining molecular weights. Part I. The principle of the method with reference to the molecular and ionic attraction of solute for solvent 103
- YAMAKAMI, K. Barger's microscopical method of determining molecular weights. Part II. Its application to caseinogen 522
- Yeast-fat, nature of (MacLean and Thomas) 483
- Yeast, fermentation of, effect of various substances on (Masters and Maughan) 586
- ZILVA, S. S. The action of ozone on the fat-soluble factor in fats 740
- ZILVA, S. S. The extraction of the fat-soluble factor of cabbage and carrot by solvents 494
- ZILVA, S. S. *see also* DRUMMOND, J. C. and HARDEN, A.











BINDING SECT. JUN 10 1964

QP      The Biochemical journal  
501  
B47  
v.14  
cop.4  
Biological  
& Medical  
Serials

PLEASE DO NOT REMOVE  
CARDS OR SLIPS FROM THIS POCKET

---

UNIVERSITY OF TORONTO LIBRARY

---

**STORAGE**

*BRITTLE JOURNAL*

A.  
19/11/91

