UNIVERSAL
LIBRARY

OU_152394

UNIVERSAL
LIBRARY

To the memory of my father

JOHN ALLPORT, M.A. (Cantab.)
1856–1939

His early teaching has been an
abiding help

COLORIMETRIC ANALYSIS

by

NOEL L. ALLPORT
F.R.I.C.

RESEARCH CHEMIST,
THE BRITISH DRUG HOUSES LTD.

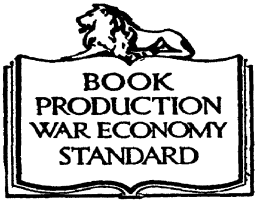
★

Second Impression



LONDON
CHAPMAN & HALL LTD
37 ESSEX STREET WC2
1947

First published, 1945
Second impression, 1947



THIS BOOK IS PRODUCED
IN COMPLETE CONFORMITY
WITH THE AUTHORIZED
ECONOMY STANDARDS

Catalogue No. 231/4

PRINTED IN GREAT BRITAIN BY WILLIAM CLOWES AND SONS, LIMITED
LONDON AND BECCLES.

PREFACE

THIS book has been written with the object of offering a practical account of colorimetric methods of analysis within the compass of a moderate sized volume. In order to achieve this, all discussion of theoretical considerations and of colour measuring instruments has been excluded and attention focussed entirely upon descriptions of the actual chemical technique. So far as I am aware there has not been any book on this subject recently published in this country, the only important works in the English language being volume one of *Photometric Chemical Analysis* by John H. Yoe, and *Methods of Colorimetric Analysis* by Foster Dee Snell and Cornelia T. Snell, both published in the United States. The former bears the date 1928 while the first volume of the latter was issued in 1935 and the second in the following year. The monumental work by F. D. and C. T. Snell clearly aimed at presenting an account of every colorimetric method which had been published to date and, in addition, it includes detailed descriptions and illustrations of many types of colorimeters. So comprehensive a book is necessarily expensive and, moreover, embraces accounts of methods having only restricted value. Therefore it seems reasonable to anticipate that a single volume of moderate size presenting the detailed technique for the more useful colorimetric determinations might be of service. In performing this task I have consistently selected for description only such methods as were definitely known from my practical experience to be reliable or, failing first hand knowledge, were accepted as suitable after consideration of all relevant circumstances. The approach has been critical throughout and, as far as possible, the limitations of the methods have been indicated in the hope that users of this book may be spared the annoyance and waste of time following attempts to conduct colorimetric determinations under conditions to which they are not applicable.

Considering the intentional omissions, it seems reasonable to maintain that questions relating to the theory of colour and colour production, whether treated from a physical, chemical or physiological standpoint, are better discussed in books especially devoted to the problems involved rather than in a treatise concerned with a branch of practical analytical chemistry. With regard to colorimeters, most analysts interested in this work will already possess instruments of their own choice or, if not, all necessary information can be readily

obtained from any laboratory furnisher. Although no systematic attempt has been made to refer to every known colorimetric test, allusions to many not actually described have been included in the monographs while references to others will be found in the sectional introductions. Methods for the colorimetric determination of hydrogen ion concentration are omitted since this subject is more appropriately discussed in books specifically concerned with that branch of chemistry. Descriptions of nephelometric methods have not been included as I hold the view that since they do not depend upon colour reactions, they cannot properly be classified under colorimetric analysis.

The writing of this book was undertaken soon after the Second World War had begun and it was thought that the continuation of hostilities might have retarded development in this branch of science, or at least have delayed the publication of new advances, thus simplifying the task of digesting contemporary material. On the contrary, although latterly it has been difficult to consult the scientific journals of the European continent, the assimilation and checking of new methods published in the United States and Great Britain have demanded considerable vigilance, often entailing the alteration of monographs while in one or two instances it has even been necessary to re-write them.

For guidance to many original papers I have derived considerable help from the books of various authors and in this connection I am particularly indebted to the works by J. H. Yoe and F. D. and C. T. Snell already mentioned. In addition, I have had frequent occasion to consult *Quantitative Clinical Chemistry* by J. P. Peters and D. D. Van Slyke (1932), *Chemical Methods in Clinical Medicine* by G. A. Harrison (2nd edition, 1937), *Recent Advances in Medicine* by G. E. Beaumont and E. C. Dodds (10th edition, 1941), and *The Chemical Analysis of Iron and Steel* by G. E. F. Lundell, J. I. Hoffman and H. A. Bright (1931). Other books from which information has been specifically derived are mentioned under the appropriate references.

My thanks are due to Mr. T. W. Jones, B.Sc., for kindly permitting me to reproduce certain paragraphs from a series of articles written by myself which were published in *The Industrial Chemist* about three years ago. I am particularly indebted to my colleague Mr. John Allen for his enthusiastic help in searching original literature and for much useful counsel in the arrangement of the subject matter. My cordial thanks are also due to Mr. A. H. A. Abbott B.Sc., A.R.I.C. for so generously giving of his time in order to prepare the indexes. In addition, I gratefully acknowledge the help given

to me by Mr. R. A. Finch, B.Sc., in checking the monographs on vitamin A and vitamin E, and I am further indebted to Mr. A. D. W. Smith for preparing the diagrams, and to Dr. D. C. Garratt, B.Sc., F.R.I.C., for his interest in the work at its inception. Finally, I am particularly desirous of recording my thanks to Mr. R. R. Bennett, B.Sc., F.R.I.C., Barrister-at-Law, a Director of The British Drug Houses Ltd. for much kindly encouragement and valuable advice.

N. L. A.

LONDON, *April*, 1944.

CONTENTS

	Page
Preface	v
General Introduction	1

SECTION I. METALS

Introduction	5
Aluminium	7
Antimony	12
Arsenic	16
Beryllium	31
Bismuth	32
Calcium	36
Chromium	38
Cobalt	43
Copper	46
Gold	50
Iron	53
Lead	60
Magnesium	70
Manganese	72
Mercury	81
Molybdenum	87
Nickel	90
Potassium	96
Silver	101
Sodium	102
Thallium	106
Tin	109
Titanium	112
Vanadium	118
Zinc	121

SECTION II. ACID RADICLES

Introduction	127
Benzoic Acid	129
Borates	135
Bromides	137

Page
Cyanides 137
p-Hydroxybenzoic Acid and its Derivatives . . . 140
Lactic Acid 144
Nitrates 145
Nitrites 152
Oxalates 155
Phosphates 155
Salicylic Acid 162
Silicates 165
Sulphates 174
Sulphides 174
Tartrates 178

SECTION III. SUBSTANCES OF CLINICAL AND BIOCHEMICAL SIGNIFICANCE

Introduction 181
Amino-acid Nitrogen (in Blood and Urine) . . . 184
Barbiturates 186
Bilirubin (in Blood Plasma or Serum) 193
Bromides (in Cerebrospinal Fluid and Blood) . . 196
Cholesterol (in Blood). 199
Colloidal Gold Reaction (on Cerebrospinal Fluid) . . 201
Creatine and Creatinine (in Tissues, Urine, Blood, and Meat Extracts) 204
Cystine and Cysteine 211
Ethyl Alcohol 214
Fructose (in Blood and Urine) 216
Glucose (in Blood) 218
Hæmoglobin and Carboxyhæmoglobin (in Blood) . . 226
Histidine (in Urine) 238
Icterus Index (of Blood Plasma or Serum) . . . 241
Lactic Acid (in Blood) 243
Metals (in Organic Material, Body Fluids, and Tissues) . 245
Non-protein Nitrogen (in Blood) 245
Phenols 247
Phenylpyruvic Acid (in Urine) 247
Phosphatase (in Blood Plasma or Serum and in Milk) . 249
Phosphates 260
Proteins (in Blood Serum, Plasma, Urine, Cerebrospinal Fluid, etc.) 260

Page
Pyruvic Acid (in Blood) 267
Silicates 269
Sulphanilamide and other Sulphonamides . . . 269
Sulphates (in Blood) 273
Suramin (in Blood) 275
Tryptophan 277
Urea (in Blood and Urine) 278
Uric Acid (in Blood and Urine) 283

SECTION IV. ALKALOIDS, HORMONES AND VITAMINS

Introduction 287
Atropine, Hyoscyamine, and Hyoscine . . . 292
Diamorphine 298
Ephedrine 299
Ergometrine and Ergotoxine 301
Morphine 310
Pilocarpine 315
Procaine 316
Quinine 318
Strychnine 321
Adrenaline 326
Androsterone (and 17-Ketosteroids) 334
Stilbœstrol 335
Carotene 338
Nicotinic Acid, Nicotinamide, and Trigonelline . . 343
Vitamin A 349
Vitamin B_1 359
Vitamin E 371

SECTION V. MISCELLANEOUS SUBSTANCES

Introduction 375
Acetone 377
Acetylene 378
Ammonia 382
Benzoyl Peroxide (in Flour) 384
Carbon (Combined, in Steel) 386
Carbon Disulphide 388

CONTENTS

	Page
Carbon Tetrachloride	389
Chlorine (Free, in Water)	391
Chloroform	394
Dulcin	395
Ethyl Alcohol	397
Formaldehyde	397
Furfuraldehyde	400
Glyceryl Trinitrate	402
Methyl Alcohol	404
Oxygen (Dissolved, in Water)	406
Phenols (in Water, Urine, and Blood)	408
Rancidity of Fixed Oils (Kreis test)	417
Saccharin	419
Tannin and Gallic Acid	421
Index to Authors	425
Index to Subjects	437

GENERAL INTRODUCTION

This book is devoted to descriptions of procedures in quantitative analysis which depend upon the measurement of the intensity of colour produced by chemical reactions. It cannot be too strongly emphasised that the degree of precision attainable by colorimetric methods is far below that which may generally be expected from determinations based upon gravimetric, volumetric or gasometric principles and it should be realised at the outset that many of the procedures described are subject to errors which may well amount to ± 5 per cent. Usually, however, the consequences of this defect are not serious, since colorimetric methods are essentially micro-chemical and are mainly employed either for the determination of substances present in traces or for the examination of extremely small samples : with the former, these errors are of little significance, while in the latter case they are tolerated by reason of the inherent difficulties involved. Besides lacking precision, colorimetric methods are probably more subject to interference by extraneous substances than procedures based upon other analytical principles and, moreover, the chemical reactions upon which the production of the colours depend are rarely specific. In spite of so many shortcomings colorimetric analysis has proved to be of immense value in all branches of chemical practice and indeed, in our present state of knowledge, it is often the only means of investigation that is available. The large volume of literature devoted to its application in almost every field of scientific research constitutes irrefutable evidence of its utility. In spite of this, there can be no doubt that unfounded claims for the accuracy and specificity of colorimetric methods, so frequently made by their sponsors, has created considerable scepticism in the minds of some analysts who thus tend to deny themselves the advantages which the principle offers.

An attempt has been made in this book to present the whole subject in what the author hopes may be its true perspective. The manner of presentation differs according to circumstances : where the colour test is of general utility the description has been supplemented by detailed procedures for its more important applications, but it has been necessary to modify this plan where the method is only suited to a specific purpose. Most of the monographs describing the tests begin with an historical allusion to the

problem under review together with the reasons dictating the selection of particular tests for description, and every account concludes with a discussion of the possibilities and limitations of the recommended methods. While the grouping of the monographs into five sections has been done to facilitate reference, it sometimes fails as a natural system, as is instanced by the significance attaching to the proportion of silicates present in certain aluminium alloys used in the manufacture of aircraft and also in human lung tissue in cases of suspected silicosis. When this division of interest occurs the descriptive matter is included in the section befitting the more usual application and a heading with cross-reference is included wherever appropriate.

The standards employed in colorimetric analysis may be classified into three types, namely : natural, in which a known weight of the pure substance being determined is submitted to the test in the same manner as the sample under examination ; artificial, in which a standard colour is produced by preparing solutions of known suitable concentrations of other substances designed to give colours closely resembling those produced by the test ; external, which embodies reference of colour intensities to a numerical scale which, when once correlated with any given test, can be employed in lieu of the actual standards. Owing to their convenience, external standards are widely used and usually it is necessary for analysts to make their own calibrations to fit the particular instrument and filters employed and, therefore, it is not generally possible to quote useful figures in accounts of the methods. In this connection the Lovibond tintometer, which is based on a system of permanent external glass standards, may be regarded as an exception since, owing to the uniformity of the standards employed, its practical convenience and its present wide distribution, investigators frequently quote colours in terms of the arbitrary Lovibond units. Therefore, much data referable to the Lovibond scale will be found in the descriptive matter of this book together with details of the standard colour discs issued for use with the Lovibond Comparator and the B.D.H. Lovibond Nessleriser. It should be clearly understood that reliance upon the Lovibond scale as a standard of reference usually leads to some loss of precision, but its deficiencies are offset by the convenience of an instrument which embodies ready-made permanent colour standards which are instantly reproducible in any laboratory equipped with a tintometer. Again, in the case of important control tests in clinical and industrial chemistry, the simplifi-

cation afforded by the use of coloured glass standards for use with the Comparator and Nessleriser is considered to be abundant justification for alluding to them in the text.

The principles of colorimetric analysis have been employed in the quantitative estimation of toxic gases in low concentrations in air and methods, embodying the use of a special hand exhaust pump for measuring samples, were developed at the request of the Home Office by the Chemical Defence Research Department for the Department of Scientific and Industrial Research, acting with the financial and technical co-operation of the Association of British Chemical Manufacturers. The analytical procedures resulting from this work were published in the years 1937 to 1939 by H.M. Stationery Office in a series of leaflets—including, where appropriate, a colour chart—under the general title " Methods for the Detection of Toxic Gases in Industry " whence the details of the tests are readily available. Table I comprises a list of the substances dealt with together with the colorimetric reactions recommended.

In the descriptions of analytical procedure which follow it should be understood that wherever possible reagents of AnalaR or equivalent grade are to be used, and when such a quality is not commercially available the analyst should satisfy himself that impurities capable of disturbing the accuracy of the colorimetric determination are not being introduced. In certain instances where the use of particular chemicals of the highest purity is absolutely essential AnalaR reagents are specified by way of special caution. Unless otherwise stated " water " refers to distilled water free from heavy metals.

The fact that any particular application of a colorimetric method is not discussed in the text should not be taken as implying that the test is unsuited to the purpose. On the contrary, it is hoped that the information included will facilitate the adoption of tests to the various special needs of analysts and research chemists.

TABLE I.—LEAFLETS ON "METHODS FOR THE DETECTION OF TOXIC GASES IN INDUSTRY," PUBLISHED BY H.M. STATIONERY OFFICE

No.	Gas or Vapour	Test Paper Used to Match Colour Chart	Reagents Employed	Standard *
1.	Hydrogen Sulphide	Lead Acetate	—	—
2.	Hydrogen Cyanide	Benzidine - Copper Acetate or Congo Red - Silver Nitrate	—	—
3.	Sulphur Dioxide	Starch - Iodate - Iodide	—	—
4.	Benzene	—	Sulphuric Acid with Formaldehyde	Sodium Nitroprusside (aq. soln.)
5.	Nitrous Fumes	—	α-Naphthylamine and Sulphanilic Acid	Methyl Orange (acidified soln. in acetone)
6.	Carbon Disulphide	—	Diethylamine and Copper Acetate	Natural
7.	Carbon Monoxide	Palladium Chloride	—	—
8.	Phosgene	*p*-Dimethylaminobenzaldehyde - Diphenylamine	—	—
9.	Arsine	Mercuric Chloride	—	—
10.	Chlorine	—	*o*-Tolidine	Potassium Dichromate (aq. soln.)
11.	Aniline	—	Bleaching Powder with Ammonia and Phenol	Toluidine Blue (aq. soln.)

* Permanent external glass standards for use with the Lovibond All-Purposes Comparator are available for all these tests. In addition, there is a similar standard for use in determining the concentration of nitrobenzene in air by the method of N. Strafford and D. A. Harper, *J. Soc. Chem. Ind.*, 1939, **58**, 169.

SECTION I

METALS

INTRODUCTION

Largely owing to the important influences exercised by the presence of certain metals on the growth of living matter, on the suitability of foodstuffs for human and animal consumption, and on the physical properties of alloys, the colorimetric determination of many of these elements has been developed with considerable success. Thus, in the case of substances like arsenic and lead which, besides being highly toxic, are also widely distributed in nature, colorimetric methods have proved to be of great value. In this connection the development of a simple and reliable method for determining traces of mercury would constitute a useful advance : the best at present available is described in the text but the procedure is complicated by the need for preliminary isolation of the metal. More effective colorimetric methods are also needed for magnesium and zinc, the determination of the latter being particularly important in view of its extraordinarily wide distribution and its significance in the metabolism of living organisms.

A multiplicity of procedures are available for the colorimetric determination of copper and iron and proposals for the use of new reagents in this connection continue to appear in periodical scientific literature with almost monotonous regularity. Needless to say no attempt has been made to include these worthless references, much less to describe the methods. Although proposals for the colorimetric determination of cadmium, tungsten and uranium have been published the methods are not reproduced here as the author's trials failed to yield satisfactory results.

After careful consideration it was decided to omit all reference to the platinum group of metals. Perhaps it is true to say that only in the case of palladium has any success attended efforts to apply colorimetric methods to the determination of these elements. In this instance, F. C. Robinson[1] has described a technique

depending upon the yellow to brown colour imparted by palladium to a mixture of nitric and sulphuric acids while Yoe and Overholser have proposed the use of *p*-nitrosodiphenylamine[2] and also *p*-nitrosodimethylaniline[3] as colorimetric reagents for the same metal. Although these tests may well be satisfactory in favourable circumstances they are all disturbed by the presence of the other platinum metals and as preliminary isolation of the palladium is therefore necessary it will generally be more satisfactory to complete the assay gravimetrically. Descriptions of the complicated technique involved in these separations, together with much detailed information concerning the analytical chemistry of many of the rarer elements, has been incorporated into an excellent treatise by Schoeller and Powell[4].

With regard to the general application of colorimetric methods to metallurgical analysis it may be helpful to refer to E. Taylor-Austin's tables of experimental data based upon the use of the Lovibond-Schofield apparatus[5]. Another valuable contribution embodying a large number of actual readings obtained with a Hilger-Spekker photo-electric absorptiometer is available in the form of two published lectures by E. J. Vaughan[6, 7]; these present graphs and readings applicable to defined procedures for the determination of manganese, vanadium, molybdenum chromium and nickel in steel while the analysis of copper and aluminium alloys is also discussed. In connection with this work J. H. High has suggested the use of a neutral filter whereby the frequent re-setting of the absorptiometer is avoided[8].

With reference to the presence, influence and determination of metals in foods and biological materials, bibliographies of the relevant literature from the beginning of the year 1921 have been published from time to time in " The Analyst " and in Table II

TABLE II.—REFERENCES TO THE BIBLIOGRAPHIES OF METALS IN FOODS AND BIOLOGICAL MATERIALS PUBLISHED IN " THE ANALYST "

Metal	Reference	Metal	Reference
Antimony . .	1934, **59**, 109	Lead . . .	1932, **57**, 775
Bismuth . .	1933, **58**, 607	Manganese . .	1933, **58**, 91
Ditto . .	1943, **68**, 115	Ditto . .	1941, **66**, 196
Ditto . .	1943, **68**, 217	Mercury . .	1933, **58**, 280
Cadmium . .	1934, **59**, 109	Nickel . . .	1933, **58**, 340
Chromium . .	1933, **58**, 341	Ditto . .	1940, **65**, 603
Cobalt . . .	1933, **58**, 340	Thallium . .	1934, **59**, 109
Ditto . . .	1940, **65**, 513	Tin . . .	1933, **58**, 398
Copper . .	1932, **57**, 709	Zinc . . .	1933, **58**, 30
Ditto . .	1942, **67**, 293	Ditto . . .	1941, **66**, 452

are given the appropriate references to that journal together with the metals discussed.

REFERENCES

1. F. C. Robinson, *Trans. Inst. Min. Met.*, 1926, **35**, 423.
2. J. H. Yoe and L. G. Overholser, *J. Amer. Chem. Soc.*, 1939, **61**, 2058.
3. L. G. Overholser and J. H. Yoe, *J. Amer. Chem. Soc.*, 1941, **63**, 3224.
4. W. R. Schoeller and A. R. Powell, " The Analysis of Minerals and Ores of the Rarer Elements," 2nd Ed., London : Charles Griffin & Company, Ltd., 1940.
5. E. Taylor-Austin, *J. Soc. Chem. Ind.*, 1941, **60**, 29.
6. E. J. Vaughan, " The Use of the Spekker Photo-Electric Absorptiometer in Metallurgical Analysis," Publication of The Institute of Chemistry : London, 1941.
7. E. J. Vaughan, " Further Advances in the Use of the Spekker Photo-Electric Absorptiometer in Metallurgical Analysis," Publication of The Institute of Chemistry : London, 1942.
8. J. H. High, *Analyst*, 1943, **68**, 78.

ALUMINIUM

Colorimetric methods for the determination of this metal depend upon the use of certain dyestuffs with which aluminium compounds form highly coloured lakes. The first dye to be proposed for this purpose was alizarin S (sodium alizarin monosulphonate)[1] while hæmatoxylin[2], quinalizarin[3] and the triphenylmethane dye eriochrome cyanine[4] have also been recommended. However, the best reagent so far discovered is the ammonium salt of aurine-tricarboxylic acid, now frequently called " aluminon." The use of this compound was originally suggested by Hammett and Sottery[5] and many papers on the test have been published. Since it is superior to all other suggested reagents, only those precedures which involve its use will be described.

Method[6]. To the solution to be tested, which may conveniently occupy a volume of about 30 ml., is added 5 ml. of concentrated hydrochloric acid, 5 ml. of glacial acetic acid and 5 ml. of a 0·2 per cent. aqueous solution of aurine-tricarboxylic acid reagent. After mixing, a concentrated solution of ammonia which has been saturated with ammonium carbonate, is slowly added until the mixture is just alkaline to litmus. The liquid is then rendered acid again by adding 5 ml. of glacial acetic acid and, after allowing to stand for 10 minutes, again neutralised with the ammonia-ammonium carbonate mixture, this time 5 ml. being added in excess. After the liquid has cooled, any pink colour produced is

matched against standards similarly prepared. Within a range from 0·02 to 0·08 mg. Al it is possible to perceive increments of 0·01 mg. A standard solution containing 0·1 mg. Al per ml. may be prepared by dissolving 1·759 gm. of aluminium potassium sulphate in water slightly acidified with sulphuric acid and diluting to 1 litre; this stock solution can then be diluted tenfold immediately before use.

Discussion. The above technique is an improved modification of the original method and is due to Scherrer and Mogerman[6]. Some investigators have advocated the use of external standards but these are inadmissible since stocks of reagent differ in colour intensity. If the sample under examination contains organic material it should be removed by wet oxidation or by incineration, in the latter case the ash being treated with hydrochloric acid to produce the test solution. Iron forms a violet-coloured lake with the reagent and, if present, should be removed either by precipitation with sodium hydroxide or, perhaps more advantageously, by extraction with ether from a solution which is 5N with respect to hydrochloric acid. The presence of phosphates or silicates may lead to low results and it is advisable that the quantity of each in the test should not exceed about 1 mg.; on the other hand, more than that amount of either calcium, magnesium, chromium, nickel, cobalt or manganese will tend to yield high results. Under the conditions of the test, salts of the heavy metals produce white turbidities or precipitates which may disturb the accuracy of the determination of aluminium, but the influence of the rare earths and most other metals except gallium and beryllium is eliminated by the presence of ammonium carbonate in the ammonia used for alkalising. The lake due to gallium dissolves only slowly in ammonium carbonate and, if present in the test solution, may be returned as aluminium. It has been suggested[6] that the interference of beryllium can be overcome by adding zirconium sulphate (up to about 40 times as much zirconium as aluminium suspected of being present) and then co-precipitating from a solution acidified with acetic acid by means of 8-hydroxyquinoline. In this way the beryllium is left in solution and the aluminium can be determined colorimetrically after collecting the precipitate and decomposing it by wet combustion. Provided the amount of zirconium does not exceed 10 mg. its presence is without influence on the accuracy of the test.*

* N. Strafford and P. F. Wyatt (*Analyst*, 1943, **68**, 319) recommend an alternative procedure in which 5 ml. of a 15·6 per cent. aqueous solution of ammonium

Application to Alloys. For the determination of small amounts of aluminium in brass, bronze and bearing metals Lundell and Knowles[7] recommend the following separation :—

About 1 g. of the alloy is dissolved in the minimum quantity of concentrated nitric acid and the mixture rendered alkaline by the addition of 30 ml. of an 8 per cent. aqueous solution of sodium hydroxide. After boiling for about a minute 20 ml. of an 8 per cent. solution of sodium sulphide (made by saturating an 8 per cent. aqueous solution of sodium hydroxide with hydrogen sulphide and diluting with an equal volume of 8 per cent. solution of sodium hydroxide) is added and the mixture passed through a hardened filter paper. The filtrate is acidified with 5N hydrochloric acid, warmed to about 60° C., that temperature being maintained until any precipitate has settled and, after the addition of a little filter paper pulp, the mixture is again filtered, the filtrate evaporated to a volume of about 30 ml. and the colorimetric test applied.

The above separation is liable to lead to loss of aluminium by adsorption and the following electrolytic method[8] is to be preferred :—

An appropriate weight of the sample is dissolved in sulphuric acid, or, if it is necessary to employ other acids to effect solution, the resulting liquid is gently fumed with not more than 5 ml. of

acetate containing 10·8 per cent. of ammonium chloride is added to the solution to be tested followed by 2 ml. of a 0·1 per cent. aqueous solution of ammonium aurine-tricarboxylate. The mixture is heated in a boiling water bath for 15 minutes, cooled to about 12° C., 1 ml. of starch glycerite (prepared by triturating 1 gm. of starch with 20 ml. of glycerin and heating till a clear mixture is obtained) added and sufficient 0·8N ammonium borate to bring the reaction to pH 7·5 ± 0·2. The solution of ammonium borate is prepared by dissolving 93 g. of boric acid in 1 litre of N ammonia, diluting to 0·8N and standardising by titrating 25 ml. diluted with water to 150 ml. with N hydrochloric acid to methyl red. Iron, if present, is removed by preliminary oxidation with bromine in the presence of hydrochloric acid followed by the addition of ammonium thiocyanate and extraction with a mixed solvent consisting of 5 vols. of amyl alcohol and 2 vols. of ethyl ether. The ammonium aurine-tricarboxylate is prepared by a slight modification of the method of J. A. Scherrer and W. H. Smith (*J. Research Natl. Bur. Standards*, 1938, **21**, 113) in which 4 g. of sodium nitrite is added gradually with stirring to 44 ml. of concentrated sulphuric acid then 12 g. of salicylic acid introduced in portions during 10 minutes : after adjusting the temperature of the mixture to between 17° and 19° C., 3·5 ml. of formaldehyde (38 per cent. solution) is added drop by drop with stirring, the agitation being continued for an hour longer and, after allowing to stand for 20 hours, the mixture is poured into 2 litres of water. The dye-acid is washed several times with water then three times alternately with boiling 0·5N hydrochloric acid and water and finally dissolved in excess of ammonia and the solution evaporated to dryness, preferably under reduced pressure.

In the same paper (*Analyst*, 1943, **68**, 319) Strafford and Wyatt describe an improved procedure for the colorimetric determination of aluminium using hæmatoxylin in which the colour of the excess reagent is removed with ammonium borate.

concentrated sulphuric acid. After diluting with water to about 25 ml. and removing any insoluble lead sulphate by filtration, the clear liquid is transferred to a mercury cathode cell. This cell, which is illustrated diagrammatically in Fig. 1, may conveniently comprise a cylindrical glass vessel with a conical base, fitted with a two-way glass stopcock, one arm of which is connected to a levelling bulb by means of rubber tubing, while the other arm is provided with a piece of glass tubing constricted in the same manner as an ordinary burette jet. A piece of platinum wire dipping into this cylindrical vessel forms a suitable anode, while

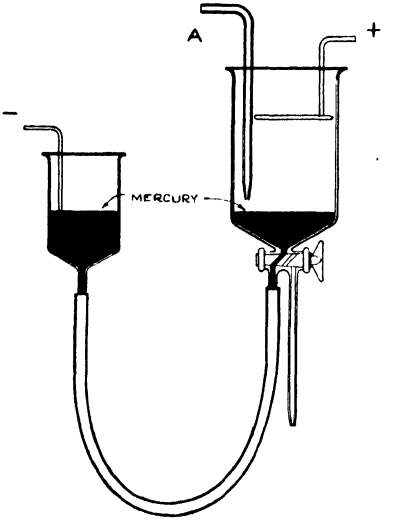


FIG. 1.—MERCURY CATHODE CELL FOR THE SEPARATION OF ALUMINIUM

The glass tube, A, serves for directing air into the solution for the purpose of agitating the liquid when a mechanical stirrer is not available. (After A. D. Melaven, *Ind. Eng. Chem., Anal. Ed.*, 1930, **2**, 180.)

the negative terminal connects with the mercury by means of a copper wire arranged in the levelling bulb. The cell is charged with mercury, so that the anode compartment contains about 50 ml., the stopcock being set to allow of communication between the two vessels ; the solution to be electrolysed is then poured on to the mercury on the anode side and the circuit closed. During the passage of the current the electrolyte should be vigorously agitated with a mechanical stirrer provided with a glass propeller. A current of 3 to 5 ampères passed for 2 or 3 hours will remove reasonable quantities of most metals, while a gram of iron or copper can be deposited completely in 1 hour. When electrolysis

is completed, the levelling bulb is lowered until the mercury falls just to the upper end of the stopcock bore, the tap being then turned through 180 degs. to permit the electrolyte to drain into a suitable vessel. A closed circuit should be maintained during the removal of the mercury, the anode being lowered, if necessary, so that it continues to dip into the electrolyte. This process effectively separates traces of aluminium from most metals, but not from beryllium, vanadium, the alkaline earths or the rare earths. Manganese is not entirely removed, but the small amounts left do not interfere with the subsequent determination of the aluminium.

Application to Food. The preliminary treatment of the sample may consist either in wet oxidation or incineration. The latter has been adopted by Monier-Williams[9] who has proposed a precise but elaborate procedure involving separation of the iron as basic acetate and final determination of the aluminium by means of 8-hydroxyquinoline. In the following method, due to Lampitt and Sylvester[10], wet oxidation is followed by separation of iron as hydroxide and it is recommended as being reasonably simple and sufficiently accurate for all ordinary purposes.

A suitable quantity of the food sample, say 10 g., is transferred to a silica flask and submitted to wet oxidation using about 7 ml. of sulphuric acid and sufficient nitric acid to destroy all organic matter. The cooled acid residue is diluted with 50 ml. of water, care being taken to ensure complete solution of the aluminium sulphate, a precaution that may well be necessary if 100 parts per million or more of aluminium is present in the sample under examination. A slight excess of ammonia is then added, and the solution boiled until the vapours no longer smell of ammonia. This operation is most important : the excess of ammonia must be removed otherwise an appreciable loss of aluminium will occur ; on the other hand, if the boiling is unduly prolonged the solution becomes definitely acid and the precipitated aluminium is redissolved. When the procedure is correctly conducted, the solution becomes neutral to methyl orange. After boiling off the ammonia the solution is filtered through a 9 cm. filter (Whatman No. 41) and washed with 10 ml. of cold water. The reaction of the filtrate should be tested with methyl orange. The precipitate is dissolved from the filter-paper with 15 ml. of hot dilute hydrochloric acid (5 ml. of 5N acid and 10 ml. of water), which is poured slowly, drop by drop, round the upper part of the paper which is then washed in a similar manner with 10 ml. of hot water. The filtrate and the

washings are collected in a silica flask, 10 ml. of 5N sodium hydroxide added, the mixture boiled, allowed to cool and filtered through a hardened 9 cm. paper (Whatman No. 54), the filtrate being collected in a 50 ml. graduated flask. The silica flask and filter paper are washed with about 20 ml. of water and the mixed filtrate and washings diluted to 50 ml. A suitable aliquot part of this solution is neutralised with hydrochloric acid and the colorimetric determination conducted in the manner already described.

REFERENCES

1. F. W. Atack, *J. Soc. Chem. Ind.*, 1915, **34**, 936T.
2. W. D. Hatfield, *Ind. Eng. Chem.*, 1924, **16**, 233.
3. I. M. Kolthoff, *Chem. Weekblad*, 1927, **24**, 447.
4. F. Alten, H. Weiland and E. Knippenberg, *Z. anal. Chem.*, 1934, **96**, 91.
5. L. P. Hammett and C. T. Sottery, *J. Amer. Chem. Soc.*, 1925, **47**, 142.
6. J. A. Scherrer and W. D. Mogerman, *J. Research Natl. Bur. Standards*, 1938, **21**, 105.
7. G. E. F. Lundell and H. B. Knowles, *Ind. Eng. Chem.*, 1926, **18**, 60.
8. A. D. Melaven, *Ind. Eng. Chem., Anal. Ed.*, 1930, **2**, 180.
9. G. W. Monier-Williams, Reports on Public Health and Medical Subjects No. 78 (1935), "Aluminium in Food."
10. L. H. Lampitt and N. D. Sylvester, *Analyst*, 1932, **57**, 418.

ANTIMONY

In order to determine this metal colorimetrically a preliminary isolation will generally be necessary. It was shown by Beam and Freak[1] that antimony could be quantitatively isolated from acid solution by deposition on copper foil after the manner of the Reinsch reaction. B. S. Evans recommended the method for the determination of antimony in copper and brass[2] and in lead[3] and used the sulphide reaction for the final colorimetric comparison. Later, S. G. Clarke introduced a new colorimetric test for antimony using potassium iodide and pyridine[4] which, although less sensitive then the sulphide method, is applicable in the presence of comparable amounts of arsenic and tin and this method was subsequently adopted for the examination of copper and its alloys by Clarke and Evans[5].

Method[4]. A copper strip (about 15×2 cm.) which has been rolled into an open flat spiral and cleaned with 5N nitric acid is introduced into the solution to be tested which should contain the

antimony in about 20 per cent. hydrochloric acid. The latter is boiled gently for 1½ to 2 hours, the solution then poured off, the copper rapidly washed with cold water (the washing must not occupy more than a few seconds) placed in a small beaker, then covered with cold water and about 1 g. of sodium peroxide immediately added. The operations, from the washing of the coil up to the addition of the peroxide, must be done rapidly, otherwise the antimony may become insoluble. After about 5 minutes the beaker is placed on a hot plate, the contents warmed until the copper darkens all over, the liquid then poured off into a flask and the copper and beaker rinsed twice with water, the washings being added to the liquid in the flask which should now contain all the antimony originally present in the test solution together with traces of copper dissolved off the foil. A current of hydrogen sulphide is passed into the solution for about 15 seconds and the mixture allowed to stand on a boiling water-bath until the precipitate of copper sulphide and any bismuth sulphide that may be present has coagulated. The liquid is filtered and the precipitate washed with a dilute solution of ammonium nitrate. To the filtrate is added about 5 ml. of concentrated sulphuric acid and the mixture boiled until white fumes begin to be evolved when a few drops of nitric acid are added and the heating continued until copious fuming occurs ; after allowing to cool the acid solution is diluted with about 15 ml. of water. The following reagents are introduced into a 100 ml. Nessler glass in the order named : 10 ml. of a 1 per cent. aqueous solution of gum acacia, 5 ml. of a 20 per cent. aqueous solution of potassium iodide, 1 ml. of a 10 per cent. aqueous solution of pyridine, 1 ml. of a tenth saturated aqueous solution of sulphur dioxide, 60 ml. of 9N sulphuric acid and finally the diluted acid solution expected to contain antimony as prepared above. The yellow colour produced is matched against a standard prepared by adding a 0·01 per cent. solution of Sb to another Nessler glass containing the same reagents with an additional 20 ml. of 9N sulphuric acid. The standard solution of antimony containing 0·1 mg. Sb per ml. is prepared by dissolving 0·267 g. of antimony potassium tartrate, $KSbO.C_4H_4O_6$, in water, adding 100 ml. of concentrated hydrochloric acid and diluting with water to 1 litre. If more than 1 mg. of Sb is present the colour becomes too deep for accurate matching while a still larger quantity produces a turbidity ; in this case 20 ml. of the coloured reaction mixture is transferred to another Nessler glass containing similar quantities of all the reagents, any turbidity thereupon

disappears and the colour may be matched against a fresh standard.

The solution of gum acacia is conveniently prepared by dissolving 10 g. of the gum in 1 litre of water, boiling in order to destroy oxidases, filtering and preserving with thymol.

Discussion. Bismuth and several of the other heavy metals yield precipitates with the reagent which are generally coloured while zinc gives a white crystalline precipitate ; traces of arsenic and tin produce no colour and do not affect the test. It is noteworthy that antimony in either state of oxidation gives the yellow colour presumably due to the reduction of pentavalent antimony to the trivalent state[4]. The accuracy of the Reinsch method of isolation is dependent upon the presence of chlorides as well as upon the acid concentration. The washing of the copper foil after the deposition of the antimony should be rapid as Clarke has shown[6] that ordinary distilled water will dissolve antimony film, an effect due to the oxygen contained in the water but, provided the washing only occupies a few seconds, it is unnecessary to use boiled water. The solubility of freshly deposited antimony in ordinary distilled water has been confirmed by Grant[7].

Application to Copper and its Alloys[5]. A 5 g. sample is dissolved in 30 ml. of 9N sulphuric acid and 15 ml. of concentrated nitric acid and evaporated until the sulphuric acid fumes strongly. After allowing the residue to cool, 300 ml. of 5N hydrochloric acid and 10 g. of sodium hypophosphite are added and the mixture boiled for 10 minutes. In the absence of any appreciable amount of arsenic the liquid may be treated with copper foil in order to isolate the antimony as described above. If a brownish-black precipitate, due to arsenic, appears during this boiling the liquid must be boiled for a further 20 minutes, cooled slightly and, after the addition of 20 ml. of benzene, well shaken in order to coagulate the precipitated arsenic. The liquid is then filtered through a wet filter paper and the precipitate washed with a minimal quantity of hot water. The colorimetric determination of the antimony is applied to the mixed filtrate and washings commencing with the introduction of the copper foil, as already described.

In the case of a tin bronze, 10 g. of oxalic acid is added before the reduction of the copper with sodium hypophosphite ; any turbidity produced may be ignored.

Application to Tin[4]. A 5 g. sample is treated with 50 ml. of concentrated hydrochloric acid, sufficient bromine being added to

maintain an excess during the reaction, then 10 g. of oxalic acid is added followed by about 350 ml. of water. The mixture is gently boiled, about 0·5 g. of sodium hypophosphite added in order to remove the excess of bromine and the colorimetric determination of the antimony applied to the solution commencing with the introduction of the copper foil as already described. Should an undue amount of arsenic be present as indicated by a black precipitate after the addition of sodium hypophosphite a further 2 g. of the latter should be added and the boiling continued for 15 minutes longer. The liquid is allowed to cool and, after the addition of 20 ml. of benzene, well shaken in order to coagulate the precipitated arsenic, the mixture is then filtered through a wet filter paper, the precipitate washed with a minimal quantity of hot water and the determination of the antimony in the combined filtrate and washings continued as described above.

Application to Lead[3]**.** A 20 g. sample is dissolved in 100 ml. of hot 5N nitric acid, and the solution then diluted with water until all the lead nitrate dissolves and the lead precipitated by the addition of 80 ml. of approximately 9N sulphuric acid. After allowing the mixture to cool, it is filtered through paper pulp and the precipitate washed twice with dilute sulphuric acid. The filtrate and washings are boiled until copious white fumes are evolved, the residue, after allowing to cool, diluted with 100 ml. of water, and 50 ml. of concentrated hydrochloric acid and 5 g. of sodium hypophosphite added. The mixture is boiled for about 15 minutes, allowed to cool, 20 ml. of benzene added, the whole well shaken and filtered through a wet filter which is afterwards washed two or three times with hot water. If any notable amount of arsenic precipitates under this treatment, it may be necessary to add more sodium hypophosphite and repeat the process to ensure all arsenic being removed. The colorimetric determination of the antimony is applied to the filtrate and washings commencing with the introduction of the copper foil as already described above.

Notes on the Applications. Although arsenic does not interfere with the colour test, undue quantities will loosen the antimony film on the copper foil, hence directions for the removal of this element have been included. Evans[8] has pointed out that cuprous chloride has no fundamental inhibitive effect upon the Reinsch reaction, but even traces of cupric chloride adversely affect the deposition of antimony upon copper. It was therefore suggested[5] that reduction of the copper by sodium hypophosphite is more expeditious than any method of actual removal of the metal. It is

essential to remove all nitric acid by fuming with sulphuric acid before applying the Reinsch reaction as it is resistant to the reducing action of sodium hypophosphite and unless removed tends to produce cupric salts. In the presence of cuprous chloride the concentration of hydrochloric acid used in the Reinsch separation is of paramount importance; it was found necessary to employ hydrochloric acid of 5N strength in order to obtain quantitative results.[5]

REFERENCES

1. W. Beam and G. A. Freak, *Analyst*, 1919, **44**, 196.
2. B. S. Evans, *Analyst*, 1922, **47**, 1.
3. B. S. Evans, *Analyst*, 1927, **52**, 565.
4. S. G. Clarke, *Analyst*, 1928, **53**, 373.
5. S. G. Clarke and B. S. Evans, *Analyst*, 1929, **54**, 23.
6. S. G. Clarke, *Analyst*, 1929, **54**, 99.
7. J. Grant, *Analyst*, 1929, **54**, 227.
8. B. S. Evans, *Analyst*, 1923, **48**, 357 and 417.

ARSENIC

The colorimetric method, generally known as the "Gutzeit" test, is now widely employed and it is generally considered to be the most convenient way of determining traces of arsenic. An extensive literature on the test has been published and numerous variations suggested. The alternative colorimetric method, depending upon the reduction of the arsenomolybdate compound has interested various investigators from time to time[1–10], but most of the procedures described are complicated since, in addition to isolating the arsenic in the trivalent state, it is necessary to oxidise to the pentavalent form before the test with ammonium molybdate can be employed. However, a procedure due to Milton and Duffield[11], which is based upon this principle, is free from many of the defects inherent in the earlier proposals and a description of the method as it is applied to organic material is given on p. 29.

In 1879 H. Gutzeit[12] proposed the use of paper impregnated with an acidified solution of silver nitrate as a means of detecting arsine but, before this, Mayençon and Bergeret[13] had suggested the introduction of mercuric chloride paper into the Marsh apparatus. The value of the mercuric chloride test was ultimately recognised, but it was not until C. A. Hill and H. S. Collins[14]

designed and recommended the simple form of apparatus, now familiar to most analysts, that the colorimetric method eventually began to displace the Marsh-Berzelius test; to-day it is established throughout the world as the readiest means of detecting and determining traces of arsenic. The following account of the general test is essentially the same as that given in the British Pharmacopœia, 1932.

Apparatus. A bottle of about 120 ml. capacity with a wide neck is fitted with a rubber bung through which passes a glass tube having a length of 200 mm. and an internal diameter of 6·5 mm. The tube is drawn out at one end to a diameter of about 1 mm. and a hole not less than 2 mm. in diameter is blown in the side of the tube near the constricted part. The tube is passed through the bung fitting the bottle so that, when inserted in the bottle containing 70 ml. of liquid, the constricted end of the tube is

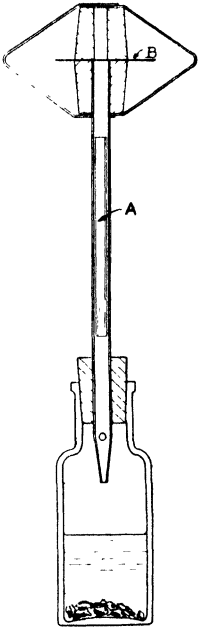


FIG. 2.—THE APPARATUS RECOMMENDED IN THE BRITISH PHARMACOPŒIA FOR DETERMINING TRACES OF ARSENIC

The hole blown in the side near the constriction prevents liquid from rising in the interior of the tube and wetting the lead paper A. The mercuric chloride paper is shown at B. (Modified from the original design of C. A. Hill and H. S. Collins, *Chem. & Drugg.*, 1905, **67**, 548.)

above the surface and the hole in the side is below the bottom of the bung. The upper end of the tube is cut off square, ground smooth and inserted into a rubber bung, provided with a hole 6·5 mm. in diameter which has been bored centrally and true, so that the ground end is flush with the upper and wider end of the bung. Another, exactly similar rubber bung, is placed in apposition to the one fitted to the upper end of the tube and the two are held tightly together with a rubber band or spring clip (Fig. 2). In order to prepare the apparatus for use, a lead paper is rolled round a piece of stout wire and inserted into the glass tube so that the upper end of the paper is not less than 25 mm. below the top of the tube and a mercuric chloride paper fitted between the two rubber bungs which are then secured together so that the borings of the bungs meet to form a true tube of 6·5 mm. diameter interrupted by a diaphragm of paper. The lead papers are made by soaking pieces of thin white filter paper, 100×10 mm., in a 10 per cent. w/v solution of lead acetate in boiled water and drying. The mercuric chloride papers are prepared by soaking pieces of thin white filter paper, not less than 25 mm. in width, in a saturated aqueous solution of mercuric chloride, pressing to remove superfluous moisture and drying in the dark at about 60° C. They should be stored in a stoppered bottle in the dark and kept away from the vapours of ammonia. Since the standard colours are produced on this paper it should conform to the specifications of weight which experience has shown to give the best results. The British Pharmacopœia defines the paper as such that the weight in grams per square metre lies between 65 and 120 and the thickness in millimetres of 400 papers should be " approximately equal, numerically, to the weight in grams per square metre."

General Test. The following reagents will be required and they should be so free from arsenic that no visible stain is produced in control tests :—

1. Concentrated Hydrochloric Acid, approximately 32 per cent. w/w HCl.

2. Solution of Stannous Chloride prepared by treating 20 g. of tin metal with 80 ml. of 6·7N hydrochloric acid (60 ml. of 32 per cent. acid and 20 ml. of water), warming until the reaction ceases, diluting with water to 100 ml., pouring off the solution from undissolved metal, mixing with an equal volume of 32 per cent. hydrochloric acid, boiling down to the original bulk and filtering.

3. Stannated Hydrochloric Acid prepared by adding 1 ml.

of the above solution of stannous chloride to 100 ml. of concentrated hydrochloric acid and mixing.

4. Zinc, Granulated. The analyst should satisfy himself that the zinc used is sensitive and it is therefore important that the same supply of metal should be used for preparing the standard stains as is employed in the actual tests.

5. Solution of Bromine made by dissolving 30 g. of bromine in water containing 30 g. of potassium bromide and diluting the solution with more water to produce 100 ml.

6. Brominated Hydrochloric Acid prepared by adding 1 ml. of the above solution of bromine to 100 ml. of concentrated hydrochloric acid and mixing.

7. Calcium Hydroxide.

The procedure is best explained by first describing the testing of the " solution to be examined " which is, in a practical sense, always the same although the preparation of this final solution, containing any arsenic originally present in the sample, varies considerably according to the circumstances.

The solution to be examined is placed in the bottle of the arsenic testing apparatus, 10 g. of zinc is added, the glass tube immediately placed in position and the reaction allowed to proceed for 40 minutes. Any yellow stain produced on the mercuric chloride paper is compared in daylight with standard stains prepared from known quantities of arsenic. These are conveniently made by introducing into each of four arsenic apparatus bottles 50 ml. water, 10 ml. of stannated hydrochloric acid and 0·2 ml., 0·5 ml., 0·8 ml. and 1 ml. respectively of a solution of arsenic containing 0·01 mg. As_2O_3 per ml. then adding to each 10 g. of zinc, immediately fitting to each of the four glass bottles the prepared glass tubes of the arsenic apparatus and allowing the reaction to proceed for 40 minutes. The standard solution of arsenic is made from a stock solution prepared by dissolving exactly 0·1 g. of arsenic trioxide in a mixture of 50 ml. of concentrated hydrochloric acid and sufficient water to produce 100 ml. ; from this strong standard the solution for use is freshly made by diluting 1 ml. with sufficient water to produce 100 ml. Standard stains are not permanent and should be prepared on the actual day of use.

Preparation of the Solution to be Examined. In the following description it is proposed to present an outline of the official instructions embodied in the British Pharmacopœia for the examina-

tion of certain medicinal chemicals, and therefore it should be clearly understood that the quantities given may need modification when testing commodities belonging to other grades of purity. In all cases the final " solution to be tested " should not contain more than 0·01 mg. of As_2O_3 since stains produced by quantities greater than this are not dependable.

In order to test an inorganic salt such as sodium sulphate, the British Pharmacopœia directs that 5 g. be dissolved in 50 ml. of water and, after adding 10 ml. of stannated hydrochloric acid, the resulting solution be examined as described above. This simple procedure will apply equally to many other freely soluble salts of the alkali metals and of magnesium and calcium. When a salt behaves as a buffer, such as potassium acetate or sodium citrate, 15 ml. of stannated hydrochloric acid should be employed. Certain freely soluble organic substances such as citric acid, lactic acid, sugars and glycols can also be examined directly and the Pharmacopœia prescribes a quantity to be taken for the test (usually 2 g., 5 g., or 10 g.) which best suits the character of the sample and the permissible limit of arsenic. In examining concentrated sulphuric acid only 8 ml. of stannated hydrochloric acid is used for 2 g. of the sample since in this case the substance under test will itself contribute to the reaction with the zinc.

Brominated hydrochloric acid must be employed in the examination of carbonates and substances likely to contain carbonates ; this modification ensures that any arsenic present is oxidised to the non-volatile pentavalent form and is therefore not lost during the evolution of the carbon dioxide. Taking a typical case, the Pharmacopœia directs that 2 g. of calcium carbonate be dissolved in 14 ml. of brominated hydrochloric acid and 45 ml. of water and the excess bromine be removed by adding a few drops of solution of stannous chloride ; the extra hydrochloric acid employed compensates for that neutralised by the substance under test. Calcium hydroxide is treated similarly excepting that 16 ml. of brominated hydrochloric acid is used.

Certain insoluble organic compounds may be prepared for the arsenic test by first igniting with calcium hydroxide and then dissolving the residue in brominated hydrochloric acid. The method, which may be typified by the Pharmacopœial instructions for the examination of acetylsalicylic acid, consists in mixing 5 g. of the organic compound to be tested with 2 g. of calcium hydroxide and 5 ml. of water in a porcelain dish, drying and gently igniting, the residue then being dissolved in 16 ml. of brominated hydrochloric

acid and 45 ml. of water and the excess bromine removed with a few drops of stannous chloride solution. This method is officially prescribed for benzoic acid, salicylic acid and hexamine, while the sodium salts of the acids are ignited without the addition of lime.

The direct method is inapplicable to nitrates and they must be decomposed by heating with a small quantity of sulphuric acid until white fumes are evolved when the acid residue may be tested for arsenic in the normal manner. Powerful reducing agents must be oxidised before the arsenic test can be applied and in the official instructions for the examination of dilute hypophosphorous acid it is directed that 5 g. of the sample be mixed with 1 g. of potassium chlorate, 10 ml. of water and 15 ml. of hydrochloric acid, the mixture allowed to stand for 1 hour, then heated gently to expel chlorine, the remaining trace of the latter being removed with a few drops of stannous chloride solution and the mixture finally diluted with 35 ml. of water.

In the examination of iron compounds it is necessary to remove the arsenic by distillation in the presence of hydrochloric acid with stannous chloride as a reducing agent. This procedure is illustrated by the official method for ferrous sulphate wherein it is directed to dissolve 5 g. in 10 ml. of water and 15 ml. of stannated hydrochloric acid contained in a small flask and then to distil 20 ml. To the distillate is added a few drops of bromine solution to remove any sulphurous acid, the excess of bromine being reduced by the addition of a few drops of stannous chloride solution and, finally, the distillate diluted with 40 ml. of water. Ferric compounds should be completely reduced to the divalent state before distillation by the addition of stannous chloride solution. The examination of copper salts follows on the same lines, sufficient stannous chloride being added prior to distillation to reduce all the copper to the monovalent state.

A similar distillation procedure is needed in the examination of bismuth salts and may be conveniently described by a consideration of the official method for the determination of arsenic present as impurity in bismuth salicylate. In this case it is necessary to destroy the organic radicle by ignition with lime and it is directed that 5 g. of the sample be mixed with 1 g. of calcium hydroxide and 5 ml. of water in a porcelain dish, the mixture dried and ignited and the residue dissolved in 20 ml. of brominated hydrochloric acid and 10 ml. of water. The solution is transferred to a small flask, sufficient stannous chloride is added to remove the excess of bromine and the liquid distilled, 22 ml. of distillate being

collected ; after the addition of a few drops of stannous chloride solution and 40 ml. of water, the resulting solution is examined by the general test in the usual way.

Owing to its use in large doses by intravenous injection in the treatment of certain widely prevalent tropical diseases it is important that tartar emetic should be essentially free from arsenic[15] and an accurate separation can be effected by double distillation. In the official procedure for testing antimony potassium tartrate 1 g. is dissolved in a mixture of 10 ml. of water and 16 ml. of stannated hydrochloric acid, the solution distilled and 20 ml. of distillate collected. After adding 1 drop of stannous chloride solution, the distillate is redistilled, 16 ml. being collected ; this is diluted with 45 ml. of water, 2 drops of stannous chloride solution added and the arsenic determined in the usual manner. The method of double distillation should be applied whenever antimony is present and the above method is applicable to many organic antimony compounds such as stibophen (Fouadin) which is included in the B.P. Third Addendum (1941).

In testing complex organic material, such as food, dyestuffs and toxicological specimens, it is essential to resort to wet oxidation, ignition with fixed alkalies being unsatisfactory in these cases. The weighed sample should be placed in a round-bottomed flask and well charred by heating with slightly diluted nitric acid, then about 7 ml. of concentrated sulphuric acid added and the heating continued, concentrated nitric acid being added from a dropping funnel in the meantime. When all visible carbon has disappeared the addition of nitric acid should be discontinued until white fumes appear ; if at this stage the sulphuric acid darkens in colour the addition of nitric acid should be resumed for a while, the process being repeated until a colourless solution in sulphuric acid is obtained. The dissolved nitrous fumes are then removed by adding water to the cooled sulphuric acid and evaporating again until white fumes appear, the treatment with water being repeated two or three times as necessary. After allowing to cool, the residue of sulphuric acid is diluted with water, 0·05 g. of sodium metabisulphite * added, the solution boiled until free from sulphur

* W. A. Davis and J. G. Maltby (*Analyst*, 1936, **61**, 96) point out that it is most important to add sulphite at this stage in order to reduce the pentavalent arsenic to the trivalent state and the writer has confirmed that when nitric acid is employed in the wet oxidation stannous chloride is inefficient as a reducing agent and it is necessary to apply some other process of reduction before proceeding to the Gutzeit test. In their paper Davis and Maltby refer to the use of " sodium bisulphite " and although this is quite general practice it would appear that the reagent is more correctly termed " sodium metabisulphite." In an investigation

dioxide, a drop of stannous chloride solution added and the test continued in the usual manner. In these circumstances it will not usually be necessary to add hydrochloric acid since the sulphuric acid will provide the necessary reaction with the zinc, but should iron or heavy metals be present in more than traces hydrochloric acid and more stannous chloride must be added, the mixture distilled and the final test applied to the distillate.

In the First Report of the Sub-Committee on the Determination of Arsenic, Lead, and other Poisonous Metals in Food Colouring Materials to the Standing Committee on the Uniformity of Analytical Methods of the Society of Public Analysts[16] it is advised that a distillation process should follow the wet oxidation so as to obtain a distillate containing all the arsenic and no disturbing impurities. The Sub-Committee recommends the addition of 5 g. of a mixture made up in the following proportions—5 g. of sodium chloride, 0·5 g. of hydrazine sulphate, 0·02 g. of potassium bromide—followed immediately by 10 ml. of concentrated hydrochloric acid ; this mixture is then distilled in an all glass apparatus into diluted nitric acid (2 ml. of concentrated acid and 10 ml. of water). When nearly all the hydrochloric acid has distilled over, a further 10 ml. of the same acid is added and the distillation continued into another portion of dilute nitric acid ; the united distillates are evaporated to dryness, water twice added to the residue and each time evaporated off and the final residue dissolved by warming in 3 ml. of concentrated sulphuric acid, the solution allowed to cool, then mixed with water and the colorimetric test for arsenic applied.

Certain substances, particularly basic dyestuffs, give trouble during the oxidation owing to their liability to violent deflagration. In such cases the Sub-Committee recommends treating 5 g. of the sample with 25–30 ml. of 30 per cent. nitric acid, gently warming until the initial vigorous reaction is over, pouring off the acid liquor, washing the residue in the flask with a very little water, pouring off the washing and mixing it with the nitric acid then adding 10 ml. of concentrated sulphuric acid to the charred residue

into the composition and evaluation of the commercially available sodium bisulphite J. B. P. Harrison and M. F. Carroll (*J. Soc. Chem. Ind.*, 1925, **44**, 127T) determined the total sulphur dioxide by adding a weighed sample to an excess of iodine and back titrating with sodium thiosulphate and evaluated the sulphur dioxide existing as metabisulphite by oxidation with hydrogen peroxide and titration of the acid produced. The analytical results obtained were shown to be incompatible with the assumption that the commercial salt is sodium hydrogen sulphite but they did accord with its being sodium metabisulphite, $Na_2S_2O_5$.

in the flask, continuing the oxidation with concentrated nitric acid and returning the poured-off acid liquor after the oxidation has reached the quiescent stage. For many other details designed to aid towards accuracy in the examination of organic substances for arsenic the Report itself should be consulted.

In a paper on the determination of arsenic in foods contaminated by war gases, H. Amphlett Williams[17] deprecates the use of nitric acid in the preliminary wet oxidation and advises digestion with a mixture consisting of 10 g. of potassium sulphate, 2 ml. of a 10 per cent. aqueous solution of copper sulphate, $CuSO_4.5H_2O$, and 20 ml. of concentrated sulphuric acid, or sufficient acid to allow 8 ml. for each gram of dry food in the portion taken. This mixture is heated until a clear liquid is obtained, the acid solution is allowed to cool, diluted with about 70 ml. of water, boiled until free from any odour of sulphur dioxide, the liquid cooled, diluted to 100 ml. and suitable aliquot portions examined by the usual Gutzeit test after adding 0·5 ml. of stannous chloride solution. In order to prevent acid being sprayed through the folded lead paper on to the mercuric chloride paper consequent upon the increased rate of hydrogen evolution due to the presence of copper sulphate, a closely packed pad of asbestos wool is inserted just below the lead paper. In the absence of chlorides this method gives satisfactory results but common salt, in quantities exceeding 0·05 g. leads to loss of arsenic. Owing to the wide distribution of sodium chloride, particularly in food, the procedure cannot be regarded as suitable for general use. In the writer's hands the method worked well, the wet oxidation, although occupying more time than the nitric-sulphuric acid method, required no manipulative attention and the final stains were highly satisfactory. Unfortunately, however, the presence of even 0·02 g. of sodium chloride resulted in a definite diminution in the intensity of stains.

Taylor and Hamence[18] recommend the use of granulated zinc that has been alloyed with 0·3 per cent. of copper in order to ensure the liberation of all the arsenic in the form of arsine. The resulting stains as obtained on mercuric chloride paper are slightly less intense than those produced when using pure zinc, but these investigators counteract this disadvantage by employing papers which have been soaked in a 5 per cent. alcoholic solution of mercuric bromide for 1 hour and then dried. In this way stains are obtained which are more intense than those resulting from application of the ordinary method and it is suggested that they are more easily assessed.

The Electrolytic Method. So long ago as 1861 C. L. Bloxam[19] proposed that the generation of the hydrogen arsenide might be effected by electrolysis using platinum electrodes. Subsequent investigators experimented with the method, but it was not until Sand and Hackford[20] suggested the use of lead electrodes that this principle was satisfactorily applied to the Marsh-Berzelius test. When using platinum electrodes the main difficulty had been the failure to effect reduction of the arsenic since, according to Sand and Hackford, " the reduction of very small quantities of arsenate to arsenite probably requires a large supertension,* for this was only accomplished by the metals having a large supertension, such as lead, zinc, and also by iron in the form of wire." The design of apparatus underwent modification in the hands of various investigators and the following is a description of that proposed by N. Evers[21] which in turn is an adaptation to the colorimetric method of that suggested by Monier-Williams[22] for use in the Marsh-Berzelius test.

The apparatus (Fig. 3) comprises a lead beaker connected by a wire to the positive pole of the current source, which accommodates a thin porous pot in which stands a glass vessel that is open at the bottom and rests on the floor of the porous pot. The upper part of the glass vessel is shaped like a two-necked Woulff's bottle, one neck of which is fitted with a rubber bung through which passes a glass rod. This rod is covered with a piece of lead foil, the latter being attached by first cutting it to an approximate L shape (A in Fig. 3), disposing the rod along the edge marked *b* and winding the foil around the rod leaving the residue of the horizontal limb to partly line the interior of the glass vessel in its lower portion while the foil is fixed to the rod by means of the rubber bung. A wire from the negative terminal of the current source is attached with a locking-nut to the foil covering that part of the rod above the rubber. A tube about 80 mm. long, filled with non-absorbent cotton wool, is attached to the other neck of the glass vessel by means of a ground glass connection and to the top end of this is joined a second tube filled with plumbised cotton wool to within 4 mm. of the end, a small plug of absorbent wool being inserted at the top. A suitable device for

* The supertension (now termed overvoltage) of an electrode is the excess of electromotive force necessary for the liberation of hydrogen at that electrode over the electromotive force required for the reversible production of hydrogen on a cathode of platinised platinum. According to W. A. Caspari (*Z. physik. Chem.*, 1899, **30**, 89) the supertension in volts of the most important metals are in the following order : platinised platinum 0·005 ; smooth platinum 0·09 ; nickel 0·21 ; copper 0·23 ; tin 0·53 ; lead 0·64 ; zinc 0·70 ; mercury 0·78.

holding the mercuric chloride paper such as has already been described is affixed at the extreme end of this tube. Although it is not mentioned in the original paper there would not seem to be any objection to this upper tube being made in accordance with the dimensions of the official Pharmacopœial apparatus so that lead papers could be employed in place of plumbised cotton wool.

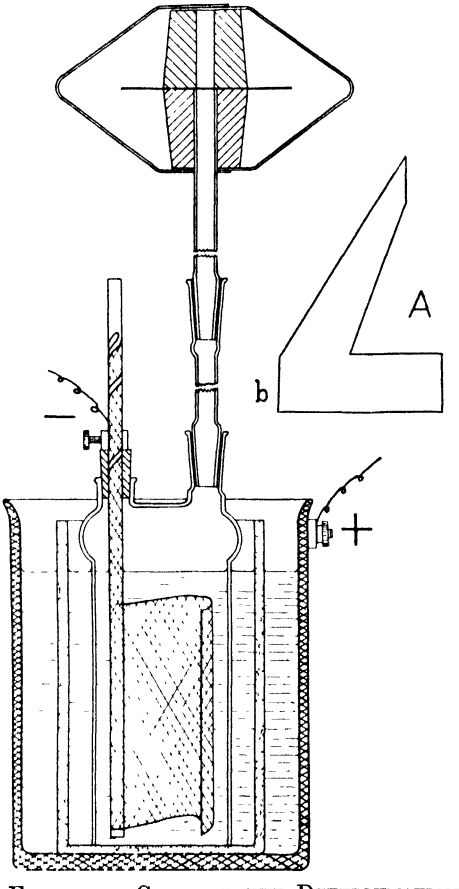


FIG. 3.—LEAD ELECTRODE CELL FOR THE DETERMINATION OF MINUTE QUANTITIES OF ARSENIC

The shape of the foil serving as the cathode is shown at A. (After N. Evers, *Yearb. Pharm.*, 1926, 540.)

Sulphuric acid of 15 to 20 per cent. by weight, containing 0·025 per cent. of cadmium sulphate, is used as the conducting liquid. The test is carried out by dissolving or mixing the required weight of substance in 35 ml. of the cadmiumated sulphuric acid. The liquid is poured into the porous pot, and

sufficient of the cadmiumated sulphuric acid is introduced into the outer vessel so that the level of the liquids is the same. The glass apparatus with the mercuric chloride paper in position is then placed inside the porous pot. A direct current of 3 to 6 ampères is passed, the potential difference between the electrodes being from 7 to 9 volts. The evolution of arsenic is generally complete after 30 minutes, but if a stain is obtained the current should be allowed to flow for a further 30 minutes. The cathode must be kept clean in order to preserve its sensitivity and from time to time the film of cadmium should be scraped off. Reversing the current will ruin the cathode. If a second test is put on immediately following a previous run, full sensitivity cannot be assured but the cathode regains its activity after standing in water. An insensitive cathode can be fully restored by washing in water, standing in very dilute nitric acid, rubbing off any deposit with cotton wool, standing in a dilute solution of cadmium sulphate and finally washing in distilled water.

The evolution of halogens during the electrolysis prevents the direct application of the electrolytic method to halogen salts. This may be overcome by using in the outer chamber (between the porous pot and the lead beaker) a 10 per cent. solution of sodium thiosulphate instead of the cadmiumated sulphuric acid. This absorbs the halogen and it is thus possible to obtain satisfactory results ; the slight precipitation of sulphur in the outer chamber can be readily washed out at the conclusion of the test.

The lead cathode was criticised by Aumonier[23], mainly on the ground of its uncertain sensitivity, and he proposed an apparatus employing a mercury cathode cell, the porous pot being impregnated with montan wax except for an annulus in contact with the conducting liquid. Later, T. Callan and R. T. Parry Jones[24] designed an improved and simplified model in which the porous pot is replaced by parchmentised paper. The cell consists of a flat-bottomed glass tube, 5 in. long and about $1\frac{3}{8}$ in. in bore having an aperture approximately 1 in. in diameter blown in the side $\frac{1}{4}$ in. from the bottom of the tube as shown in Fig. 4. The aperture is covered by a piece of parchmentised paper (prepared by rapidly immersing No. 1 Whatman filter paper in pure 80 per cent. sulphuric acid for a few seconds and then washing with water and storing under water until required) which acts as an efficient diaphragm of very low electrical resistance between the anolyte and the catholyte. The aperture is flanged, as indicated in the diagram, in order that the parchment may be held tightly in

position by means of a rubber band. Mercury, previously purified by spraying through a long column of dilute nitric acid, is placed in the bottom of the tube, so that its surface is just below the level of the aperture in the side of the tube. Electrical

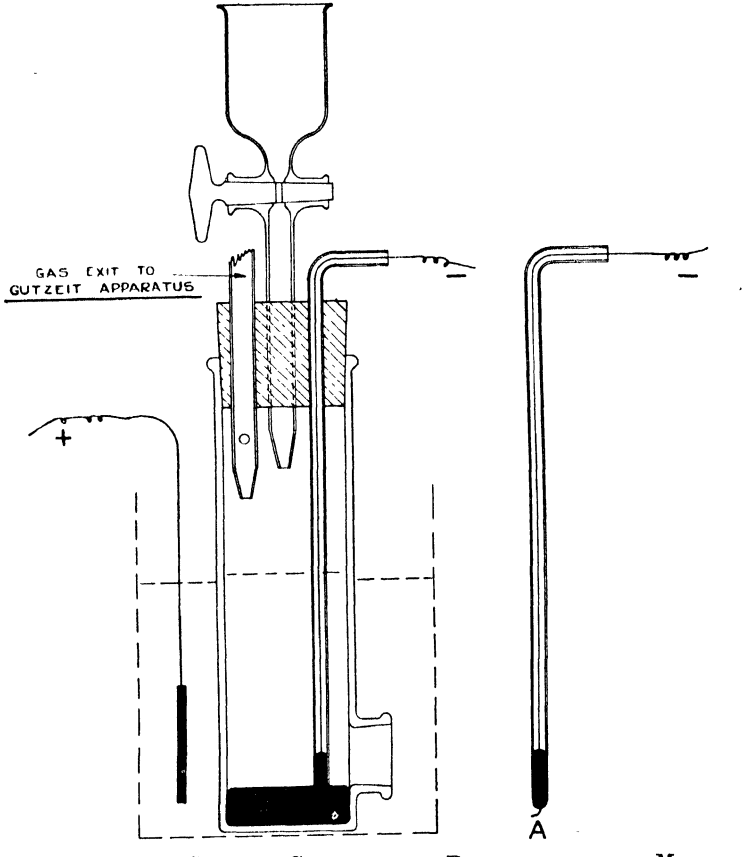


FIG. 4.—MERCURY CATHODE CELL FOR THE DETERMINATION OF MINUTE QUANTITIES OF ARSENIC

In addition to the above simple cell with rubber bung, which is fully described in the text, two all glass types have been recommended, the one with a separate unit carrying the tap funnel and attached to the cell reservoir by a ground glass joint while in the other design the cell is constructed all in one piece. (After T. Callan and R. T. Parry Jones, *Analyst*, 1930, **55**, 90.)

connection is made with the mercury by means of a narrow glass tube, into the lower end of which is sealed a small piece of platinum wire as shown at A in Fig. 4. The whole of the exposed portion of the platinum wire dips below the surface of the mercury in the cell. A length of copper wire, dipping into this tube and making

contact with the end of the platinum wire by means of a short column of mercury, forms the negative connection of the apparatus. The narrow tube, carrying the cathode lead, passes through a rubber stopper which closes the upper end of the cathode cell. This rubber stopper carries a glass tap funnel, through which the electrolyte or test solution may be introduced, and also an exit tube having a small side hole near the bottom and connecting directly with the Gutzeit arsenic detector. The cathode cell is immersed in the electrolyte of the anode cell, which is itself surrounded by a cooling bath kept constantly at about room temperature. The anode consists of a sheet of platinum foil (about 2 in. × 1 in.) welded to a short length of stout platinum wire. A 20 per cent. w/v aqueous solution of sulphuric acid is employed as electrolyte and a suitable current is 2 ampères for 15 minutes followed by 4 ampères for a further 15 minutes. It is claimed that when used in conjunction with mercuric chloride paper, stains are sharply defined, of good colour and equal in intensity to those given using a lead cathode in its most sensitive condition.

Arsenomolybdate Method of Milton and Duffield[11]. The sulphuric acid residue resulting from the wet oxidation of the sample is diluted to about 100 ml. with water, 1 ml. of 50 per cent. aqueous solution of potassium iodide and 1 ml. of 40 per cent. stannous chloride in concentrated hydrochloric acid added and the mixture heated to boiling. The resulting arsenite solution is made up to a known volume, an aliquot portion (expected to contain not more than 0·1 mg. As_2O_3) transferred to the bottle A (Fig. 5), 2 ml. of concentrated sulphuric acid added followed by 10 ml. of concentrated hydrochloric acid, 1 ml. of 40 per cent. solution of stannous chloride, sufficient water to bring the volume of the mixture to about 50 ml. and, finally, 5 g. of zinc shot. The rubber stoppers are quickly pressed home and the evolved gases passed into a mixture of 5 ml. of 0·02N iodine and 1 ml. of N sodium bicarbonate contained in tube C. After 30 minutes tube C is removed and to its contents is added 2 ml. of an acid molybdate solution (made by mixing equal volumes of exactly 13N sulphuric acid and a 9·5 per cent. aqueous solution of sodium molybdate, $Na_2MoO_4.2H_2O$) and then 0·1 ml. of a 5 per cent. aqueous solution of sodium metabisulphate (free from iron). To the resulting colourless solution is added 0·9 ml. of water and 1 ml. of a 0·2 per cent. solution of stannous chloride freshly made by appropriately diluting the 40 per cent. solution with water. The intensity of

the blue colour, which fully develops in 5 minutes and is thereafter stable for some hours, is measured by means of a Hilger-Spekker absorptiometer using the red filter No. 2. A blank determination on the reagents employed is also conducted and the corresponding absorptiometer reading deducted from that obtained for the sample. Any form of colorimeter may be used provided allow-

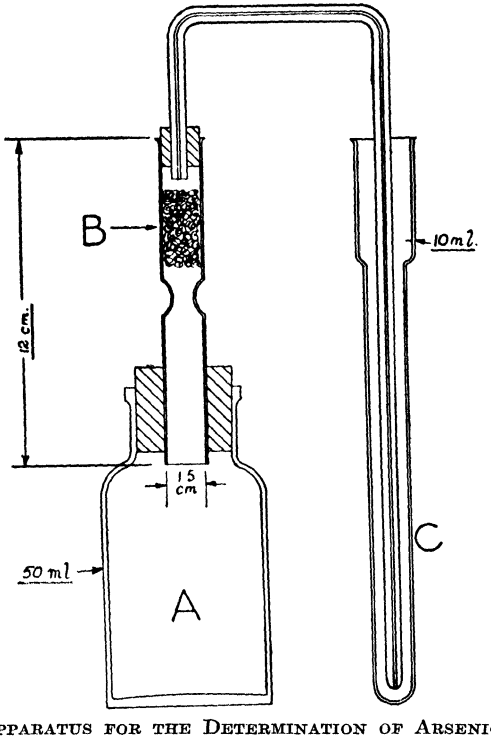


FIG. 5.—APPARATUS FOR THE DETERMINATION OF ARSENIC BY THE ARSENOMOLYBDATE METHOD

Arsine is generated in the bottle A, freed from hydrogen sulphide by passing over glass wool soaked in lead acetate solution at B and thence passed into the gas absorption tube C, containing a solution of iodine and sodium bicarbonate, by means of a capillary tube of 4 mm. external and 0·5 mm. internal diameter. (After R. F. Milton and W. D. Duffield, *Analyst*, 1942, **67**, 279.)

ance is made for colour produced by the blank. The effective range for colorimetric determinations extends from 0·001 to 0·1 mg. As_2O_3.

REFERENCES

1. G. Denigès, *Compt. rend.*, 1920, **171**, 802.
2. T. Kuttner and H. R. Cohen, *J. Biol. Chem.*, 1927, **75**, 517.
3. E. Truog and A. H. Meyer, *Ind. Eng. Chem., Anal. Ed.*, 1929, **1**, 136.
4. E. H. Maechling and F. B. Flinn, *J. Lab. Clin. Med.*, 1930, **15**, 779.
5. C. Zinzadze, *Ann. Agron.* (*N.S.*), 1931, **1**, 321.

6. R. B. Deemer and J. A. Schricker, *J. Assoc. Off. Agr. Chem.*, 1933, **16**, 226.
7. C. Zinzadze, *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 230.
8. H. J. Morris and H. O. Calvery, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 447.
9. A. L. Chaney and H. J. Magnuson, *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 691.
10. M. B. Jacobs and J. Nagler, *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 442.
11. R. F. Milton and W. D. Duffield, *Analyst*, 1942, **67**, 279.
12. H. Gutzeit, *Pharm. Ztg.*, 1879, **24**, 263.
13. — Mayençon and — Bergeret, *Compt. rend.*, 1874, **79**, 118.
14. C. A. Hill and H. S. Collins, *Chem. & Drugg.*, 1905, **67**, 548.
15. R. Griffiths Jones, *Lancet*, 1926, **210**, 194.
16. *Analyst*, 1930, **55**, 102.
17. H. Amphlett Williams, *Analyst*, 1941, **66**, 228.
18. G. Taylor and J. H. Hamence, *Analyst*, 1942, **67**, 12.
19. C. L. Bloxam, *Quart. J. Chem. Soc.*, 1861, **13**, 12 and 338.
20. H. J. S. Sand and J. E. Hackford, *J. Chem. Soc.*, 1904, **85**, 1018.
21. N. Evers, *Yearb. Pharm.*, 1926, 540.
22. G. W. Monier-Williams, *Analyst*, 1923, **48**, 112.
23. F. S. Aumonier, *J. Soc. Chem. Ind.*, 1927, **46**, 341T.
24. T. Callan and R. T. Parry Jones, *Analyst*, 1930, **55**, 90.

BERYLLIUM

A method for the colorimetric determination of this metal using quinalizarin (1:2:5:8-tetrahydroxy-anthraquinone) has been proposed[1] but it is subject to severe limitations. The determination relies upon the principle of matching tints and not on a comparison of the same tint at varying intensities.

Method[1]. Two standard solutions are required : (1) pure beryllium nitrate in 0·25N sodium hydroxide of such strength that 1 ml. contains 0·1 mg. BeO ; and (2) a freshly prepared 0·05 per cent. solution of quinializarin in 0·25N sodium hydroxide. A portion, say 10 ml., of this dye solution, 1 ml. of which is theoretically equivalent to 0·0332 mg. Be, is diluted twentyfold with 0·25N sodium hydroxide to form solution A. Another solution, B, is similarly prepared but an excess of the standard beryllium solution (1) is also included so that the maximum colour is developed. This should be a pure blue in contrast to the purple tint of solution A. The standard beryllium solution (1) is now added in small quantities to solution A and a sample withdrawn after each addition and compared in a colorimeter with a sample of solution B, the sample from A being returned to the bulk before each addition of standard beryllium solution. The operations

are repeated until the blue tint of B is matched by that of A. The unknown solution, which should be 0·25N with respect to sodium hydroxide, is tested by being added to another 10 ml. portion of standard dye solution (2) and the mixture made up to 200 ml. with 0·25N alkali. This solution, C, is matched against B in precisely the same manner as A. The volume of standard beryllium solution (1) added to C will be less than that added to A by an amount corresponding to the quantity of beryllium present in the unknown.

Discussion. The above procedure is not applicable in the presence of magnesium, zirconium, cerium, thorium or some of the rare earth metals. According to Fischer[1] determinations of beryllium are possible in the presence of relatively large quantities of aluminium provided the latter is included in the standard solution and sufficient sodium hydroxide is added to redissolve the precipitate of alumina.

In order to determine beryllium in beryl, 0·2 g. of the finely powdered mineral is fused with sodium carbonate, the melt taken up with hydrochloric acid, the silica rendered insoluble by evaporation, the residue treated with dilute acid, filtered, the filtrate made up to 200 ml. with alkali and portions tested as described above. If ferric hydroxide is present in the final solution it is allowed to settle and the clear liquid used for the determination.

To determine beryllium present in alloys of copper or nickel, 0·1 g. of sawings is dissolved in a mixture of cold hydrochloric acid and hydrogen peroxide, the solution evaporated to dryness on a boiling water-bath and the residue dissolved in a little water. The solution is treated with potassium cyanide until the precipitate first formed has redissolved and the liquid is colourless and it is then diluted in a graduated flask with 0·25N sodium hydroxide and an aliquot portion assayed colorimetrically.

REFERENCE

1. H. Fischer, *Z. anal. Chem.*, 1928, **73**, 54.

BISMUTH

The yellow colour produced by the reaction between bismuth salts and potassium iodide in acid solution[1] forms the basis of the most satisfactory colorimetric method for the determination of

this metal. The various suggestions to increase the sensitivity of the test by the introduction of quinine[2–5] or cinchonine[6] do not appear to yield consistent results. With acid solutions containing bismuth, thiourea produces a yellow colour[7] and the reaction has been applied quantitatively[8, 9] but, as the method is less sensitive than the potassium iodide test, is similarly disturbed by the presence of other metals, and since it is essential to work in dilute nitric acid of precise concentration, it will not be described.

Method. The solution under examination is transferred to a Nessler glass, 3 ml. of 5N sulphuric acid added, and the mixture diluted to 50 ml. To this is added 2 ml. of a 10 per cent. aqueous solution of potassium iodide and 0·2 ml. of dilute sulphurous acid (containing 0·5 per cent. SO_2). Any yellow colour produced is matched against standards similarly prepared. A standard bismuth solution containing 0·05 mg. Bi per ml. is prepared by dissolving 0·05 g. of the pure metal in 10 ml. of concentrated sulphuric acid and diluting to 1 litre with water. Satisfactory comparisons can be made within the range 0·01 to 0·2 mg. Bi.

Method Using Permanent External Standards. The test can be applied with the aid of the B.D.H. Lovibond Nessleriser for which two discs of standards are available, the one covering the range from 0·01 to 0·05 mg. Bi while the other contains stronger colours equivalent to quantities up to 0·2 mg. Bi. The method adopted for their standardisation is precisely that given above.

Discussion. Under the conditions of the test many substances form precipitates or liberate free iodine, producing yellow solutions ; consequently, it is necessary to exercise particular care to ensure that no interfering substances are present. In general, chlorides, nitrates, aluminium, magnesium, zinc and small quantities of copper, lead, ferrous iron, manganese, arsenic and cadmium do not disturb the accuracy of the determination. Silver, tin, antimony, mercury, nitrites and oxidising agents must be absent. The presence of sulphurous acid ensures that the yellow colour is not wholly, or in part, due to free iodine liberated by the presence of an oxidising agent.

This test is applied as the final stage in the determination of traces of bismuth liable to be present in copper. The procedure is an important one owing the profound influence which even minute amounts of bismuth exercise upon the electrical properties of copper and the method recommended by the Fiscal Policy Joint Committee, Brass, Copper and Nickel Silver Industries[10] is given below.

Application to Copper.[10] For samples which may contain up to 0·005 per cent. of bismuth 2 g. of drillings is dissolved in 20 ml. of nitric acid (50 per cent.) contained in a 250-ml. round-bottomed flask, the solution boiled until it is viscous, 3 ml. of concentrated sulphuric acid then added and the heating continued until white fumes are freely evolved. After allowing to cool, 30 ml. of distilled water and 1 g. of citric acid, previously dissolved in 20 ml. of water, are added and the mixture rendered slightly alkaline with ammonia (s.g. 0·880). The cooled liquid is transferred to a separator, 50 ml. of a freshly prepared 20 per cent. aqueous solution of potassium cyanide added, the mixture diluted to about 200 ml. with water, and the bismuth extracted by shaking with three successive 10 ml. portions of a fresh 0·1 per cent. chloroformic solution of diphenylthiocarbazone. The chloroformic extracts are washed with 50 ml. of water contained in a second separator and transferred to a convenient flask or beaker, nearly all the solvent evaporated, 1 ml. of concentrated sulphuric acid added, the flask or beaker heated over a flame until white fumes are evolved and the organic matter oxidised by the cautious addition of 30 per cent. hydrogen peroxide. Heating is continued until the acid is colourless and white fumes are again evolved. After allowing to cool, 15 ml. of water is added, any precipitated lead sulphate filtered off, the solution transferred to a 50 ml. Nessler glass, 10 ml. of a 2 per cent. aqueous solution of potassium iodide added, dilute sulphurous acid (containing 0·5 per cent. SO_2) introduced drop by drop until any colour due to free iodine is removed, 1 drop being added in excess, and the volume made up to 30 ml. A blank solution is prepared by adding to a second Nessler glass 10 ml. of dilute sulphuric acid (10 per cent.), 5 ml. of water and volumes of the other reagents similar to those used in the assay solution. The yellow colour of the latter is then matched by adding a standard bismuth solution (0·05 g. of metal dissolved in 10 ml. of concentrated sulphuric acid and diluted to 1 litre with water) to the control.

Notes on the Application to Copper. Silver, nickel, arsenic and phosphorus do not interfere with the method. If tin is present it will not dissolve during the initial treatment with nitric acid and, in this case, the metastannic acid should be filtered off, ignited, fused with potassium bisulphate, the fused mass leeched out with water and the aqueous extract combined with the original solution of copper. Generally, three extractions with diphenylthiocarbazone solution are sufficient to remove all bis-

muth but should there be any doubt, owing to the third extract still exhibiting a colour change (which may be due to zinc), further extractions should be made and separately examined for bismuth. If a precipitate of lead sulphate is obtained the filter paper containing it should be ignited, the residue dissolved in 10 ml. of hot concentrated hydrochloric acid and to the cooled solution 1 g. of tartaric acid, 1 ml. of concentrated nitric acid and 1 ml. of concentrated sulphuric acid added, the mixture then cautiously heated until the volatile acids have been expelled and the residue becomes quiescent. After diluting with 15 ml. of water the solution is filtered and the filtrate combined with the main solution of bismuth which is to be submitted to the colorimetric determination.

Where the content of bismuth is less than 0·002 per cent. a larger sample should be treated and, similarly, should the concentration of bismuth exceed 0·005 per cent. an aliquot part of the initial solution in nitric acid should be taken for the assay.

This method is similar to one suggested by Haddock[11] who, however, has modified the colour test by including 2 ml. of a 30 per cent. solution of hypophosphorous acid, added after the sulphurous acid and before the potassium iodide, the latter being introduced last of all.

This method of isolating bismuth will be applicable in many other connections and particularly in the examination of organic material. The quantity of potassium cyanide used in the above procedure is sufficient to combine with 2 g. of copper but in cases where only traces of other metals are present it is only necessary to employ a few drops of this reagent.

Rapid Method for Gunmetal, Brass and Bronze[12]. About 2 g. of the sample contained in a tall 250 ml. beaker is dissolved by gentle heating in a mixture of 14 ml. of 5N hydrochloric acid and 6 ml. of nitric acid (s.g. 1·2, made by adding 100 ml. of acid s.g. 1·42 to 155 ml. of water). When the solution is complete it is made alkaline with approximately N ammonia, then the mixture is rendered clear by the addition of 9N sulphuric acid and 10 ml. of the acid is added in excess. After allowing to cool, sufficient 60 per cent. aqueous solution of potassium iodide is introduced to precipitate all the copper. (For 1 g. of copper 6 g. of potassium iodide is required.) A solution of 5 g. of sodium hypophosphite dissolved in 20 ml. of water is added, the mixture allowed to stand for 10 minutes, or longer if necessary, until completely bleached, then diluted to 200 ml. with water and 100 ml.

filtered into a Nessler glass. If any bismuth is present, it will be seen as the yellow iodide. Into another Nessler glass is introduced 5 ml. of 9N sulphuric acid, 1 g. of potassium iodide, 1 g. of sodium hypophosphite and sufficient water to bring the volume of the liquid to within a few ml. of the graduation mark ; standard bismuth solution (made as described in the section dealing with the general method but containing 0·1 mg. Bi per ml.) is then added until the colour matches that in the first Nessler glass after the volume of the two solutions has been equalised.

Notes on the Rapid Method. The use of sodium hypophosphite to reduce the iodine liberated from the potassium iodide by the copper is the particular feature of this method, which was evolved by H. R. Fitter[12]. If sodium thiosulphate is employed for this purpose sulphur separates in the final matching solution while if sulphurous acid be used the excess sulphur dioxide reacts with the potassium iodide to form a colour similar to that of bismuth iodide. The method is valuable as a sorting test for samples of gunmetal, some of the specifications for which demand a bismuth content of less than 0·01 per cent. Iron, arsenic, manganese and small amounts of lead and antimony do not interfere.

REFERENCES

1. F. B. Stone, *J. Soc. Chem. Ind.*, 1887, **6**, 416.
2. P. Aubrey, *J. Pharm. Chim.*, 1922, [vii], **25**, 15.
3. L. Cuny and G. Poirot, *J. Pharm. Chim.*, 1923, [vii], **28**, 215.
4. G. Ciogolea, *Bul. Soc. Chim. România*, 1928, **10**, 55.
5. C. Laporte, *J. Pharm. Chim.*, 1923, [vii], **28**, 304.
6. F. Feigl and F. Neuber, *Z. anal. Chem.*, 1923, **62**, 369.
7. K. A. Hofmann and K. L. Gonder, *Ber.*, 1904, **37**, 242.
8. C. Mahr, *Z. anal. Chem.*, 1933, **94**, 161.
9. S. L. Tompsett, *Analyst*, 1938, **63**, 250.
10. Report of The Chemical Sub-Committee, Fiscal Policy Joint Committee, Brass, Copper and Nickel Silver Industries, 1935. Abstracted *Analyst*, 1935, **60**, 554.
11. L. A. Haddock, *Analyst*, 1934, **59**, 163.
12. H. R. Fitter, *Analyst*, 1938, **63**, 107.

CALCIUM

Most methods for the colorimetric determination of calcium depend upon the preliminary precipitation of an insoluble salt followed by the application of a colour reaction to the acid radicle

but none of these methods can be considered as being particularly satisfactory. Thus, a procedure has been proposed for determining calcium in blood depending upon precipitation of the phosphate from dilute alcoholic solution followed by isolation of the precipitate and application of the molybdenum blue test[1]. Although this method has enjoyed some favour, the determination is more usually conducted by precipitating as oxalate and titrating the latter with 0·01N potassium permanganate in accordance with the technique due to Kramer and Tisdall[2, 3] or by one of the suggested modifications of this process[4, 5]. A colorimetric method depending upon the formation of the calcium salt of picrolonic acid (1-*p*-nitrophenol-3-methyl-4-nitro-5-pyrazolone) although not generally capable of yielding results of precision, may occasionally prove to be useful.

Method[6]. To about 5 ml. of the solution to be tested, which should be rendered neutral, is added a few drops of a 10 per cent. alcoholic solution of thiosalicylic acid and the mixture diluted to 10 ml., or other suitable volume, and 2 ml. transferred to a centrifuge tube followed by 6 ml. of a 0·25 per cent. aqueous solution of picrolonic acid. After allowing the tube to stand in ice-water for at least 4 hours the precipitated calcium picrolonate is separated by centrifuging, the precipitate washed three times with anhydrous ether, then dissolved in hot water and the solution diluted to 50 ml. To an amount of the solution expected to contain between 0·02 and 0·15 mg. of calcium is added 1 ml. of saturated bromine water. The mixture is heated on a water-bath for 10 minutes then 10 ml. of alcohol added to remove the excess of bromine and finally, after allowing to cool, the reddish-yellow colour is developed by the addition of 2 ml. of 2N sodium hydroxide. A standard solution of calcium may be prepared by dissolving 0·0729 g. of calcium oxalate, $CaC_2O_4.H_2O$, in 25 ml. of 2N nitric acid and diluting to 1 litre with water : each ml. of this solution will contain 0·02 mg. Ca in 0·05N nitric acid.

Discussion. The thiosalicylic acid (*o*-thiol-benzoic acid) used in the test serves to obviate any interference due to the presence of iron or aluminium. The determination is not disturbed by magnesium or the alkali metals but heavy metals, together with barium and strontium, will interfere since they also form insoluble picrolonates.

REFERENCES

1. J. H. Roe and B. S. Kahn, *J. Biol. Chem.*, 1929, **81**, 1.
2. B. Kramer and F. F. Tisdall, *J. Biol. Chem.*, 1921, **47**, 475.

3. B. Kramer and F. F. Tisdall, *J. Biol. Chem.*, 1923, **56**, 439.
4. E. P. Clark and J. B. Collip, *J. Biol. Chem.*, 1925, **63**, 461.
5. J. W. Trevan and H. W. Bainbridge, *Biochem. J.*, 1926, **20**, 423.
6. F. Alten, H. Weiland and E. Knippenberg, *Biochem. Z.*, 1933, **265**, 85.

CHROMIUM

The methods depending upon oxidation to chromate or dichromate followed by measurement of the colour due to the resulting anion are probably the best although generally of limited application. The oxidation to chromate is accomplished by means of persulphate in the presence of silver nitrate acting as a catalyst and this principle has been applied by Hillebrand[1] for the determination of chromium present in rocks. If permanganate be employed as the oxidising agent, dichromate is produced and a method embodying this procedure has been developed by B. S. Evans[2] for the determination of traces of chromium in steel. The organic compound diphenyl-carbazide serves as a delicate reagent for the detection of chromates with which it forms a magenta colour in acid solution[3–5]. This reaction is extremely sensitive and if conducted in the presence of dilute sulphuric acid will detect 1 part of chromium in 150,000,000 parts of solution. For quantitative work this delicacy of response is a disadvantage since it involves working with very high dilutions with consequent loss of precision. Furthermore, the sensitivity varies appreciably with the reaction of the solution under test; it is reduced to about 1 part in 70,000,000 parts if acetic acid is used in place of sulphuric acid. Again, the presence of many other metals particularly mercury, silver and copper as well as high concentrations of dissolved salts will lead to interference. Nevertheless, a promising method involving the use of diphenyl-carbazide has been proposed for the determination of chromium in steel[6] and another procedure is described for the corresponding examination of plant ash and kindred material[7]: in the former, oxidation is effected by permanganate and the iron then removed with sodium carbonate while in the latter, the chromium is first isolated by precipitation with ammonia and then converted to chromate with persulphate. These methods are best illustrated by descriptions of their applications to particular products, whence appropriate modifications to suit other purposes will in many cases be apparent.

Method for Chromium in Steel (using Permanganate)[2]. About 4 g. of the sample is dissolved in 50 ml. of boiling 5N sulphuric acid : if ferrous sulphate tends to crystallise out, the solution should be diluted with a little water. To this solution 5 to 10 ml. of concentrated nitric acid is added, the mixture boiled until red fumes are dispelled then 25 g. of diammonium hydrogen phosphate added followed by 250 ml. of water (and more nitric acid if ferric phosphate separates) and the whole again heated to boiling. While still boiling a saturated aqueous solution of potassium permanganate is added a few drops at a time until an excess is present as indicated by a permanent precipitate of manganese dioxide, or by a persistent red colour, and then a further excess of 0·3 ml. is added and the boiling continued for 15 minutes. In the meantime, 120 ml. of a 20 per cent. aqueous solution of sodium hydroxide is transferred to a large beaker, about 0·5 ml. of saturated permanganate solution added and the mixture boiled for some minutes ; if the purple colour of the permanganate changes to green more should be added until a purple tint persists. When the mixture containing the sample has been boiled for 15 minutes about 10 ml. of a 5 per cent. aqueous solution of manganese sulphate is added to the hot sodium hydroxide and then the hot acid liquid is poured slowly into the alkali with constant stirring. The resulting mixture is transferred to a 500-ml. graduated flask, allowed to cool and tested with litmus paper to ensure that it is strongly alkaline ; if not, more sodium hydroxide is added, the liquid transferred to a 500-ml. graduated flask, allowed to cool, rendered acid with 10 ml., or a sufficiency, of glacial acetic acid, diluted to the mark with water, shaken and finally filtered through a dry paper.

This procedure removes all coloured ions except those of chromium, nickel and cobalt : if none of these is present the solution should be almost colourless. In the absence of the two latter metals, 100 ml. of the filtrate is transferred to a Nessler glass and a lesser volume of water is added to a second Nessler followed by the addition of 20 ml. of 5N sulphuric acid to each then 0·01N potassium dichromate is run into the blank until its colour matches that of the test, the volume of the two solutions being equalised before making the final comparison. There is usually a very small blank which may be determined by adding just sufficient dilute ferrous sulphate solution to both solutions to remove the colour due to dichromate and then estimating any tint which still persists in the sample solution in terms of 0·01N

potassium dichromate by adding the latter to a fresh mixture of water and acid as before: this blank reading, which usually approximates to 0·25 ml., is deducted from the original reading. If the quantity of chromium in the sample is large enough to colour the solution in the reduced form it is sufficiently accurate to assume that the blank due to extraneous colour is 0·25 ml. The reading in terms of 0·01N potassium dichromate multiplied by the factor 0·0217 gives the percentage of chromium in the steel when working on a sample weighing 4 g.

If nickel or cobalt be present it is necessary to remove them before applying the colour test by adding a slight excess of sodium hydroxide to the filtrate previously heated to boiling and, after allowing to cool, filtering off the precipitate and proceeding with the colorimetric determination as already described.

Discussion. In developing the above method Evans undertook special experiments to prove that, under the conditions of the test, chromium is completely oxidised to dichromate, because certain text-books state that permanganate will not effect this in strongly acid solution. Vanadium, tungsten and molybdenum do not interfere with the determination of chromium. The action of the ammonium phosphate is not fully understood since it was found that substitution of sodium phosphate led to bad results, while ammonium acetate was ineffective. It appears that one function of the ammonium phosphate is to combine with the iron thus preventing the formation of ferric hydroxide which would induce precipitation of basic chromate. Soon after the details of the above procedure had been published Evans added a further note[8] suggesting a modification whereby diphenyl-carbazide might be employed for the final colorimetric determination of the chromium. Later, Agnew[6] proposed the following alternative and simpler procedure for the same purpose depending upon permanganate oxidation and the colour reaction using diphenyl-carbazide. The method is applicable without modification to steels containing molybdenum, vanadium, tungsten, cobalt and nickel, as much as 15 per cent. of the last-mentioned metal in the sample under examination not disturbing the determination of even traces of chromium.

Method for Chromium in Steel (using Permanganate and Diphenyl-carbazide)[6]. About 1 g. of the sample, accurately weighed, is dissolved in 35 ml. of 2N sulphuric acid, 5 ml. of nitric acid (s.g. 1·2) added and the mixture boiled until free from nitrous fumes. After allowing to cool the solution is diluted with water

to exactly 200 ml. and 40 ml. of this treated with 3 drops of a saturated aqueous solution of potassium permanganate, the mixture boiled for a few minutes and the excess permanganate reduced by the cautious addition of concentrated hydrochloric acid. The mixture is at once cooled under the tap, the iron, etc., precipitated with a slight excess of a saturated aqueous solution of sodium carbonate, the liquid diluted to 100 ml. with water and the whole shaken and filtered. A measured quantity of this filtrate, say 50 ml. (corresponding to about 0·1 g. of the sample), is acidified by the careful addition of 20 ml. of 5N sulphuric acid, 5 ml. of a freshly prepared 0·1 per cent. alcoholic solution of diphenyl-carbazide added and the mixture transferred to a 100-ml. Nessler glass. Into another Nessler glass are poured 20 ml. of 5N sulphuric acid, 5 ml. of diphenyl-carbazide solution and about 70 ml. of water and the determination then completed by adding 0·001N potassium dichromate to this mixture until the colour matches that derived from the sample. The calculation may be simplified by employing a solution containing 0·02 mg. Cr per ml. which can be prepared by diluting 11·54 ml. of 0·1N potassium dichromate to 1 litre with water.

Method for Chromium in Plant Ash, Soil and Rocks (using Persulphate and Diphenyl-carbazide)[7]. To the plant ash, ignited soil extract or powdered rock, contained in a platinum crucible, is added 10 ml. of hydrofluoric acid and 4 drops of concentrated sulphuric acid and the mixture is evaporated to dryness on a sand-bath ; about 6 g. of potassium pyrosulphate is added and the mixture fused until the melt is clear. The cooled melt is dissolved in about 150 ml. of dilute hydrochloric acid, about 2 ml. of a 5 per cent. aqueous solution of ferric sulphate and a few drops of concentrated nitric acid added, the liquid boiled for a minute and then ammonia added drop by drop until the mixture is just alkaline to litmus. The precipitate is collected on a filter, washed with a hot 2 per cent. aqueous solution of ammonium sulphate, dissolved in dilute sulphuric acid and the iron reprecipitated with ammonia and washed. The precipitate is returned to the original beaker, the small amount adhering to the filter paper being removed with hot dilute sulphuric acid to bring the total quantity to 4 ml. of concentrated acid. The solution, which should now be free from chlorides and contain no more than a trace of manganese, is diluted to 80 ml., heated to boiling, treated with 5 drops of concentrated nitric acid, 1 ml. of a 2·5 per cent. solution of silver nitrate and 20 ml. of a freshly prepared 10 per cent. aqueous

solution of ammonium or potassium persulphate. The beaker is covered with a clock glass and the mixture boiled for 10 minutes ; the final volume should not be less than 70 ml. Iron and aluminium are then precipitated by adding solid sodium carbonate to the cold solution a little at a time until a slight excess is present, the mixture is transferred to a graduated flask, diluted to 100 ml. with water, mixed and filtered through a dry filter paper into a dry flask. To 50 ml. of this filtrate is added 10 ml. of 10N sulphuric acid and 5 ml. of a freshly prepared 0·1 per cent. alcoholic solution of diphenyl-carbazide. The liquid is diluted to 70 ml. and the violet colour compared with that of standards. To prepare the latter, appropriate quantities up to about 5 ml. of a solution of potassium dichromate containing 0·002 mg. per ml. (11·54 ml. of 0·01N diluted to 1 litre) are mixed with 50 ml. of water, 10 ml. of 10N sulphuric acid and 5 ml. of the reagent and the solutions diluted to 70 ml.

Discussion. The above procedure is due to van der Walt and van der Merwe[7] and it has been applied to investigations on soils by Davidson and Mitchell[9] ; however, it should be observed that it is not applicable in the presence of manganese. If the method be compared with that described for the examination of steel using permanganate and diphenyl-carbazide, it will be noted that the recommended standard solution of potassium dichromate in the latter process is ten times as strong as that described for the determination of chromium in plant ash. Provided attention be paid to maintaining the same concentration of sulphuric acid in the sample and standards fairly reliable results are obtainable at either concentration but with the stronger solution more chromium will be required to effect a sensible alteration in the depth of colour than is the case when using the weaker standards : in the latter the tint approximates to a pale violet while in the stronger series a full magenta is produced.

REFERENCES

1. W. F. Hillebrand, *U.S. Geol. Surv. Bull.*, 1919, No. 700, 182.
2. B. S. Evans, *Analyst*, 1921, **46**, 38.
3. P. Cazeneuve, *Compt. rend.*, 1900, **131**, 346.
4. H. Weber, *Z. anal. Chem.*, 1902, **41**, 568.
5. N. M. Stover, *J. Amer. Chem. Soc.*, 1928, **50**, 2363.
6. W. J. Agnew, *Analyst*, 1931, **56**, 24.
7. C. F. J. van der Walt and A. J. van der Merwe, *Analyst*, 1938, **63**, 809.
8. B. S. Evans, *Analyst*, 1921, **46**, 285.
9. A. M. M. Davidson and R. L. Mitchell, *J. Soc. Chem. Ind.*, 1940, **59**, 232.

COBALT

Two methods will be considered ; first, the one depending upon the formation of the blue ammonium cobaltothiocyanate[1] and its extraction with an organic solvent and, second, the quantitative application of the delicate colour reaction given by nitroso-R salt[2] which, as a reagent for colorimetric work, may be said to have superseded α-nitroso-β-naphthol[3, 4].

Method Using Ammonium Thiocyanate. The solution to be examined, preferably containing between 0·05 and 0·5 mg. Co, should occupy a volume of about 20 ml. and be of about normal acidity with respect to hydrochloric acid. Sufficient solid ammonium thiocyanate is added to bring the concentration of this salt in the solution to 30 per cent. and this is followed by the addition of sodium pyrophosphate in such quantity that the final mixture contains about 5 per cent. The solution is transferred to a separator and the cobaltothiocyanate completely extracted by shaking with successive 10-ml. portions of a mixed solvent consisting of equal volumes of amyl alcohol and amyl acetate. The extracts are transferred to a Nessler glass, diluted to a convenient volume with the solvent and the greenish-blue colour matched against that of standards similarly prepared. A standard solution containing 0·1 mg. Co per ml. is made by dissolving in water 0·263 g. of freshly ignited cobalt sulphate, adding a little dilute sulphuric acid and diluting to 1 litre with water.

Discussion. The blue colour is probably due to an undissociated salt having the constitution $(NH_4)_2[Co(SCN)_4]$; if the aqueous layer containing this blue compound be diluted with additional water it becomes pink due to the liberation of cobaltous ions, hence it is essential to maintain a high concentration of ammonium thiocyanate which on no account should fall below 25 per cent.[5]. Sodium pyrophosphate is added in order to inhibit interference by ferric iron[6] ; the same reagent, added in solid form to the completed standard colours, preserves them from deterioration for many months[5]. Although this method may not always be capable of a high degree of precision, it is undoubtedly the best available for the determination of traces of cobalt in nickel and, indeed, the reaction has been recommended for the complete separation of the two metals by Rosenheim and Huldschinsky[7] who employed a mixed solvent consisting of 25 volumes of ether and 1 volume of amyl alcohol and isolated the metals by elec-

trolysis preparatory to weighing them. The test is not disturbed by most of the commonly occurring metals provided none is present in sufficient quantity to form a precipitate with the pyrophosphate.

Approximate Determination of Cobalt in Nickel Salts[8]. A weighed quantity of the sample, up to 5 g., is dissolved in 10 ml. of water, 1 ml. of 5N hydrochloric acid and 1 drop of 0·1N potassium permanganate added; after mixing, 5 ml. of an approximately 7·5M aqueous solution of ammonium thiocyanate is added, the mixture transferred to a separator and shaken with 10 ml. of a solvent comprising equal volumes of amyl acetate and amyl alcohol. After allowing to separate, the aqueous layer is discarded, the solvent layer shaken with a mixture of 5 ml. of an approximately M aqueous solution of ammonium phosphate, $(NH_4)_2HPO_4$, and 5 ml. of 7·5M aqueous solution of ammonium thiocyanate. After separation, the lower layer is rejected, the solvent layer washed once with a mixture consisting of 1 ml. of M ammonium phosphate, 2 ml. of 7·5M ammonium thiocyanate and 1 ml. of water. After separation, the presence of cobalt in quantities greater than 0·02 mg. Co is indicated by a blue coloration in the amyl acetate-amyl alcohol layer.

Method Using Nitroso-R Salt. The sample to be tested should be in the form of a solution in diluted hydrochloric or sulphuric acid. To 5 ml. of the solution to be examined is added 2 g. of sodium acetate, $NaC_2H_3O_2.3H_2O$, and 3 drops of phenolphthalein indicator; the mixture is warmed on a boiling water-bath, rendered slightly alkaline with 5N sodium hydroxide and the reaction adjusted with N sulphuric acid and finally with 0·05N sulphuric acid until the pink colour of the phenolphthalein is only just discernible. To this solution, the volume of which should not exceed 10 ml., is added 1 ml. of a freshly prepared 0·1 per cent. solution of nitroso-R salt, the mixture boiled for 1 minute and, while still boiling, 2 ml. of nitric acid (s.g. 1·42) slowly added and the boiling continued for a further minute; finally, the solution is quickly cooled, diluted to 50 ml. with water and the reddish-orange colour compared with standards made from cobalt sulphate solution. The useful range of the test lies between 0·001 and 0·03 mg. Co.

Discussion. Nitroso - R salt (sodium 1 - nitroso - 2 - hydroxy - naphthalene-3:6-disulphonate) was first applied to quantitative work by Stare and Elvehjem[9]. Following the discovery of the toxic symptoms arising in sheep and cattle due to a deficiency of cobalt

in certain pastures the problem assumed importance and has been the subject of several investigations[10–14]. The above method is a modification of that recommended by McNaught[15] and the method was successfully employed by Davidson and Mitchell for researches on the cobalt content of soils[16]. More recently, McNaught has published a modification[17] of the original procedure in which it is directed that the reagent be added before neutralisation. While this is doubtless an improvement on the technique given in McNaught's original paper the present author, as a result of many trials, is able to recommend the test in the form described above.

The preliminary procedure will vary according to the character of the sample under investigation. Organic material is best submitted to wet oxidation using sulphuric acid and nitric acid and, after the complete removal of the latter, the remaining concentrated sulphuric acid diluted with water and the solution examined for cobalt. In any case, the sample which is to be tested should be in the form of a solution in diluted hydrochloric acid or diluted sulphuric acid. Accurate adjustment of the reaction of the solution prior to the addition of the reagent is essential for the development of concordant colours. The test is applicable in the presence of moderate quantities of nickel but the colour is disturbed by a large preponderance ; preliminary separation by means of dimethylglyoxime may sometimes be satisfactory but often leads to loss of cobalt by adsorption : in such cases it is better to employ the thiocyanate method. Heavy metals and iron interfere ; the former can be removed from an acid solution with hydrogen sulphide and the latter may be extracted with ether from a solution which is 5N with respect to hydrochloric acid.

Sylvester and Lampitt[18] describe a method for the separation of cobalt from the ash of foods which involves precipitation with α-nitroso-β-naphthol, together with any iron and copper that may be present, followed by wet oxidation of the precipitate and extraction under specified conditions with diphenylthiocarbazone. After wet oxidation of the second residue the cobalt is determined colorimetrically with nitroso-R salt. This procedure eliminates any possible disturbance due to the presence of a relatively large quantity of calcium.

Method Using Nitroso-R Salt and Permanent External Standards. This test can be performed with the help of the B.D.H. Lovibond Nessleriser and two discs are available which together cover a range from 0·001 to 0·03 mg. Co. Excepting that it is necessary

to prepare a control test in order to compensate for the slight tint due to the reagents, the procedure employed in conjunction with these standards is identical with that described above.

REFERENCES

1. H. W. Vogel, *Ber.*, 1879, **12**, 2314.
2. H. S. van Klooster, *J. Amer. Chem. Soc.*, 1921, **43**, 746.
3. F. W. Atack, *J. Soc. Chem. Ind.*, 1915, **34**, 641.
4. E. G. Jones, *Analyst*, 1918, **43**, 317.
5. A. D. Powell, *J. Soc. Chem. Ind.*, 1917, **36**, 273.
6. G. Romijn, *Chem.-Ztg.*, 1911, **35**, 1300.
7. A. Rosenheim and E. Huldschinsky, *Ber.*, 1901, **34**, 2050.
8. "AnalaR Standards for Laboratory Chemicals," issued by The British Drug Houses, Ltd., and Hopkin and Williams, Ltd., London, 1944.
9. F. J. Stare and C. A. Elvehjem, *J. Biol. Chem.*, 1932, **99**, 473.
10. E. B. Kidson, H. O. Askew and J. K. Dixon, *N.Z. J. Sci. Tech.*, 1936, **18**, 601.
11. K. J. McNaught, *N.Z. J. Sci. Tech.*, 1937, **18**, 655.
12. H. O. Askew and J. K. Dixon, *N.Z. J. Sci. Tech.*, 1937, **18**, 707.
13. S. W. Josland, *N.Z. J. Sci. Tech.*, 1937, **19**, 31.
14. S. W. Josland and K. J. McNaught, *N.Z. J. Sci. Tech.*, 1938, **19**, 536.
15. K. J. McNaught, *Analyst*, 1939, **64**, 23.
16. A. M. M. Davidson and R. L. Mitchell, *J. Soc. Chem. Ind.*, 1940, **59**, 232.
17. K. J. McNaught, *Analyst*, 1942, **67**, 97.
18. N. D. Sylvester and L. H. Lampitt, *J. Soc. Chem. Ind.*, 1940, **59**, 57.

COPPER

Of the numerous reagents proposed for the colorimetric determination of this metal the most generally useful is sodium diethyl-dithio-carbamate[1, 2] which produces a brown colour when added to alkaline solutions containing traces of copper. The compound is soluble in organic solvents[3] and a useful application of the test, due to L. A. Haddock and N. Evers[4], is based upon extraction of the copper complex with carbon tetrachloride followed by measurement of the intensity of the colour by means of the Lovibond tintometer. Dithio-oxamide (rubeanic acid), which was originally suggested as a delicate reagent for nickel, cobalt and copper[5] is sometimes useful for the colorimetric determination of the last mentioned metal[6]; if the solution to be examined is slightly acidified with acetic acid neither nickel nor cobalt interfere.

Method Using Sodium Diethyl-dithio-carbamate[4]. The solution to be tested, which should be neutral and preferably occupy a

volume of about 10 ml., is transferred to a small separator, 10 ml. of a 20 per cent. w/v aqueous solution of citric acid added followed by 6 ml. of an aqueous solution of ammonia (10 per cent. w/v NH_3). After mixing, 10 ml. of a freshly prepared 0·1 per cent. aqueous solution of sodium diethyl-dithio-carbamate is added followed immediately by 2·5 ml. of carbon tetrachloride. The mixture is vigorously shaken, allowed to separate and the lower layer run into a dry 10-ml. measure. The extraction is repeated with three further portions each of 2·5 ml. of carbon tetrachloride which are transferred to the same 10-ml. measure. If the final portion of carbon tetrachloride is almost colourless the liquid in the measure is, if necessary, diluted to 10 ml. with more solvent then rendered clear by shaking with a small quantity of anhydrous sodium sulphate and the intensity of the brown colour measured by means of a Lovibond tintometer using a 1-cm. cell. The intensity of the yellow component of the colour is correlated with the quantity of copper present in accordance with the values quoted in Table. If the fourth portion of carbon tetrachloride shows more than a trace of colour the extraction of the copper compound is continued with further quantities of 2·5 ml. of solvent, the mixed extracts diluted to 20 ml. and then clarified and the colour measured as before, the value of the yellow component being referred to the quantity of copper in accordance with the appropriate data in Table III.

TABLE III.—RELATION BETWEEN THE QUANTITY OF COPPER AND THE COLOUR PRODUCED BY THE METHOD DUE TO L. A. HADDOCK AND N. EVERS (*Analyst*, 1932, **57**, 495)

10 ml. of Carbon Tetrachloride		20 ml. of Carbon Tetrachloride	
Copper mg.	Colour observed in 1 cm. cell : Lovibond Yellow Units	Copper mg.	Colour observed in 1 cm. cell : Lovibond Yellow Units
0·005	0·7	0·05	3·1
0·01	1·2	0·06	4·2
0·02	2·5	0·07	5·1
0·03	4·2	0·08	6·3
0·04	6·4	0·09	7·4
0·05	8·2	0·10	8·3

Method Using Sodium Diethyl-dithio-carbamate and Permanent External Standards. The above technique using 10 ml. of solvent has been applied to the standardisation of a Lovibond Comparator

disc covering a range from 0·0025 to 0·05 mg. Cu. If the colour produced in the test is deeper than the standards the determination should be repeated using a smaller quantity of the original liquid under examination previously diluted to 10 ml. with distilled water.

Discussion. The particular merit of this method lies in its applicability in the presence of iron. The quantity of citric acid specified is sufficient to inhibit interference from 0·1 g. of trivalent iron, but, provided the amount of citric acid is increased, it is possible to determine traces of copper in the presence of even more iron. Ferrous iron, if present, must be oxidised since the test is inapplicable in the presence of more than 1 mg. of the divalent metal. Even so small an amount of copper as 0·005 mg. may be determined with reasonable accuracy when associated with as much as 0·25 g. of ferric iron.

Since the colour due to copper is stable for at least an hour the determination may be conducted by directly matching the test against a series of natural standards. According to Haddock and Evers[4], the determination is applicable in the presence of nitrates in amounts up to the equivalent of 0·75 mg. NO_3', sodium phosphate equivalent to 0·25 g. P_2O_5 and, provided the reaction of the mixture before extraction does not exceed *p*H 9, calcium phosphate equivalent to 0·3 g. $Ca_3(PO_4)_2$. In the absence of iron, 0·5 g. SO_2 present as sulphurous acid does not interfere. The method works well in the presence of aluminium and zinc in quantities up to 0·2 g. of the former or 0·1 g. of the latter. Determinations can be conducted in the presence of tin, and, if iron is absent, stannic salts as well as aluminium and zinc can be dissolved directly in an excess of sodium hydroxide and ammonia and the procedure for the extraction of the copper applied without modification. Chromium must be in the chromic condition; otherwise the solution should be reduced by the addition of sulphurous acid until no colour is given when the reaction mixture is spotted on to an external indicator consisting of a solution of diphenylamine in sulphuric acid. After reduction the solution containing the chromium should be heated to boiling with 2 g. of citric acid and, after allowing to cool, rendered alkaline with excess of ammonia. If this preliminary procedure is applied traces of copper may be accurately determined in the presence of 0·2 g. Cr. If no more than a trace of iron is present copper may be determined in the presence of manganese (up to 0·2 g.) by adding 2 g, of citric acid and 5 ml. of a 5 per cent. solution of sulphur dioxide.

followed by excess of ammonia. A faint pink colour is extracted from solutions containing manganese, but the relation between the intensity of the yellow component and the amount of copper present is not disturbed. Antimony, bismuth, cadmium, lead and mercury all yield white turbidities with sodium diethyldithio-carbamate and hence the presence of small amounts of any of these should not interfere with the determination of copper.

T. P. Hoar prefers to prevent precipitation of the copper compound of the reagent by addition of gum and to measure the colour of the aqueous mixture directly[7]. Two procedures are described, one in which interference by iron is prevented by the inclusion of citrate and another in which sodium pyrophosphate is used. At best, the methods are only suitable for cases where the amount of iron present does not exceed the copper by more than a hundredfold and they are more readily influenced by the presence of other substances than the procedure involving extraction of the copper complex.

In the examination of organic material, dry ashing may be employed in the preliminary procedure without loss of copper provided the temperature of the muffle does not exceed 565° C.[8] and this course was adopted in an investigation of the copper content of milk[9]. For the determination of copper in tomato purée Cockburn and Herd[10] adopt wet oxidation and draw attention to the danger of using flasks previously used for nitrogen determinations to which copper sulphate has been added to serve as a catalyst. It is important to eliminate all trace of nitrous acid while, if the wet oxidation is to be followed by direct measurement of the colour of the copper complex in the aqueous liquid, it is necessary to guard against the presence of yellow nitro compounds by treating the sulphuric acid digest with ammonium oxalate[11].

Method Using Dithio-oxamide[6]. A suitable quantity of the neutral solution under examination containing between 0·0025 and 0·1 mg. Cu is transferred to a Nessler glass and 1 ml. of 5N acetic acid, 5 ml. of a 20 per cent. w/v aqueous solution of ammonium acetate and 1 ml. of gum acacia solution added in succession ; the mixture is diluted with distilled water to 50 ml. and 0·5 ml. of a 0·2 per cent. solution of dithio-oxamide in alcohol (90 per cent.) added. The olive green colour, which develops to its full intensity in about 5 minutes and is then stable for some time, is matched against standards similarly prepared. The solution of gum acacia is prepared by dissolving 1 g. of the gum

in 100 ml. of water, boiling to destroy oxidases, filtering and preserving with thymol.

Method Using Dithio-oxamide and Permanent External Standards. Two standard discs for use with the above technique and covering a range from 0·0025 to 0·1 mg. Cu, are issued for use with the B. D. H. Lovibond Nessleriser.

Discussion. Colorations or precipitates are also produced in the presence of platinum, palladium and silver, while iron, if present in more than a faint trace, gives rise to a brown tint and interferes with the determination of copper. Traces of mercury and most other heavy metals tend to prevent the full development of the colour due to copper but small amounts of aluminium, bismuth, chromium, calcium, manganese and the alkali metals do not interfere.

REFERENCES

1. M. Delépine, *Bull. Soc. chim.*, 1908, [iv], **3**, 652.
2. T. Callan and J. A. R. Henderson, *Analyst*, 1929, **54**, 650.
3. F. Grendel, *Pharm. Weekblad*, 1930, **67**, 913, 1050 and 1345.
4. L. A. Haddock and N. Evers, *Analyst*, 1932, **57**, 495.
5. P. Rây and R. M. Rây, *Quart. J. Indian Chem. Soc.*, 1926, **3**, 118.
6. N. L. Allport and G. H. Skrimshire, *Quart. J. Pharm.*, 1932, **5**, 461.
7. T. P. Hoar, *Analyst*, 1937, **62**, 657 and 788.
8. T. H. Gebhardt and H. H. Sommer, *Ind. Eng. Chem., Anal. Ed.*, 1931, **3**, 24.
9. L. W. Conn, A. H. Johnson, H. A. Trebler and V. Karpenko, *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 15.
10. T. Cockburn and M. Herd, *Analyst*, 1938, **63**, 482.
11. C. E. Sage and S. G. E. Stevens, *Analyst*, 1938, **63**, 719.

GOLD

The early proposals[1–3] for the colorimetric determination of this metal depend upon the production of a gold sol using stannous chloride as the reducing agent. According to the concentration, the colour produced varies from pale yellow, for just detectable amounts, through red to the purple of Cassius. The method was originally employed qualitatively to prove the presence of gold in sea water[4]. Many other reagents have been suggested, including acetylene[5], phenylhydrazine hydrochloride[6], *m*-phenylene-diamine[7], benzidine[8], *o*-tolidine[9], formaldehyde[10], *p*-dimethylamino-benzal-rhodanine (*p*-dimethylamino-benzylidine-rhodanine)[11] and mercurous chloride[12]. In the present author's experience the

stannous chloride reaction is uncertain in action and is readily disturbed by the presence of dissolved electrolytes and, for general use, formaldehyde would seem to be a much more satisfactory reducing agent, while, of the reagents depending upon the oxidising property of the auric ion *o*-tolidine is probably the best.

Method Using Formaldehyde[10]. The neutralised solution to be examined is transferred to a Nessler glass, diluted to 50 ml. with water, 1 ml. of a 20 per cent. aqueous solution of sodium hydroxide added followed by 1 ml. of formaldehyde solution (40 per cent. w/v). After standing 5 minutes the colour is matched against standards similarly prepared. The standard solution is conveniently prepared by dissolving 5 mg. of pure gold metal in 10 ml. of aqua regia, boiling to remove oxides of nitrogen, cooling and diluting with water to 1 litre : this standard contains 5μg. Au per ml. In the presence of 1 part per million of gold this test produces a pale violet while 5 parts per million gives a deep slate colour. The tint, which is reasonably permanent, may be modified by the presence of dissolved electrolytes and it is advisable that the standards should have approximately the same composition as the sample.

Application to Cyanide Solutions. Potassium bromate is added at the rate of about 1 g. per 50 ml. of sample followed by approximately 2 ml. of sulphuric acid (90 per cent.). The excess of bromine is removed by boiling, the mixture allowed to cool, diluted to 50 ml. and the test for gold applied as described above.

In the following alternative procedure the gold is isolated, then redissolved in aqua regia preparatory to applying the colorimetric test[2]. A special copper reagent is first prepared by dissolving 10 g. of copper sulphate, $CuSO_4.5H_2O$, in 100 ml. of water, adding 20 g. of sodium chloride and 1 g. of copper metal turnings ; after boiling for 10 minutes the mixture is allowed to cool and acidified by the addition of 5 ml. of acetic acid (30 per cent.). A suitable proportion of the solution under test is acidified with hydrochloric acid, the mixture boiled for a few minutes, then 5 to 10 drops of the copper reagent added and the liquid tested by spotting on to paper moistened with potassium ferrocyanide in order to ensure that an excess of copper is present as indicated by the appearance of a reddish brown colour. To the still boiling liquid 5 drops of a 2 per cent. aqueous solution of sodium sulphide is added and after 5 minutes the precipitate allowed to settle, the supernatant liquor decanted through a filter and the precipitate dissolved in 25 ml. of

a 5 per cent. aqueous solution of sodium cyanide containing a little added sodium hydroxide. The solution is poured through the filter and the latter washed with a little water. To the combined filtrate and washings, which may be cloudy, is added 2 g. of zinc dust and the mixture heated to 45° C. for 30 minutes in order to precipitate the gold. The supernatant liquor is decanted through a filter, the excess zinc dissolved in 10 ml. of 5N hydrochloric acid and the solution passed through the filter. The gold thus collected is dissolved off the filter with warm aqua regia (made by mixing 2·5 ml. of concentrated nitric acid with 7·5 ml. of concentrated hydrochloric acid) the acid solution boiled to remove oxides of nitrogen, diluted with a suitable quantity of water and the colorimetric test applied.

Application to Organic Material[11]. The sample is submitted to wet oxidation in the usual way with sulphuric and nitric acids, the resulting clear liquid being evaporated to dryness. The residue, which contains the gold as metal, is treated with aqua regia, the acid extract boiled to remove the oxides of nitrogen then suitably diluted with water and the colour test applied.

Method Using *o*-Tolidine[9]. To the solution to be examined which should be neutral or only slightly acid with hydrochloric acid is added one twenty-fifth of its volume of a 0·1 per cent. solution of *o*-tolidine in N hydrochloric acid. The yellow solution produced is matched against standards similarly prepared.

Discussion. The formaldehyde method is not applicable in the presence of platinum, palladium, silver, mercury, arsenic, vanadium, tin, molybdenum or tungsten but small amounts of lead zinc or aluminium do not interfere. The *o*-tolidine test gives a just perceptible colour in the presence of 0·5 part per million of gold. Ferric salts, ruthenium, osmic acid, vanadic acid and tungstic acid give a yellow colour, copper gives a green colour while aluminium, antimony, bismuth, cadmium, chromium, cobalt, iridium, lead, mercury, manganese, nickel, platinum, rhodium, tin, uranium and zinc do not disturb the reaction. The liquid under examination should be only slightly acid, since high concentrations of free mineral acid depress the sensitivity. In testing solutions that have been obtained by the use of aqua regia special care should be taken to guard against the possible presence of free chlorine or of nitrous acid, since both give a yellow colour with *o*-tolidine, while the latter also reduces auric compounds.

REFERENCES

1. H. R. Cassel, *Eng. Mining J.*, 1903, **76**, 661.
2. A. Prister, *J. Chem. Met. Mining Soc., S. Africa*, 1903, **4**, 235 and 455.
3. C. B. Brodigan, *Met. Chem. Eng.*, 1914, **12**, 460.
4. E. Sonstadt, *Chem. News*, 1872, **26**, 159.
5. R. N. Maxson, *Z. anorg. Chem.*, 1906, **49**, 172.
6. E. Pozzi-Escot, *Ann. chim. anal.*, 1907, **12**, 90.
7. J. A. Siemssen, *Chem.-Ztg.*, 1912, **36**, 934.
8. G. Malatesta and E. di Nola, *Boll. chim. farm.*, 1912, **52**, 461.
9. W. B. Pollard, *Analyst*, 1919, **44**, 94.
10. J. A. Müller and A. Foix, *Bull. Soc. chim.*, 1922, **33**, 717.
11. B. K. Merejkovsky, *Bull. Soc. Chim. biol.*, 1933, **15**, 1336.
12. G. G. Pierson, *Ind. Eng. Chem., Anal. Ed.*, 1934, **6**, 437.

IRON

Numerous methods for the colorimetric determination of iron are available but it will only be necessary to describe a selected few for, while certain methods may be of value for particular purposes, one or other of the following more generally successful procedures can usually be applied to most cases that arise. The thiocyanate method for ferric iron[1] is still widely employed and an extensive literature on the test has been published ; here, it is proposed to describe a technique which, in the author's experience, has proved to be satisfactory for many purposes. The method using thioglycollic acid[2, 3] which was first applied to quantitative work by Lyons[4] is generally more serviceable : in the presence of ammonia the reagent produces a purple colour with a trace of either ferrous or ferric iron. For the colorimetric determination of iron in the divalent state the method employing $\alpha\alpha'$-dipyridyl (2:2'-bipyridine) is useful. The intense red complex formed by the interaction of ferrous iron with this compound was first described by Blau[5] and the reagent has been recommended for the determination of traces of iron in organic material by R. Hill[6]. A method proposed by Yoe[7] using 7-iodo-8-hydroxyquinoline-5-sulphonic acid (ferron) merits description here because it responds only to trivalent iron. Finally, for certain purposes the well-known test for ferric iron using sodium salicylate serves for quantitative work and a procedure due to R. O. Scott[8] is included here.

Method Using Thiocyanate. The sample solution to be tested, preferably containing between 0·002 and 0·025 mg. Fe, should be approximately neutralised, brought to a volume of 5 ml., trans-

ferred to a small stoppered separator and then acidified by the addition of 1 ml. of 5N hydrochloric acid. The iron is fully oxidised by adding 1 drop of 0·1N potassium permanganate, then 5 ml. of approximately 7·5M ammonium thiocyanate solution (57 per cent. w/v) added followed by 10 ml. of a mixed solvent consisting of equal volumes of amyl acetate and amyl alcohol. The mixture is vigorously shaken then allowed to separate, the aqueous layer rejected and the red colour of the solvent matched against standards similarly prepared. A standard stock solution of ferric iron containing 1 mg. Fe per ml. may be prepared by dissolving 8·63 g. of ferric ammonium sulphate, $Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$, in water, adding 50 ml. of concentrated nitric acid and diluting to 1 litre with more water.

Method Using Thiocyanate and Permanent External Standards. A standard disc covering the range from 0·002 to 0·025 mg. Fe is available for use with the Lovibond Comparator. The technique to be employed with these standards is the same as described above with the addition that a blank test using 5 ml. of distilled water is prepared for the purpose of interposing between the source of light and the coloured glasses.

Discussion. Any heavy metals that are present may be expected to interfere with the determination of iron by this method, but, in general, they may be easily removed by precipitation as sulphides from acid solution. Aluminium and magnesium do not interfere but where appreciable quantities of these metals are present it is advisable to compare the colours obtained with standards containing approximately the same proportion of other metals as are present in the test solutions. For the determination of traces of iron in substances containing zinc the salicylate method (p. 57) is recommended. Under the conditions of the above test a yellow colour is produced by titanium but in some cases it may still be possible to determine iron by using standards containing approximately the same concentration of the interfering element as the test mixtures. The presence of phosphates or fluorides leads to disturbance in the production of colour due to iron but it may sometimes be possible to minimise this by increasing the concentration of hydrochloric acid used in the test.

The particular feature of the technique described above is the enormous excess of ammonium thiocyanate employed : experience has shown that this is essential for the attainment of reliable results and the uncertainties of the method so frequently reported in the past are largely attributable to an inadequate concentration

of this reagent. Various proposals[9–13] have been made in regard to the solvent employed for extracting the colour but, in the author's experience, a mixture of amyl acetate and amyl alcohol has proved to be the most satisfactory.

Method Using Thioglycollic Acid. The solution to be examined, which should be neutral or slightly acid, may conveniently be diluted to 50 ml. with water then 2 ml. of a 20 per cent. aqueous solution of citric acid and 0·1 ml. of thioglycollic acid added and the mixture rendered slightly alkaline with ammonia. The purple colour due to either ferrous or ferric iron which develops to its maximum intensity in about 5 minutes and remains stable for many hours is matched against standards similarly prepared from a solution of ferric ammonium sulphate and containing between 0·002 and 0·06 mg. Fe.

Method Using Thioglycollic Acid and Permanent External Standards. Two discs, together covering the range from 0·002 to 0·06 mg. Fe, are issued for use with the B.D.H. Lovibond Nessleriser and they have been standardised by the method given above. A disc containing nine standards ranging from 0·01 to 0·1 mg. Fe is also available in the Comparator series, the test being performed using approximately one-fifth the quantities of solution and reagents specified in the foregoing description.

Discussion. The reaction is not appreciably disturbed by relatively large amounts of lead, silver, tin, mercury, copper, cadmium, chromium, manganese, aluminium, calcium, magnesium or the alkali metals but any significant quantity of zinc depresses the intensity of the colour due to iron while uranium, cobalt and nickel interfere. Sulphates, phosphates and nitrates do not disturb the test but if sulphites are present it will be necessary to apply a preliminary oxidation with potassium permanganate and to remove the excess of the latter by treatment with oxalic acid before determining the iron. The test is not applicable in the presence of cyanides. In general, thioglycollic acid is the most useful of the reagents proposed for the colorimetric determination of iron. In a detailed study of the method Swank and Mellon[14] have defined the possibilities and limitations of the method but as their technique did not include the use of citric acid they experienced precipitation of hydroxides in the presence of aluminium, chromium and magnesium. The reagent has been recommended by Tompsett for the determination of iron in biological material[15] while the application to blood has been the subject of a paper by Burmester[16].

Method Using αα′-Dipyridyl. In order to conduct a determination the following reagents should be prepared :—

1. A 1 per cent. Solution of αα′-Dipyridyl in 0·1N hydrochloric acid.
2. Strong Standard Iron Solution made by dissolving 0·4318 g. of ferric ammonium sulphate, $Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$, in a little water, adding 10 ml. of concentrated hydrochloric acid and diluting to 1 litre.
3. A Diluted Standard Iron Solution A containing 0·008 mg. Fe per ml., made by mixing 160 ml. of the strong iron solution with 2 ml. of a 5 per cent. aqueous mercuric chloride solution and diluting to 1 litre with water.
4. A Diluted Standard Iron Solution B, made by diluting 100 ml. of standard iron solution A to 1 litre with water.

A series of permanent natural standards is made in tubes of uniform bore using 0·2 to 6 ml. of standard iron solution B and 0·6 to 1 ml. of standard iron solution A. To each is added 2 ml. of the αα′-dipyridyl reagent, 1 ml. of a freshly prepared 10 per cent. aqueous solution of sodium sulphite, 0·1 ml. of 5N hydrochloric acid and then sufficient of the sodium sulphite solution to produce 10 ml. After shaking, 0·1 ml. of 5 per cent. mercuric chloride solution is added and the tubes sealed.

The sample liquid to be tested is treated in the same manner as the standard solutions and the red colour produced compared in the usual way.

Discussion. The concentration of free hydrochloric acid in the final test mixture is not highly critical. Iron in the ferric state does not give any colour with αα′-dipyridyl. A pale blue tint is produced in the presence of cupric ions but this is barely perceptible even when the concentration of the copper is 1000 times that of the iron just needed to produce a visible pink coloration ; nevertheless, it is important to ensure the absence of monovalent copper since this yields an intense colour with the reagent. If other heavy metals, or zinc, are present it is necessary to use an excess of αα′-dipyridyl so that all the metals may combine with the reagent. The method is especially useful for the examination of natural waters and it has been successfully applied to the determination of iron in biological material. By omitting the treatment with sulphite, ferrous iron can be detected and determined to the exclusion of ferric iron. Koenig and Johnson state that for cases where organic material is wet oxidised prior to the

determination of iron by αα′-dipyridyl the accuracy of the colorimetric test, when conducted on a 100-ml. scale, is not disturbed by the presence of up to 10 ml. of 6N hydrochloric acid, 2 ml. of 36N sulphuric acid, 40 ml. of 0·6N trichloracetic acid and 5 ml. of 9N perchloric acid[17].

If the natural standards prepared as described are stored away from bright light they will remain stable for many months. The popularity of this test is limited by the high cost of αα′-dipyridyl which is an extremely difficult compound to prepare.*

Method Using 7-Iodo-8-hydroxyquinoline-5-sulphonic Acid (Ferron)[2]. To the liquid to be examined is added a buffer solution containing 1·02 per cent. of potassium hydrogen phthalate and 0·131 per cent. of hydrochloric acid (HCl) in sufficient quantity to bring the reaction of the mixture to *p*H 2·5 ; to the mixture is added 0·25 ml. of a 0·2 per cent. aqueous solution of the reagent followed by sufficient water to produce 50 ml. of liquid. The stable green colour due to the presence of ferric iron is matched against standards similarly prepared.

Discussion. More careful control of experimental conditions is necessary with this method than with the one using thioglycollic acid, the colour being dependent on the concentration of reagent and the reaction of the medium. Aluminium combines with ferron to form an almost colourless complex and thus disturbs the test for iron[18] : chromium, cobalt and copper also interfere, and, to a lesser extent, nickel and uranium. Of the commonly occurring anions, citrates, cyanides, fluorides, orthophosphates, pyrophosphates, oxalates and tartrates all greatly decrease the intensity of colour produced by ferric iron. In the absence of interfering substances the green colour due to the presence of 1 part per million of trivalent iron in the reaction mixture is readily distinguishable from the yellow tint of the reagent. Summarising, it may be stated that the use of ferron constitutes a specific test for ferric iron in the sense that no other commonly occurring metal gives a green colour but not in the sense that it is free from interference by other metals.

Method Using Sodium Salicylate[8]. The iron solution, containing

* The synthesis suggested by J. P. Wibaut and J. Overhoff (*Rec. trav. chim. Pays-Bas*, 1928, **47**, 761) in which 2-bromopyridine is boiled with cymene in the presence of copper powder for 90 minutes, the solvent removed by steam distillation, the residue rendered alkaline and the αα′-dipyridyl distilled and recrystallised from dilute alcohol is probably the most satisfactory. The 2-bromopyridine may be prepared from 2-aminopyridine (obtained from pyridine and sodamide) by heating with cuprous bromide and hydrobromic acid; however, the yield is low, much of the bromine migrating to the β position.

between 0·03 and 0·4 mg. Fe is treated with 4 ml. of approximately 5N hydrochloric acid followed by 5 ml. of a 1 per cent. aqueous solution of sodium salicylate, and then approximately 9N ammonia drop by drop until the amethyst colour of the iron complex just changes to yellow. After adding 2 drops further of ammonia solution, approximately 8N acetic acid is added, at first drop by drop until the mixture just becomes pink, and then 5 ml. in excess ; finally, the solution is diluted with water to 50 ml. and the intensity of the solution immediately measured by means of a photometer or tintometer. The measuring instrument is calibrated by applying the test to appropriate quantites of a standard solution containing 0·1 mg. Fe per ml. made by dissolving 0·863 g. of ferric ammonium sulphate, $Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$, in slightly acidulated water and diluting to 1 litre.

Discussion. As above described, this method is not suitable for direct visual matching against natural standards and the originator of the technique employed a Hilger-Spekker photo-electric absorptiometer with No. 5 green filters having a transmission band of 5200 to 5400 Å and using 4-cm. cells, the scale reading being set to 1·0 with distilled water. Scott found that the colour produced by the test reaches maximum intensity in 30 to 45 minutes, depending upon the amount of iron present, then fades slightly to a value constant for many hours. Thus, if measurement of the colours produced be deferred for about an hour satisfactory results may be obtained by the method of direct comparison. The iron present should not exceed 0·5 mg. in 50 ml. of final reaction mixture while accurate measurements cannot be made when less than 0·01 mg. is present. It is important to note that comparable quantities of ferrous iron also give the amethyst colour due to the trivalent metal since the former is oxidised in the course of the test.

Titanium imparts a yellow colour to the final reaction mixture, making it useless for visual comparison, but, if a photometer is used, iron may be determined with fair accuracy provided the quantity of titanium does not exceed 0·3 mg. The influence of other substances is minimised by measuring the colour at zero time and disturbance by interfering ions is not appreciable provided the following amounts present in the final solution are not exceeded : $Al^{\cdots}$ 3·5 mg., $Mn^{\cdot\cdot}$ 12 mg., SiO_3'' 3 mg., PO_4''' 4 mg., F' 0·1 mg. and free chlorine 2 mg.

Application of the Sodium Salicylate Method to the Determination of Iron in Zinc[8]. The sample (2 g.) is dissolved in 20 ml. of

concentrated hydrochloric acid containing a little nitric acid, the solution treated with 2 ml. of a 1 per cent. aqueous solution of uranyl nitrate and ammonia added in slight excess. After bringing the mixture to the boil and allowing to stand for 20 minutes the precipitate of uranium hydroxide, together with entrained ferric hydroxide, is filtered off and dissolved in 10 ml. of 5N hydrochloric acid, the solution transferred to a Nessler glass, ammonia added until the liquid is almost alkaline followed by 1 ml. of a 10 per cent. aqueous solution of sodium salicylate and more ammonia, this time in slight excess as shown by the yellow colour due to uranium. Finally the mixture is acidified with 8N acetic acid until the amethyst colour due to iron is developed, at which stage the yellow uranium colour is destroyed; 10 ml. is then added in excess and the colour matched against standards containing the same quantity of uranium, preferably after standing 1 hour.

Notes on the Application. The above technique is Scott's modification of a method due to Agnew[19]. In conducting the original procedure there is some difficulty in deciding when the solution containing the iron and uranium has been nearly neutralised, and also a liability for salicylic acid to precipitate if the sodium salicylate is added in the absence of sufficient ammonia. By adding a 1 per cent. solution of sodium salicylate before the ammonia, as recommended by Scott, both these difficulties are overcome. The salicylate method is useful for the determination of traces of iron in zinc since in the presence of this metal the thioglycollic acid procedure is inapplicable.

REFERENCES

1. H. Ossian, *Pharm. Centralb.*, 1837, **13,** 205.
2. R. Andreasch, *Ber.*, 1879, **12,** 1391.
3. P. Claesson, *Ber.*, 1881, **14,** 412.
4. E. Lyons, *J. Amer. Chem. Soc.*, 1927, **49,** 1916.
5. F. Blau, *Monatsh.*, 1898, **19,** 647.
6. R. Hill, *Proc. Royal Soc.*, 1930, **107 (B),** 205.
7. J. H. Yoe, *J. Amer. Chem. Soc.*, 1932, **54,** 4139.
8. R. O. Scott, *Analyst*, 1941, **66,** 142.
9. L. Andrews, *Chem. News*, 1894, **70,** 165.
10. K. Heise, *Farbe u. Lack*, 1931, 464.
11. A. Bernhard and I. J. Drekter, *Science*, 1932, **75,** 517.
12. G. Barkan, *Klin. Wochenschr.*, 1932, **11,** 598.
13. H. W. Winsor, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9,** 453.
14. H. W. Swank and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.*, 1938, **10,** 7.
15. S. L. Tompsett, *Biochem. J.*, 1934, **28,** 1536.
16. B. R. Burmester, *J. Biol. Chem.*, 1934, **105,** 189.
17. R. A. Koenig and C. R. Johnson, *J. Biol. Chem.*, 1942, **143,** 159.
18. H. W. Swank and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9,** 406.
19. W. J. Agnew, *Analyst*, 1928, **53,** 30.

LEAD

There can be little doubt that in the absence of interfering metals the sulphide method is the best for colorimetric work and the conditions governing its application are now well established. During recent years the complementary problem of isolating lead from various materials preparatory to its determination as lead sulphide has received much attention. In discussing this method of determination Warington[1] stressed the importance of making colorimetric comparisons with standards containing the same quantity of dissolved salts as are present in the solution under examination. Later, it was shown by C. A. Hill[2] that "the colour given by a definite quantity of lead sulphide in a solution of any given salt is sensibly the same for different concentrations of the salt, provided that the concentration vary between certain limits." Hence, in determining lead present as impurity in soluble colourless salts, it is now customary to treat a solution containing 7 or 12 g. of the salt under examination with the appropriate reagents and match the resulting brown colour against a series of standards similarly prepared and each containing 2 g. of the same substance. This principle was embodied in the official tests for medicinal chemicals and the following account is based on the description included in the British Pharmacopœia, 1932.

Sulphide Method. The test is conveniently described by first giving as an example the simple case of determining the lead present as impurity in crystalline sodium sulphate. To do this, two solutions of the sample are made, the one containing 12 g. in a little hot water and the other 2 g. also dissolved in hot water. These solutions are transferred to Nessler glasses and 5 ml. of acetic acid (33 per cent. w/w) added to each. The solution containing the larger quantity of sample is conveniently termed the primary, while the other is referred to as the auxiliary solution. Both solutions are rendered alkaline with dilute ammonia (10 per cent. w/w NH_3) and 1 ml. of a 10 per cent. aqueous solution of potassium cyanide is added to each. If the colours of the solutions differ, the tints are equalised by the addition of a few drops of a highly diluted solution of burnt sugar or other non-reactive substance of similar colour. The two solutions are diluted to 50 ml. and 2 drops of a 10 per cent. aqueous solution of sodium sulphide, $Na_2S.9H_2O$, is added to the primary solution and the

latter stirred until uniform : if lead is present a brown colour is produced. A standard solution of lead, containing 0·01 mg. Pb per ml., is added to the auxiliary, the quantity used being such as experience suggests will be sufficient to give a colour equal in intensity to that of the primary solution after 2 drops of sodium sulphide have been added. Having in this manner made an approximate estimation of the quantity of standard lead solution needed to balance the primary colour, the test should be repeated, this time adding to the auxiliary an amount of standard lead solution such that when sodium sulphide is added the intensity of the brown colour will be equal to that in the primary.

The Pharmacopœia directs that the potassium cyanide solution be prepared by dissolving 10 g. in 90 ml. of water, adding 2 ml. of a 3 per cent. solution of hydrogen peroxide, allowing to stand for 24 hours and finally diluting to 100 ml. This procedure ensures freedom from sulphide which is liable to be present. No darkening should occur when 2 ml. of the cyanide solution is mixed with 5 ml. of dilute ammonia, 40 ml. of water and 5 ml. of standard lead solution. The standard lead solution is made by dissolving 0·160 g. of lead nitrate in a mixture of 50 ml. of concentrated nitric acid and sufficient water to produce 100 ml. and then diluting 10 ml. of this to 1 litre with water.

Discussion. It will be evident from the above example that the lead is being determined on 10 g. of the sample and the result should be calculated accordingly. A determination should not be accepted as accurate if more than 15 ml. of standard lead solution is required to effect a colour match and, in such cases, the difference in weight between the primary and auxiliary solutions should be reduced, say, to 5 g. by taking 7 g. for the former and 2 g. for the latter. Similarly, it is necessary to take smaller quantities in the examination of substances which are not sufficiently soluble to allow of 12 g. being dissolved in 50 ml. It is important that the standard lead solution be added to the auxiliary solution before the sulphide and hence the need for conducting the test twice unless the first estimate should prove to be correct. Provided not more than 15 ml. of standard lead solution is employed the use of a protective colloid such as gum acacia is unnecessary.

The potassium cyanide is included in order to prevent interference by copper and by slight traces of iron since with both metals it forms complex cyanides[3]. In the case of samples containing heavy traces of iron it is sometimes possible to prevent

disturbance by using more cyanide and heating the alkaline solutions in a boiling water-bath for some time before proceeding to test for lead. If iron is interfering with the test for lead the fact will be revealed by the appearance of a greenish tinge in the colour produced by adding sodium sulphide.

Application to Tartar Emetic. Obviously the test is inapplicable in the presence of metals giving dark coloured sulphides but it can be applied to antimony potassium tartrate. Owing to its parenteral application for certain tropical diseases this compound is officially required to contain not more than 5 parts per million of lead and the test is conducted by dissolving 2 g. of the sample in water, adding 7 ml. of a 20 per cent. aqueous solution of sodium hydroxide, 1 ml. of potassium cyanide solution and finally 2 drops of sodium sulphide solution. An auxiliary solution is similarly prepared using 1 g. of tartar emetic ; not more than 0·5 ml. of standard lead solution should be required in the latter to match the tint produced in the primary solution.

Application to Stibophen. This is the official name for sodium-antimony-bispyrocatechol-3:5-sodium disulphonate (also known under the trade name Fouadin) and the test for lead present as impurity which is included in the B.P. Third Addendum (1941) is given here as an example of the method as applied to an organic antimony compound.

To prepare the primary solution 1 g. is heated in a round-bottomed flask with 10 ml. of concentrated nitric acid and 5 ml. of concentrated sulphuric acid. After the first reaction has subsided, heating is continued until white fumes are evolved and the liquid is clear and almost colourless. After allowing to cool, the residual acid is diluted with 15 ml. of water, 1 g. of tartaric acid added, the liquid cooled and then neutralised by the addition of 20 per cent. aqueous solution of sodium hydroxide, a further 10 ml. of alkali being added in excess. The mixture is again cooled, transferred to a 100-ml. Nessler glass, 1 ml. of potassium cyanide solution added, the liquid diluted to the mark with water and 2 drops of sodium sulphide solution added. An auxiliary solution is prepared by dissolving 1 g. of tartaric acid in 50 ml. of water, adding 12·5 ml. of 20 per cent. aqueous solution of sodium hydroxide followed by 1 ml. of potassium cyanide solution and finally diluting to 100 ml. with water. It is officially required that not more than 0·5 ml. of standard lead solution should be added to the auxiliary solution in order to produce a mixture that, after the addition of sodium sulphide, yields a colour equal

in intensity to that produced in the primary solution, thus indicating that the stibophen does not contain more than 5 parts per million of lead.

Application to Calcium Phosphate. In order to determine lead present as impurity in calcium phosphate the following special procedure due to Nicholls[4] may be applied. A stock solution of ammonium citrate is made by dissolving 400 g. of citric acid in water, gradually adding about 340 ml. of ammonia (s.g. 0·880) and diluting to 1 litre. The test is carried out by dissolving 1 g. of the sample to be tested in 10 to 15 ml. of 2N hydrochloric acid, adding about 30 ml. of the stock solution of ammonium citrate, treating the mixture with 2 ml. of a 10 per cent. solution of potassium cyanide which has been made faintly alkaline with ammonia and finally adding 2 drops of 10 per cent. aqueous solution of sodium sulphide. Any colour is compared with that produced by adding sulphide to an auxiliary solution containing the same quantities of the reagents and an amount of standard lead solution estimated by trial to give the same tint.

An alternative method for the determination of lead in calcium phosphate using the diphenylthiocarbazone extraction is described on p. 67.

Application to Zinc and Compounds of Zinc. It was shown by T. T. Cocking[5] that, with suitable modifications, the colorimetric test could be applied directly to compounds of zinc. In a simple case, such as zinc sulphate, where only a minute trace of lead is present, the primary solution is prepared by dissolving 7 g. of the sample in water, adding 2 g. of ammonium acetate, excess of ammonia, and 2 ml. of potassium cyanide solution and finally a little very dilute hydrogen sulphide water. The auxiliary solution is prepared similarly, starting with 2 g. of the sample, and the determination is conducted in the manner already described.

In the case of metallic zinc, zinc oxide and zinc carbonate, however, a special procedure has to be followed, as these substances may be more heavily contaminated with lead and hence only a small quantity can be used for the test. For this process a solution of zinc ammonium acetate free from lead is required. This is prepared by dissolving 100 g. of zinc oxide in acetic acid (33 per cent. w/w), rendering the solution strongly alkaline with ammonia and filtering. A little ammonium sulphide (sufficient to precipitate all the lead and only a small portion of the zinc) is now added, the mixture warmed, filtered, and finally diluted to

1 litre. The filtrate should be tested for freedom from lead by adding to a portion dilute hydrogen sulphide water. In order to determine lead present as impurity in zinc oxide, 1 g. (or a suitable quantity) of the sample is dissolved in 33 per cent. w/w acetic acid, the solution diluted to 100 ml. with water and 5 ml. transferred to a Nessler glass together with 20 ml. of the zinc ammonium acetate solution (equivalent to 2 g. of zinc oxide); ammonia, potassium cyanide and very dilute hydrogen sulphide water are successively added and any brown colour produced is matched by the addition of lead to an auxiliary made by adding ammonia and potassium cyanide solutions to 20 ml. of the zinc ammonium acetate. The amount of lead which it is found necessary to add to the latter in order that there may be equality of colour upon the addition of sulphide is the quantity present in 0·05 g. of the zinc oxide. After standing some time the test solution will become cloudy from precipitation of zinc sulphide, but it remains perfectly clear for such time as amply suffices for completing the determination. If the solution of sulphide added be too concentrated, a cloudiness is produced at once and in this case the test is useless and must be discarded.

The presence of chlorides is inadmissible and on no account must hydrochloric acid be employed to dissolve the sample. Acetic acid should be used wherever possible and metallic zinc should be dissolved in nitric acid. This limitation is necessitated by the readiness with which lead is precipitated as the basic lead chloride, $PbCl_2.3PbO.4H_2O$, in ammoniacal solution.

Application to Food, Dyestuffs and Complex Organic Materials Generally. Obviously in these cases it is necessary to isolate the lead prior to its colorimetric determination as sulphide. This is most conveniently done by wet oxidising the sample, adding to the acid residue water, citric acid, excess of ammonia and potassium cyanide and extracting the lead by shaking with successive portions of a 0·1 per cent. chloroformic solution of diphenylthiocarbazone. The mixed chloroformic extracts are evaporated to dryness, the excess of reagent removed by wet oxidation and the lead in the residue determined by the sulphide colorimetric method[6]. This process separates lead from all other metals excepting thallium and bismuth; the latter may be removed by precipitating the lead as sulphate from the residue following the second wet oxidation. Monier-Williams[7] has described the full procedure for the examination of foods and has embodied the separation from bismuth according to the technique recom-

mended by Francis, Harvey and Buchan[8], together with the special method for the determination of lead in bone material developed by Roche Lynch, Slater and Osler[9]. This procedure is of wide application and is reproduced here.

A suitable weight of the sample, usually 5 g., but varying with the nature of the material and the amount of lead present, is transferred to a 250-ml. round-bottomed flask of Pyrex glass and submitted to wet oxidation using 5 ml. of concentrated sulphuric acid and a sufficiency of nitric acid which is added slowly from a tap-funnel. When oxidation is complete the cooled residue is diluted with water and again heated until the sulphuric acid fumes freely. After allowing to cool, the acid solution is diluted with about 20 ml. of water, 2 g. of citric acid is added and the solution boiled for a few minutes. Usually at this stage the liquid will be clear or it may contain a slight residue of silica but, if the original sample contained much calcium phosphate, a considerable precipitate of calcium sulphate will separate and in that event the special procedure described later must be employed.

If necessary, the solution should be filtered through a 7-cm. Whatman filter No. 44 which has been washed with hot approximately 5N hydrochloric acid and the residue on the filter extracted several times by pouring 10 ml. of boiling 5N hydrochloric acid over it, the same 10 ml. of acid being again heated and re-passed through the filter, the latter finally washed with hot water and the filtrate and washings concentrated to about 50 ml. and cooled. The solution is now neutralised to litmus paper by the cautious addition of ammonia (s.g. 0·880) and then 0·5 ml. added in excess, the mixture cooled, 1 ml. of a 10 per cent. aqueous solution of potassium cyanide AnalaR added, the liquid transferred to a separator and at once extracted with three successive 10-ml. portions of a 0·1 per cent. w/v solution of diphenylthiocarbazone in chloroform. If much lead is present, as indicated by the green reagent solution changing to a reddish hue, further extractions may be necessary. Each extract is washed in turn with 10 ml. of water in a smaller separator, the chloroformic layer being finally transferred to a 50-ml. conical flask of Pyrex glass and the solvent evaporated. The residue from the chloroform is now wet oxidised by adding 0·7 ml. of concentrated sulphuric acid, heating until white fumes are evolved and then adding nitric acid drop by drop until all organic matter is destroyed. The residual sulphuric acid is diluted with about 5 ml. of water and reheated until it fumes freely. The whole of the lead originally present in

the sample is now in the small flask together with about 0·5 ml. of sulphuric acid.*

To this acid residue is added 15 ml. of a mixture of 1 volume of absolute alcohol and 2 volumes of water and the flask set aside overnight. The next day, whether any precipitate is visible or not (0·02 mg. of lead shows) the solution is filtered through a 5-cm. Whatman filter No. 44 which has been washed with hot, approximately 5N hydrochloric acid and then with hot water just before use. The residue is washed three times with a few ml. of a mixture containing 20 ml. of water, 10 ml. of absolute alcohol and 1 ml. of concentrated sulphuric acid. The lead sulphate is dissolved by boiling 10 ml. of a 10 per cent. aqueous solution of ammonium acetate in the flask in which the precipitation was carried out and passing the hot solution through the filter. This operation is repeated, the same 10 ml. of solution being again boiled and passed through the filter : the latter is finally washed three times with about 5 ml. of hot water containing a little ammonium acetate. A rough estimate of the quantity of lead present having been formed from the appearance of the sulphate precipitate, the whole, or a suitable aliquot part of the solution, is transferred to a 50-ml. Nessler glass, rendered alkaline with ammonia, 1 ml. of potassium cyanide added, the mixture diluted to the mark, 2 drops of sodium sulphide solution added and any colour produced matched in the usual manner using an auxiliary solution containing 1 g. of ammonium acetate. A blank experiment should be performed on the apparatus and reagents used and the lead so found deducted from that found in the sample.

If the sample under examination contains much calcium phosphate (e.g. bone material or baking powder) the calcium sulphate which precipitates after the wet oxidation may retain lead. In such cases the acid mixture, suitably diluted with water, is trans-

* If bismuth is known to be absent the colorimetric determination of the lead can be carried out at once by diluting the acid residue with water, adding 2 g. of ammonium acetate, rendering alkaline with ammonia and, after transferring to a Nessler glass, adding 1 ml. of potassium cyanide and 2 drops of sodium sulphide solution and matching the colour produced in the ordinary way using an auxiliary solution containing 2 g. of ammonium acetate. Alternatively, it may be necessary to dilute the ammoniacal solution to a known volume and to operate on an aliquot part. Using this procedure, many analysts have failed to obtain a colourless primary solution : this is due to the manner in which the residue left after evaporation of the chloroformic extracts is wet oxidised. In order to prevent the formation of stable and coloured nitro-derivatives of diphenylthiocarbazone it is essential to heat to fuming with sulphuric acid before adding nitric acid, an important detail which was not adequately emphasised in the original paper on the method (*Analyst*, 1932, **57**, 440). D. C. Garratt (*Analyst*, 1935, **60**, 817) has suggested the use of 30 per cent. hydrogen peroxide in place of nitric acid for this stage of the process and this modification works well.

ferred to a 100-ml. centrifuging tube, the flask being washed out with a little ammonium acetate solution. The precipitate is separated by centrifuging, the clear liquid decanted back into the original round-bottomed flask, the residue in the centrifuge tube washed twice with two 20-ml. portions of hot water and the washings added to the original supernatant liquid. To the solid residue in the centrifuge tube is added 4 g. of potassium or sodium carbonate (anhydrous), 90 ml. of hot water and the tube immersed in a boiling water-bath for 4 hours, the contents being frequently stirred. The sides of the tube are washed down with water, the mixture cooled and then centrifuged and the supernatant liquid added to the solution in the original round-bottomed flask. The residue of insoluble carbonate is washed on the centrifuge once with cold water, and this washing also added to the liquid in the original flask. The clear solution in the round-bottomed flask contains the " soluble lead " and phosphate present in the original sample and is frequently coloured by iron salts. It is treated with 2 or 4 g. of citric acid, according to the amount of iron present, and then rendered alkaline with ammonia (s.g. 0·880), 0·5 ml. being added in excess and, after adding 1 ml. of potassium cyanide solution, extracted with diphenylthiocarbazone as described above. The residue of calcium carbonate in the centrifuge tube contains the " insoluble lead." It is mixed with about 50 ml. of water and acetic or hydrochloric acid is added until the carbonate is dissolved. The solution is boiled to remove carbon dioxide, using a flask of Pyrex glass, 2 g. of citric acid added followed by ammonia (s.g. 0·880) until an excess of 0·5 ml. is present and 1 ml. of potassium cyanide solution. The lead is extracted with diphenylthiocarbazone and the chloroformic solution combined with that derived from the " soluble " portion, the subsequent separation as sulphate and colorimetric determination being conducted as already described. The results obtained should be corrected by subtracting the value found by control experiments for any lead present in the reagents used.

The diphenylthiocarbazone extraction is recommended for the determination of lead present as impurity in acid calcium phosphate[10]. The test is conducted by dissolving 2 g. of the sample in 10 ml. of concentrated hydrochloric acid, adding to the cooled liquid 40 ml. of the ammonium citrate solution employed by Nicholls (see p. 63) and 15 ml. of ammonia (s.g. 0·880). After allowing the mixture to cool and then adding 1 ml. of a 10 per cent. aqueous solution of potassium cyanide solution the lead is

extracted by shaking with three portions (10 ml., 5 ml. and 5 ml.) of a 0·1 per cent. chloroformic solution of diphenylthiocarbazone and the determination completed in the manner already described. The change of the green colour of the diphenylthiocarbazone to red due to the presence of lead is not clearly apparent owing to the presence of the large amount of ammonium citrate. If more than 200 parts per million of lead is present in the acid calcium phosphate it is advisable to operate on a smaller sample.

Sulphide Method Using Permanent External Standards. A disc for use with the B.D.H. Lovibond Nessleriser is issued which has been standardised by a method based upon the recommendations in the Second Report to the Analytical Methods Committee of the Society of Public Analysts of the Sub-Committee on the Determination of Arsenic, Lead and other Poisonous Metals in Food Colouring Materials[11]. In order to obviate possible disturbance due to the presence of aluminium the procedure includes the use of ammonium citrate. It is conducted in the following manner :—

To a suitable quantity of the solution under examination (which should be approximately neutral and not exceed 25 ml. in volume) contained in a Nessler glass is added 10 ml. of a 10 per cent. w/v solution of ammonium acetate, 5 ml. of a 10 per cent. w/v solution of ammonium citrate, 5 ml. of dilute ammonia solution (10 per cent. w/w NH_3) and 1 ml. of 10 per cent. aqueous solution of sodium sulphide, $Na_2S.9H_2O$, and the mixture stirred. A control is prepared in another Nessler glass using the same quantities of all the reagents and diluting to 50 ml. with distilled water and the tint in the primary solution matched against the permanent glass standards of the Nessleriser disc in the usual way. The markings on the disc represent the actual amount of lead (Pb) producing the colour in the test and it embraces a range from 0·01 to 0·1 mg. Pb. The solution of ammonium citrate is conveniently prepared by dissolving 8·75 g. of citric acid (AnalaR) in water, neutralising with ammonia and diluting with water to 100 ml.

Diphenylthiocarbazone Method. Many papers have been published on the use of diphenylthiocarbazone for the colorimetric determination of lead as distinct from its use for isolating the metal from other substances[12–22]. In most cases the procedures described are extremely complicated and their application is mainly restricted to biochemical investigations requiring the determination of minute quantities of lead. The following procedure,

which includes the preliminary isolation, may be of service and is primarily intended for application to biological material.

A suitable quantity of the substance is submitted to wet oxidation by heating with sulphuric acid and 30 per cent. hydrogen peroxide. The cooled residue is diluted with water and 2 g. of citric acid dissolved in the liquid which is then rendered alkaline with ammonia. After adding 1 ml. of a 10 per cent. aqueous solution of potassium cyanide, the lead is extracted by shaking with three separate portions of a 0·1 per cent. solution of diphenylthiocarbazone in chloroform, using 10 ml. for the first extraction and 5 ml. of the second and third extractions. After washing each chloroformic extract with 10 ml. of water, the solvent is distilled off and the organic matter in the residue is destroyed by heating with 1 ml. of sulphuric acid and a little 30 per cent. hydrogen peroxide. To the acid residue is added 10 ml. of water, 1 ml. of glacial acetic acid, 5 ml. of ammonia (s.g. 0·880), 0·5 ml. of a 6 per cent. aqueous solution of sulphurous acid and, finally, sufficient water to produce 25 ml. An aliquot part of this solution (say 10 ml.) is transferred to a small separator containing 5 ml. of a 1 per cent. aqueous solution of potassium cyanide and the mixture is vigorously shaken with exactly 10 ml. of a 0·005 per cent. solution of diphenylthiocarbazone in carbon tetrachloride. After allowing to separate, the lower layer is drawn off and the excess greenish coloured reagent removed from the carbon tetrachloride by shaking with five successive 10-ml. portions of a 1 per cent. aqueous potassium cyanide solution. The residual pink liquid is then washed once with water and the intensity of the colour matched against standards similarly prepared. The variation in intensity of tint is best discerned between 0·005 and 0·07 mg. Pb. The standard solution should be prepared by dissolving 0·183 g. of lead acetate, $Pb(C_2H_3O_2)_2.3H_2O$, in water containing 5 ml. of glacial acetic acid, diluting with distilled water to 1 litre and, just before use, diluting 10 ml. of this to 100 ml., so that the final solution contains 0·01 mg. Pb per ml.

Discussion. In applying this test it is difficult to obtain a satisfactory blank owing to its high degree of sensitivity. Furthermore, although the treatment with cyanide eliminates the influence of most other metals it does not entirely prevent the response due to zinc which also gives a red colour with the reagent and traces of which are widely distributed in both biological and mineral substances. Proposals have been made for the use of this reagent titrimetrically and these are probably superior to the

colorimetric methods but their description is outside the scope of this book.

REFERENCES

1. R. Warington, *J. Soc. Chem. Ind.*, 1893, **12**, 97.
2. C. A. Hill, *Chem. & Drugg.*, 1905, **77**, 388.
3. F. L. Teed, *Analyst*, 1892, **17**, 142.
4. J. R. Nicholls, *Analyst*, 1931, **56**, 594.
5. T. T. Cocking, *Chem. & Drugg.*, 1906, **79**, 507.
6. N. L. Allport and G. H. Skrimshire, *Analyst*, 1932, **57**, 440.
7. G. W. Monier-Williams, Reports on Public Health and Medical Subjects No. 88 (1938), "Lead in Food."
8. A. G. Francis, C. O. Harvey and J. L. Buchan, *Analyst*, 1929, **54**, 725.
9. G. Roche Lynch, R. H. Slater and T. G. Osler, *Analyst*, 1934, **59**, 787.
10. D. W. Kent-Jones and C. W. Herd, *Analyst*, 1933, **58**, 152.
11. *Analyst*, 1935, **60**, 541.
12. H. Fischer and G. Leopoldi, *Angew. Chem.*, 1934, **47**, 90.
13. O. B. Winter, H. M. Robinson, F. W. Lamb and E. J. Miller, *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 265.
14. S. L. Tompsett and A. B. Anderson, *Biochem. J.*, 1935, **29**, 1851.
15. F. Morton, *Analyst*, 1936, **61**, 465.
16. P. A. Clifford and W. J. Wichman, *J. Assoc. Off. Agr. Chem.*, 1936, **19**, 130.
17. A. V. Evlanova, *J. Appl. Chem. Russ.*, 1936, **9**, 1690.
18. J. Cholak, D. M. Hubbard, R. R. McNary and R. V. Story, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 488.
19. D. M. Hubbard, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 493.
20. H. A. Liebhafsky and E. H. Winslow, *J. Amer. Chem. Soc.*, 1937, **59**, 1966.
21. D. O. Shiels, *J. Ind. Hyg.*, 1938, **20**, 581.
22. K. Bambach, *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 400.

MAGNESIUM

p-Nitro-benzene-azo-resorcinol, often referred to as magneson I and now well known as a useful reagent for the qualitative detection of magnesium[1] has, so far, not been successfully applied to quantitative work and the same may be said of the α-naphthol analogue, or magneson II[2]. Titan yellow has been suggested[3] as a suitable reagent for the colorimetric determination of this metal but the gradation of colour is only perceptible within inconveniently narrow limits. The azo dye, tropæolin OO (the sodium salt of phenylamino-benzene-azo-benzene sulphonic acid) forms a salt with magnesium which is insoluble in acidulated water but dissolves in concentrated sulphuric acid to form a deep bluish-red colour and it has been recommended for the determination of magnesium in blood[4]. However, heavy metals and iron interfere

and the method is not suitable for general application. Other proposals include indirect methods in which the magnesium is precipitated and isolated as the phosphate and the acid radicle determined colorimetrically[5–7] or, alternatively, the 8-hydroxyquinoline (oxine) complex converted to a dye by coupling with diazobenzene sulphonic acid[8]. Of these, the latter is the more promising and, although somewhat complicated, is worthy of description here since no simple method is available for this important metal. Other metals which are precipitated by 8-hydroxyquinoline under the conditions of the test will interfere but calcium and barium are conveniently removed as oxalate, while iron and aluminium are first precipitated by the reagent from acid solution.

Method[8]. The reagent solution is prepared by adding 4 g. of 8-hydroxyquinoline to 8 ml. of glacial acetic acid and pouring the mixture into 200 ml. of boiling water, stirring, and allowing to cool. To 1 ml. of the solution to be tested, which should be free from ammonium ions and acidified with acetic acid, is added 0·5 ml. of a saturated aqueous solution of sodium acetate and 0·5 ml. of the 8-hydroxyquinoline reagent. After standing for 3 hours in order to allow the iron and aluminium to precipitate, 0·2 ml. of a saturated solution of sodium oxalate is added, the mixture heated in a boiling water-bath for 30 minutes and then centrifuged. The clear supernatant liquid is transferred to another tube, the precipitate washed with a little cold water and the washings added to the liquid in the second tube. To this is then added 1 ml. of a saturated aqueous solution of sodium tartrate, 0·3 ml. of the 8-hydroxyquinoline reagent and 1 ml. of 2N sodium hydroxide. After standing overnight the mixture is heated for 30 minutes in a boiling water-bath then centrifuged, the magnesium precipitate is washed with two 2-ml. portions of dilute ammonia then dissolved in 5 ml. of hot N hydrochloric acid, the solution transferred to a 50-ml. flask and diluted to about 30 ml. with water. To this is added 0·5 ml. of a solution of 0·86 g. of sulphanilic acid in 100 ml. of 30 per cent. acetic acid and 0·5 ml. of a freshly prepared aqueous solution of sodium nitrite containing 0·29 g. in 100 ml. The mixture is allowed to stand for 10 minutes then made alkaline with 10 ml. of 2N sodium hydroxide and the solution diluted to 50 ml. with water. The stable red colour is compared with standards prepared by similarly treating a solution containing 0·01 mg. per ml. Mg, this quantity being taken as approximating to the middle of the working range.

A standard solution is conveniently made by dissolving 1·014 g. of magnesium sulphate, $MgSO_4.7H_2O$, in faintly acidulated water, making up to 1 litre then, just prior to use, diluting 10 ml. of this stock solution to 100 ml. with water.

Discussion. The preliminary treatment for the removal of iron and aluminium may not always be necessary, although generally it will be advisable to ensure the absence of calcium by including the addition of sodium oxalate. Direct preparation of the standard colours from an equivalent amount of 8-hydroxyquinoline leads to a slightly different tint and is, therefore, inadmissible. Manganese, copper, zinc and titanium interfere with the determination of magnesium by this method.

REFERENCES

1. K. Suitsu and K. Okuma, *J. Soc. Chem. Ind.* (*Japan*), 1926, **29**, 132.
2. J. V. Dubský and A. Okáč, *Chem. Listy.*, 1930, **24**, 492.
3. I. M. Kolhoff, *Chem. Weekblad*, 1927, **24**, 254.
4. K. Lang, *Biochem. Z.*, 1932, **253**, 215.
5. A. P. Briggs, *J. Biol. Chem.*, 1922, **52**, 349.
6. W. Denis, *J. Biol. Chem.*, 1922, **52**, 411.
7. A. P. Briggs, *J. Biol. Chem.*, 1924, **59**, 255.
8. F. Alten, H. Weiland and B. Kurmies, *Angew. Chem.*, 1933, **46**, 697.

MANGANESE

The usual method of determining traces of this metal involves oxidation of the divalent element to the heptavalent permanganic acid and measurement of the resulting pink colour. For this purpose four reagents are in current usage. The first of these to be proposed was lead dioxide in the presence of nitric acid and the method is frequently alluded to as Crum's test after its originator[1]; it was developed on a quantitative basis by Pichard[2]. Uncertainties attend upon the use of lead dioxide, much depending upon the manner in which the reagent is prepared and this disadvantage was met by alternative recommendations. Bismuth tetroxide was suggested by Schneider[3] while Reddrop and Ramage[4] advised the more easily prepared sodium bismuthate; this latter was first employed for the colorimetric determination of manganese, as distinct from volumetric methods, by Dufty[5] in 1901. In the same year H. Marshall[6] discovered that manganous salts could be oxidised to permanganates by means of

ammonium or potassium persulphate in the presence of silver nitrate as a catalyst and this reaction was applied to colorimetric work for steel analysis by Walters[7]. Lastly, Willard and Greathouse[8] recommended the use of potassium periodate for the oxidation; this is superior to the persulphate method since the reaction proceeds with a greater degree of certainty and the tendency for the permanganate colours to fade is minimised.

The use of these reagents will be illustrated by describing examples of their application to the determination of manganese in various types of material. Lead dioxide is still favoured by some steel analysts but, apart from this, is not often employed. Sodium bismuthate is extensively used, particularly in the iron and steel industry, but the determinations are more usually completed by volumetric methods. The other reagents have been extensively applied to the colorimetric determination of manganese in a variety of substances.

In a different category from the above is the extremely sensitive test for manganese using formaldoxime hydrochloride[9]. This method has received some attention during recent years[10–13] mainly in applications to biochemical work but, since iron interferes its utility is severely limited; furthermore, as the test is conducted in alkaline solution difficulties are likely to arise due to the precipitation of manganese phosphate[14] and this can only be circumvented by a somewhat complicated technique[15].

Application of the Lead Dioxide Method to Steel. A weighed sample is introduced into 15 ml. of nitric acid (s.g. 1·2, made by diluting 100 ml. of nitric acid s.g. 1·42 with 155 ml. of water), the acid boiled until the oxides of nitrogen have been expelled, 15 ml. of hot water and 2 to 3 g. of lead dioxide added and the liquid again boiled for about 3 minutes. The mixture is centrifuged, the supernatant liquid poured off, diluted to an appropriate volume and the colour matched against standards. As an alternative to centrifuging, the liquid may be diluted to a known volume, well mixed, filtered, or the excess lead dioxide allowed to settle and an aliquot part of the solution decanted off, and its colour matched. The standards should contain the same amount of iron as the sample and this may conveniently be added in the form of ferric ammonium sulphate, an appropriate quantity being treated with nitric acid and lead dioxide as above and a standard solution of potassium permanganate added. The latter is made by diluting 100 ml. of a 0·144 per cent. solution to 1 litre with water which has been slightly acidulated with sulphuric acid; this contains

0·05 mg. Mn per ml. The convenient working range of the test lies between 0·01 and 0·1 mg. Mn.

Discussion. The colorimetric method is disturbed by the presence of chromium and this limitation applies irrespective of which oxidising agent is used to give the permanganate colour. Similarly, nickel and cobalt will interfere but other metals met with in steel are not likely to cause trouble. When possible, it is a good practice to prepare the standards by applying the test to material of known manganese content and having in other respects a composition similar to that of the sample under examination. Apart from the known uncertainties of the test the method is not ideal for colorimetric work owing to the need for separating the solution from the large proportion of undissolved lead oxides.

Application of the Bismuthate Method to Steel. A suitable weighed quantity of the sample is dissolved in about 50 ml. of nitric acid (s.g. 1·5) and the solution boiled to expel oxides of nitrogen. After allowing to cool, 0·5 g. of sodium bismuthate is added and the liquid boiled until any pink colour initially formed disappears due to permanganate being converted to manganese dioxide which is precipitated. If no pink coloration is produced the addition of more sodium bismuthate should be tried. A saturated aqueous solution of potassium metabisulphite (chloride free) is added, a few drops at a time until the solution is quite clear, the mixture again boiled for about 2 minutes, cooled to 15° C. and a further 0·5 g. of sodium bismuthate added. After standing 10 minutes to allow the pink colour to develop the excess sodium bismuthate is removed by filtering through asbestos, 3 per cent. nitric acid being used for washing. The mixed filtrate and washings are diluted to an appropriate volume and matched against a standard solution of potassium permanganate (0·00144 per cent. in slightly acidulated water : equivalent to 0·005 mg. Mn per ml.). Standards are best prepared from steel of known manganese content and of otherwise similar composition to the sample under examination : failing this, the standard permanganate solution may be added to a manganese-free iron salt so that the standards shall contain an amount of iron approximately equal to the amount of sample under test.

Discussion. The method given above for the determination of manganese in steel is based upon the proposals of Blair[16] and Blum[17]. It is more usual to complete the determination volumetrically but where the proportion of manganese is small the colorimetric procedure may prove to be convenient. Preliminary

oxidation at boiling temperature followed by reduction and re-oxidation in the cold is necessary since the bismuthate, when dissolved in hot dilute nitric acid imparts a slight straw tint to the solution while oxidation is not complete if the initial treatment is conducted at room temperature. Sodium bismuthate is not so satisfactory as persulphate or periodate for colorimetric work and, according to Davidson and Capen[18], the bismuthate method yields slightly low results when applied to the examination of plant material.

Application of the Persulphate Method to Steel and Iron. An appropriate, accurately weighed, quantity of the sample to be examined and an equal amount of a standard steel or iron are each dissolved in 10 ml. of nitric acid (s.g. 1·2, made by diluting 100 ml. of nitric acid s.g. 1·42 with 155 ml. of water), nitrous fumes being expelled by warming. To each solution, contained in a suitable tube, is added 10 ml. of a 0·2 per cent. aqueous solution of silver nitrate and then, immediately, 1 g. of ammonium or potassium persulphate. The mixtures are heated by immersing the tubes in a boiling water-bath, the heating being continued for about a minute after oxidation has begun and, after cooling, the colours compared. The amount of sample to be taken for the determination will vary according to the expected manganese content as indicated in Table IV. In the case of cast iron, 1 g. should be dissolved in 30 ml. of nitric acid (s.g. 1·2), the solution filtered and diluted to 100 ml. with more nitric acid. For the determination, 20 ml. should be treated as described above.

TABLE IV.—QUANTITY OF SAMPLE TO BE TAKEN FOR THE DETERMINATION OF MANGANESE IN STEEL BY THE PERSULPHATE METHOD

Expected Manganese Content of Steel per cent.	Amount of Sample to be Taken g.
0·1 to 0·2	0·15
0·2 „ 0·4	0·1
0·4 „ 0·8	0·07
0·8 „ 1·2	0·05
1·2 „ 1·6	0·03

Discussion. When the manganese content is low and a relatively large amount of iron is present in the solution to be matched it may prove advantageous to remove the colour due to iron by adding phosphoric acid to both sample and standard. The interference of chromium may be overcome by precipitating

with sodium bicarbonate[19]. To accomplish this, the sample, say 1 g., is dissolved in 20 ml. of dilute sulphuric acid (100 ml. of concentrated acid in 1 litre) and the resulting solution diluted to about 100 ml. with boiling water. To this is added from a burette sufficient 8 per cent. aqueous solution of sodium bicarbonate (chloride free) to form a permanent precipitate and then an additional 4 ml. or sufficient to precipitate all the chromium present. The mixture is boiled for 1 minute, filtered as rapidly as possible and the filtrate, which may tend to become cloudy due to oxidation and hydrolysis of the iron, is examined for manganese. Other elements which may be present in steel such as aluminium, titanium, zirconium, phosphorus, vanadium, tungsten, copper and tin as well as some iron will be precipitated by the sodium bicarbonate but the loss of manganese due to adsorption is only slight.

Application of the Persulphate Method to Organic Material Containing Large Amounts of Calcium and Chlorides[20]. The sample, which may consist of the whole or part of a test animal, is incinerated for 8 hours in a silica beaker in a silica lined muffle furnace at a temperature slightly less than 700° C. The ash is dissolved in 10 ml. of concentrated nitric acid, 4 drops of concentrated sulphuric acid are added and the solution evaporated to dryness over an open flame in order to remove chlorides. The residue is dissolved in 12 ml. of 25 per cent. nitric acid and the solution diluted to a suitable volume, say 20 ml. or 50 ml. according to the amount of manganese expected to be present. A suitable aliquot part of this solution is transferred to a conveniently graduated tube and 0·2 ml. of a 0·25 per cent. aqueous solution of silver nitrate is added followed by 0·25 g. of potassium persulphate and the mixture diluted to 10 ml. The tube is immersed in a boiling water-bath and, if manganese is present, the stable pink permanganic acid colour will develop within a few minutes. The colour is compared with that produced by standards similarly prepared. The best colour gradations are obtained when the amount of manganese in the final 10 ml. of solution does not exceed 0·02 mg. of manganese.

Discussion. The above procedure was developed by T. W. Ray[20]. In treating the ash with nitric acid only 4 drops of sulphuric acid should be employed ; a greater quantity is undesirable since it will form more calcium sulphate than can be dissolved. The amount of nitric acid used in dissolving the chloride-free residue must not be greater than 15 per cent. of the final volume

since it has been found[21] that this is near the upper limit for developing the maximum intensity of the permanganic acid colour. Ray observes that although the oxidation can be conducted in the presence of either nitric, sulphuric or phosphoric acid the resulting colour has a different tint with each acid and it is therefore important that the same acid should be employed in making the standards as is used for the sample.

Application of the Persulphate Method to Soils. About a gram, or other suitable quantity, of the sample is ignited in a platinum dish, hydrofluoric acid and a little concentrated sulphuric acid added to the inorganic residue and the dish heated on a sand-bath until white fumes are evolved. After cooling, a little water is added, the liquid warmed until all salts are dissolved, the solution filtered into a 100-ml. graduated flask, 1 ml. of a 0·5 per cent. aqueous solution of silver nitrate added followed by 1 g. of ammonium persulphate. The flask is immersed in a boiling water-bath for 30 minutes, the contents cooled, diluted to volume and any pink permanganate colour matched against standards similarly prepared from a solution of manganous sulphate. The content of manganese in the sulphate used to make the standard solution should be determined gravimetrically since the salt may contain a variable proportion of water. An alternative way to prepare the sample consists in fusing the ignited residue with a mixture of 10 parts of potassium pyrosulphate and 1 part of sodium fluoride and extracting the cooled melt with hot water. The test for manganese is then conducted on the resulting solution.

Discussion. It has been observed by Hough[22] that titanium inhibits the development of the permanganate colour in the persulphate test and this investigator recommends the use of potassium periodate as the oxidising agent when determining manganese in soils which contain any appreciable quantity of titanium. Attempts to remove the latter by repeatedly evaporating the ignited residue from soil samples with hydrofluoric acid without the addition of sulphuric acid were not successful.

General Procedure for the Periodate Method[8]. The material to be tested is brought into a solution containing in 100 ml. not less than 10 ml. of concentrated sulphuric acid or 20 ml. of concentrated nitric acid or 5 ml. of phosphoric acid (s.g. 1·75) or equivalent mixtures of these acids. The solution should be freed from reducing agents by boiling with nitric acid and, if carbon compounds are present as in steel, about a gram of potassium persulphate should also be added. If chloride is present it should

be removed by adding both nitric acid and sulphuric acid and the mixture evaporated until white fumes are evolved. Finally, to the acid solution thus prepared 0·2 to 0·4 g. of potassium or sodium periodate is added, the mixture brought to the boil and the containing vessel then immersed in a boiling water-bath for 30 minutes. After cooling, the solution is diluted to an appropriate volume and the pink colour matched against standards. It will generally be satisfactory to employ a solution of ordinary potassium permanganate (0·0144 per cent. containing 0·05 mg. Mn per ml.) for preparing the standards, but, where a greater degree of precision is desired, manganous sulphate solution of known strength should be added to manganese-free material which is otherwise of the same chemical nature as the sample under examination.

Discussion. In the presence of a considerable quantity of iron either sulphuric acid or phosphoric acid must be present since ferric periodate is insoluble in fairly concentrated nitric acid but readily soluble in the other acids. With the exception of chromium, nickel and cobalt, the common metals do not interfere excepting in so far as they may impart a tint to the solution. The colour of ferric salts may be removed by the addition of phosphoric acid, but the colour due to other metals must be compensated by adding comparable amounts to the standard. A number of metals such as silver, lead, bismuth and mercury form iodates or periodates which are insoluble in dilute acids, but by using a high concentration of acid these remain in solution, since the amount of periodate added is small. If a reducing agent, such as a ferrous salt, is present, free iodine will be liberated from the periodate and thus disturb the test; in these circumstances a preliminary boiling with nitric acid is essential. For a consideration of the effect of varying the acid concentration of the test solution the notes on the application of the method to organic material should be consulted (*vide infra*). There is little doubt that for colorimetric work potassium periodate is the best reagent; the course of the reaction is more regular than in the persulphate procedure while the colour produced is identical with that of a permanganate standard and shows little tendency to fade. The high cost of potassium or sodium periodate is apparently the only disadvantage to the method.

Application of the Periodate Method to Organic Material: Plant or Animal Tissue.[23] The material is incinerated in a silica basin at

a low red heat, a little concentrated hydrochloric acid added to the residue and evaporated off, a few ml. of 12N sulphuric acid and 4 drops of concentrated nitric acid added and the mixture again evaporated by gentle ignition. To the residue, 2·5 ml. of 12N sulphuric acid is added followed by a little water and the mixture heated over a Bunsen flame until white fumes are evolved. This operation removes all trace of chlorides. After allowing to cool, the acid is diluted with water and filtered into a 50-ml. flask, 2 small pieces of pumice (previously purified by boiling with dilute sulphuric acid and a little periodate) added and the liquid boiled down to a volume of about 10 ml. To this solution which is now at the optimum concentration of acid for the oxidation (about 2N) is added 0·3 g. of potassium (or sodium) periodate the liquid brought to the boil and the flask immersed in a boiling water-bath for 30 minutes. Any pink colour, resulting from manganese present in the sample, is then matched against standards, or, if the colour is too deep for direct comparison, an appropriate aliquot part is diluted with 2N sulphuric acid that has been boiled with a little periodate.

The standards are prepared from a stock solution made by dissolving 0·144 g. of potassium permanganate in about 100 ml. of water and reducing, either by passing in sulphur dioxide or, alternatively, by adding sulphuric acid and sodium sulphite, boiling until reduction is complete and all excess sulphur dioxide is removed, allowing to cool and diluting to 1 litre (1 ml. of this solution contains 0·05 mg. Mn). The standard solution for comparison is prepared from the stock solution by oxidising 20 ml. with periodate as described above and diluting to 1 litre with 2N sulphuric acid which has been previously boiled with a little periodate. Thus, 1 ml. of this final solution contains 0·001 mg. Mn and 7 to 10 ml. give convenient colour intensities for matching while a detectable pink colour can be obtained when 1 ml. is diluted to a volume of 10 ml. and increments of 0·2 ml. are perceptible. The standard colours are stable for weeks if kept free from dust.

A special procedure is necessary for substances containing much calcium. For example, in order to determine manganese in milk 100 ml. of the sample is evaporated in a silica basin over a water-bath, the residue incinerated, the ash treated with a little hydrochloric acid, the latter evaporated off, the residue moistened with a little water, broken up as finely as possible and 25 ml. of boiling 12N sulphuric acid added, the mixture being stirred mean-

while. After allowing to stand for several hours (overnight if possible) the liquid is filtered through a hard filter paper into another silica basin, the insoluble residue being washed with about six portions of 12N sulphuric acid each of about 5 ml. The filtrate and washings are evaporated to dryness and the manganese then determined in the residue as described above commencing with the addition of 2·5 ml. of 12N sulphuric acid so as to ensure that the optimum acid concentration is obtained in the solution prepared for the oxidation.

In cases where the ratio of iron to manganese is high, e.g. in blood and spleen, it is advisable, after the evaporation with sulphuric acid and concentrated nitric acid, to add 2 ml. of phosphoric acid (s.g. 1·75) and 0·5 ml. of 12N sulphuric acid in place of 2·5 ml. of the latter. This modification minimises the formation of the yellow tint due to the iron.

Discussion. The above technique is due to M. B. Richards[23] and the main endeavour has been to remove chlorides and to ensure that the correct concentration of sulphuric acid is obtained in the final solution prior to the oxidation with periodate. It was observed that when the concentration of acid is low the colour develops rapidly but is yellowish and tends to fade. The rate of oxidation appears to be at a minimum when the concentration of acid approximates to 4N but above this the colour appears more readily as the strength of the acid increases but when the latter is much more than 6N the tendency to fading is again manifested. At the concentration chosen, namely about 2N, the oxidation is reasonably expeditious and no tendency to fading is observed.

Some interesting results of the manganese content of various biological substances as determined by this method are quoted by Richards. Thus, various species of liver were found to contain from 0·139 to 0·416 mg. Mn per 100 g. of moist tissue. Corresponding figures found for kidney were 0·157 to 0·174 and for spleen 0·018 while the content of manganese in milk is given as 0·0041 mg. Mn per 100 g. The value for potatoes varied from 0·473 to 1·18 calculated on the dried material. The figure given for tapioca is 0·682 and the average content of several samples of grass is quoted as 61·4 mg. Mn per 100 g. of dry matter.

REFERENCES

1. W. Crum, *Annalen*, 1845, **55**, 219.
2. P. Pichard, *Compt. rend.*, 1872, **75**, 1821.
3. L. Schneider, *Dingl. polytech. J.*, 1888, **269**, 224.
4. J. Reddrop and H. Ramage, *J. Chem. Soc.*, 1895, **67**, 268.

5. L. Dufty, *Chem. News*, 1901, **84**, 248.
6. H. Marshall, *Chem. News*, 1901, **83**, 76.
7. H. E. Walters, *Chem. News*, 1901, **84**, 239.
8. H. H. Willard and L. H. Greathouse, *J. Amer. Chem. Soc.*, 1917, **39**, 2366.
9. A. Bach, *Compt. rend.*, 1899, **128**, 363.
10. G. Denigès, *Compt. rend.*, 1932, **194**, 895.
11. E. Kahane, *Ann. Chim. anal.*, 1935, **17**, 175.
12. C. P. Sideris, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 445.
13. G. H. Wagenaar, *Pharm. Weekblad*, 1938, **75**, 641.
14. A. C. Wiese and B. C. Johnson, *J. Biol. Chem.*, 1939, **127**, 203.
15. C. P. Sideris, *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 307.
16. A. A. Blair, *J. Amer. Chem. Soc.*, 1904, **26**, 793.
17. W. Blum, *J. Amer. Chem. Soc.*, 1912, **34**, 1395.
18. J. Davidson and R. G. Capen, *J. Assoc. Off. Agr. Chem.*, 1931, **14**, 547.
19. G. E. F. Lundell, J. I. Hoffman and H. A. Bright, *Ind. Eng. Chem.*, 1923, **15**, 1064.
20. T. W. Ray, *J. Biol. Chem.*, 1940, **134**, 677.
21. T. W. Ray, *J. Lab. Clin. Med.*, 1938, **23**, 1304.
22. G. J. Hough, *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 408.
23. M. B. Richards, *Analyst*, 1930, **55**, 554.

MERCURY

The accurate determination of minute quantities of this metal is a complicated procedure. Many colorimetric methods have been suggested, such as formation of the colloidal sulphide[1, 2], the use of ammonia and potassium iodide[3], phosphomolybdo-tungstic acid[4], diphenyl-carbazide[5] or diphenyl-carbazone[6] but none of these can be recommended. More recently, *p*-dimethylamino-benzal-rhodanine (*p*-dimethylamino-benzylidine-rhodanine) which under certain conditions, gives a brick-red colour in the presence of mercuric ions, has been proposed for colorimetric determinations[7]. A method, suitable for the determination of mercury in organic material, has been ably worked out by N. Strafford and P. F. Wyatt[7] which involves wet oxidation of the sample, precipitation and decomposition of mercury sulphide, separation of the mercury by electrolysis and the final colorimetric determination. This procedure, which is presented in full, may readily be adapted to suit particular requirements.

Method for Organic Material[7]. The wet oxidation is conducted in a 100-ml. Kjeldahl flask the neck of which carries a side-arm fitted with a glass-tapped funnel of about 15 ml. capacity as shown in Fig. 6. A condenser with a bulb of about 150 ml. capacity fits into the mouth of the flask by means of a ground glass joint. The lower end of this condenser reaches nearly to the bottom of a trap

consisting of a 250-ml. wide-mouthed conical flask containing 40 ml. of 5M sodium hydroxide, and closed by a tightly fitting 2-holed rubber stopper. A second condenser leads from this trap, its lower end reaching nearly to the bottom of another trap consisting of a 100-ml. conical flask containing 25 ml. of water, 1 ml. of 0·04M copper sulphate and 0·05 g. of paper pulp. The latter is prepared by dividing a 12·5-cm. No. 44 Whatman paper

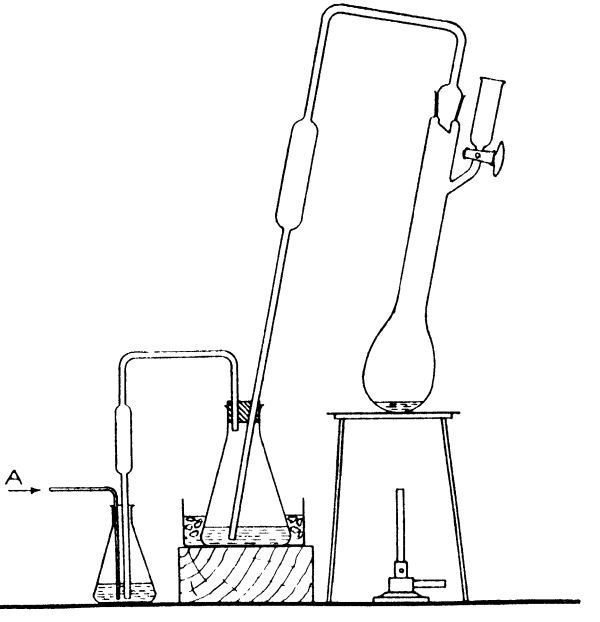


FIG. 6.—APPARATUS FOR THE WET OXIDATION OF ORGANIC MATERIAL AS USED FOR THE DETERMINATION OF MERCURY

The first trap is charged with 40 ml. of 5M sodium hydroxide while the second contains 25 ml. of water, 1 ml. of 0·04M copper sulphate and paper pulp with hydrogen sulphide passing in at A during the digestion. (After N. Strafford and P. F. Wyatt, *Analyst*, 1936, **61**, 528.)

into sixteen segments and disintegrating one of these by breaking it up with a glass rod in a little boiling water.

A weighed portion of the sample is transferred to the dry Kjeldahl flask which is then connected to the condensers and traps. The first trap is kept cooled during the wet oxidation by means of a bath of ice and water while a steady stream of hydrogen sulphide is passed through the liquid in the second trap. From 10 to 15 ml. of concentrated sulphuric acid is run into the Kjeldahl flask from the tap funnel and the latter, after being closed, is charged with 30 per cent. hydrogen peroxide. The flask is sup-

ported on an asbestos ring about 1·5 cm. in diameter and is heated by means of a small Bunsen flame. About 1 ml. of hydrogen peroxide is added from time to time and the heating continued until the oxidation of the sample is complete, the stream of hydrogen sulphide through the second trap being continuously maintained during the process. While the oxidised mixture in the flask is still being heated to boiling, 30 to 40 ml. of water is slowly added from the tap funnel and after boiling has been continued for a further 15 minutes the acid is allowed to cool, the tap being opened at intervals in order to equalise the pressure. The current of hydrogen sulphide is now stopped, the apparatus disconnected, the alkaline contents of the first trap transferred to a 600-ml. beaker surrounded with ice and water, the contents of the Kjeldahl flask cautiously added and the latter together with the first trap and its condenser washed out with a little water. The contents of the second trap and the washings from its condenser are mixed and the liquid then set aside.

Strong solution of ammonia (s.g. 0·880) is added to the contents of the beaker until a drop of the solution spotted on to Congo red paper gives only a faint greyish-black ring or spot, then a cold saturated aqueous solution of potassium permanganate is added drop by drop until the pink colour produced by the last drop fades out only slowly on stirring. The contents of the second trap are then added, the latter being rinsed with a little water, the mixed liquid (containing also any insoluble material remaining after the wet oxidation) then diluted to about 400 ml. and a fairly rapid stream of hydrogen sulphide passed in for 20 minutes. After allowing to stand for a further 20 minutes the mixture is filtered through a Gooch crucible with a pad consisting of 0·05 g. of paper pulp prepared as described above, the pad being firmly pressed down with a flattened glass rod while suction is applied. The beaker is washed out with hydrogen sulphide solution, the inner surface wiped with a small piece of filter paper, which is then added to the contents of the crucible ; the filter is washed with a little more hydrogen sulphide solution and finally with four or five 2-ml. portions of acetone in order to remove any wax-like organic matter which has distilled over during the decomposition. The crucible and its contents are dried at 60° C. for 10 minutes, a small perforated porcelain disc placed on the precipitate, the whole introduced into an all glass continuous extraction apparatus *

* Alternatively, the crucible may be placed on a glass tripod in a beaker without spout containing carbon disulphide and the mouth of the beaker closed

similar in design to that illustrated in Fig. 7 and the precipitate extracted for 1 hour with carbon disulphide. After allowing to dry, the precipitate and paper pad are detached from the crucible

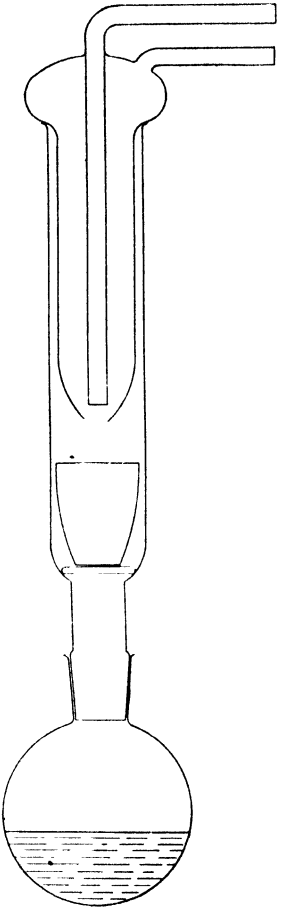


FIG. 7.—APPARATUS RECOMMENDED FOR THE EXTRACTION OF FREE SULPHUR FROM PRECIPITATED MERCURIC SULPHIDE

The Friederich condenser is a separate unit and rests upon the lip of the extraction tube which is attached to the flask containing carbon disulphide by means of an all-glass joint.

by means of a wire and transferred to a 100-ml. Kjeldahl flask, the inside of the crucible and the perforated disc cleaned with a fragment of filter paper, this being added to the contents

by a bolt-head flask containing cold water. When the beaker is warmed on a water-bath the vapour of the solvent condenses on the bottom of the flask and drips continuously in a fine stream through the crucible.

of the flask which are then treated with about 1 ml. each of concentrated sulphuric acid and 1 ml. of concentrated nitric acid. The mixture is heated gently over a low flame until the paper and precipitate are completely oxidised. If oxidation is difficult a further small quantity of nitric acid (up to 2 ml.) may be added, the solution being heated until the sulphuric acid is *slightly* fuming, due regard being paid to the possibility of losing mercury by volatilisation as a result of overheating. Finally, 2 ml. of water is added and the liquid boiled gently until nitrous fumes disappear.

The liquid, including any insoluble silica derived from the sample, is transferred to an electrolysis vessel, consisting of a flat-bottomed glass tube 7 cm. high and 2 cm. internal diameter with a small lip, and the flask washed out with three 2-ml. portions of water. The solution is rendered slightly alkaline with ammonia until the blue colour of the cuprammonium compound appears (the vessel and its contents being cooled in ice-water in the meantime) then just acidified with N nitric acid, a further 1 to 2 ml. added in excess and the solution diluted to 20 ml. with water. The vessel is closed with a cover-glass provided with two holes 1 cm. apart for the accommodation of the electrodes, the cathode being a piece of platinum foil (5 cm. × 2 cm.) bent into semi-cylindrical form and supported by a stout platinum wire welded to its upper edge, and the anode being a platinum wire spiral of the same length as the cathode. The solution is electrolysed at 3 to 4 volts with an initial current of about 0·05 ampère and after 16 to 20 hours the electrodes are washed with cold water, the flow of current broken, the cathode detached and transferred to a test tube about 8·5 cm. long and 1·5 cm. in diameter.

The deposited mercury is dissolved from the cathode by adding 5 ml. of N nitric acid and immersing the test tube in a boiling water-bath for 15 minutes. After being cooled to room temperature the acid solution is transferred to a 100-ml. Nessler glass and the test tube and electrode washed with cold water. The liquid is diluted to 95 ml. with water and stirred, 3 ml. of a 0·02 per cent. solution of *p*-dimethylamino-benzal-rhodanine in alcohol (95 per cent.) added, the volume of the mixture adjusted to 100 ml. with water and, after standing for 5 minutes, the intensity of the brick-red colour matched against standards in which the colour has been developed simultaneously with that of the sample under examination. A standard solution of mercuric nitrate is made by accurately weighing about 0·5 g. of clean, dry mercury,

dissolving it in 5 ml. of concentrated nitric acid diluted with water, boiling to remove nitrous fumes and making up to 250 ml. A suitable quantity of this stock solution is then further diluted to produce a standard containing 0·1 mg. or 0·01 mg. Hg per ml. according to requirements. The colours for comparison are prepared by transferring suitable known amounts of standard mercuric nitrate solution into 100-ml. Nessler glasses, adding 1 ml. of 0·04M copper nitrate and 5 ml. of N nitric acid, diluting to 95 ml. and stirring, adding 3 ml. of *p*-dimethylamino-benzal-rhodanine reagent, diluting to 100 ml. with water and mixing. The best gradation of colour is obtained with amounts of mercury ranging from 0·01 to 0·2 mg. in 100 ml. of solution.

Discussion. The colour due to mercury which is produced by the reagent may be developed in neutral or acetic acid solution, but is then largely masked by the strong yellow tint of the excess *p*-dimethylamino-benzal-rhodanine. By conducting the test in the presence of a controlled amount of nitric acid the yellow colour of the excess reagent is discharged, whilst that due to the mercury persists. The reagent colour is just completely suppressed in 0·1N nitric acid but this degree of acidity is not ideal for the formation of the mercury complex, whence it has been necessary to effect a compromise by conducting the colorimetric determination in the presence of 0·05N nitric acid. Since the mercury complex is produced as a colloidal suspension there is a marked tendency for precipitation to occur on standing, hence it is important that the colours in the test and standards be developed simultaneously. According to Strafford and Wyatt[7] the use of gum acacia as a protective colloid sometimes led to unreliable results. In order that the colour may develop satisfactorily it is necessary to ensure that there is no local over-concentration of nitric acid before adding the reagent.

Sulphate and halogens ions, even in traces, interfere with the formation of the mercury complex hence the electrolytic separation is essential. The only metals, other than mercury, which give a positive reaction with the reagent, under the conditions described, are silver, cuprous copper, gold, platinum and palladium ; divalent copper does not interfere. Experiment has shown that there is no danger of platinum derived from the electrodes entering the final test solution. Removal of free sulphur from the precipitate of the mixed sulphides of copper and mercury is necessary since some of the latter will be retained by the globule of sulphur which otherwise remains after the treat-

ment with the sulphuric acid-nitric acid mixture. The presence of the copper previously added as a " carrier " actually facilitates the electrolytic separation of the mercury and minimises the danger of loss of mercury by volatilisation from the cathode. The two traps which are attached to the apparatus used for carrying out the wet oxidation of organic material are essential if loss of mercury due to volatilisation is to be avoided.

An entirely different method for the colorimetric determination of mercury, which has been applied to urine by Hubbard[8], consists in initial wet oxidation of the sample with sulphuric acid aided by the periodic addition of solid potassium permanganate, volatilisation being checked by a water-cooled condenser, dilution of the digest with water and extraction of the mercury and any copper which may be present by shaking with a chloroformic solution of di-β-naphthylthiocarbazone. The mercury is separated from the copper and removed from this chloroformic layer by shaking the latter with an acid aqueous solution of sodium thiosulphate. This aqueous extract, containing the mercury, is then submitted to wet oxidation and the diluted digest again extracted with a chloroformic solution of di-β-naphthylthiocarbazone and the red component due to the mercury in the greenish coloured reagent measured by means of a photoelectric spectrophotometer. The procedure is stated to be suitable for quantities of mercury between 0·005 and 0·05 mg. The author has not had experience of this method and the reagent is not at present commercially available but references to the details for its preparation are given in Hubbard's paper.

REFERENCES

1. A. Heinzelmann, *Chem.-Ztg.*, 1910, **35**, 721.
2. C. Kohn-Abrest, *Ann. Chim. anal. Chim. appl.*, 1925, **30**, 353.
3. L. L. Lloyd and W. M. Gardner, *J. Soc. Chem. Ind.*, 1912, **31**, 10.
4. V. Ciocalteu and C. Titel, *Compt. rend. Soc. Biol.*, 1933, **112**, 621.
5. P. Menière, *Compt. rend.*, 1908, **146**, 754.
6. A. Stock and E. Pohland, *Z. angew. Chem.*, 1926, **39**, 791.
7. N. Strafford and P. F. Wyatt, *Analyst*, 1936, **61**, 528.
8. D. M. Hubbard, *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 768.

MOLYBDENUM

The thiocyanate reaction, first described by A. D. Braun[1], is the best for colorimetric work. Other methods which have been suggested for quantitative application include the use of hydrogen

peroxide[2], with which molybdates produce a brownish-red colour in faintly ammoniacal solution, and tannic acid[3], which yields an orange colour in the presence of molybdenum ; however, these are not so satisfactory, the former being uncertain in action while the latter suffers interference from the presence of iron and numerous other metals.

Method. To the neutralised solution to be examined, containing up to about 1 mg. of molybdenum, is added 8 ml. of concentrated hydrochloric acid, 5 ml. of a 2 per cent. aqueous solution of stannous chloride and 5 ml. of a 10 per cent. aqueous solution of potassium thiocyanate ; the mixture is diluted to 50 ml. with water and the light-brown or reddish-brown colour produced, which attains its maximum intensity in 5 minutes, is matched against standards similarly prepared. The colour is readily soluble in ether and it is the usual practice to extract with this solvent. A standard solution, containing approximately 0·2 mg. Mo per ml., may be made by dissolving 0·5 g. of sodium molybdate in 1 litre of water containing 5 ml. of concentrated sulphuric acid and determining its exact strength by passing 100 ml. through a Jones zinc reductor[4] into a vessel containing solution of ferric sulphate and phosphoric acid and titrating the resulting mixture with 0·05N potassium permanganate ; under these conditions, 1 ml. of 0·05N permanganate is equivalent to 0·0016 g. Mo. A blank run on the reductor should be carried out using similar quantities of sulphuric acid and water. Alternatively, the molybdenum solution may be standardised gravimetrically by precipitation as lead molybdate and the ammonium salt may be employed in place of sodium molybdate. The test is sensitive to 0·001 mg. Mo.

Discussion. This colour test is singularly free from disturbance due to the presence of other substances and it is applicable in the presence of iron, nickel, chromium, vanadium, silicates and phosphates. Preliminary separations or modifications to the test may be necessary if insoluble thiocyanates are formed ; thus, if an appreciable quantity of copper is present the cuprous thiocyanate should be filtered off before the extraction by ether of the colour due to molybdenum. Hurd and Allen[5] made an exhaustive study of the conditions governing the colour development and found that with low concentrations of acid (0·5 per cent.) the colour reaches a maximum in 8 minutes and then fades, but with 5 per cent. of hydrochloric acid some fading occurs in the first few minutes but thereafter the colour remains almost

constant ; these experiments were conducted in the presence of 1 per cent. of potassium thiocyanate and 0·8 per cent. of stannous chloride. A higher concentration of hydrochloric acid induces fading. Not less than 0·6 per cent. of potassium thiocyanate should be present in the final mixture and the concentration of stannous chloride should exceed 0·1 per cent. If sulphuric acid is employed the final mixture should contain 10 per cent. of acid. These investigators found that extraction of the colour with butyl acetate as suggested by James[6] was liable to lead to anomalous results. Extraction of the colour with a solvent is advisable since it obviates any possible bleaching effect due to the excess stannous chloride which slowly reduces the coloured pentavalent molybdenum thiocyanate ; at the same time the sensitivity of the test is increased. In the method as described above, the ether used as a solvent for the colour should be tested for freedom from peroxide by shaking a sample with a freshly prepared mixture of potassium iodide and starch solution. The formation of a blue colour indicates the presence of ether peroxide and in this event the ether should be shaken with a 10 per cent. aqueous solution of sodium thiosulphate and redistilled.

The method has been applied to the determination of molybdenum present as impurity in tungsten[7] but it finds its chief application in steel analysis where it is particularly useful when the concentration of molybdenum is less than 1 per cent. The following procedure is a slight modification of that recommended by T. R. Cunningham and H. L. Hamner[8].

Application to Steel. An appropriate weight of the sample, say 0·5 g. or, if the expected molybdenum content is less than 0·1 per cent., 1 g., is transferred to a 150-ml. beaker, 25 ml. of approximately 7N sulphuric acid added and the contents warmed to about 60° C. When the reaction has ceased, 3 ml. of 30 per cent. hydrogen peroxide is added and the mixture boiled for 5 minutes. If carbon is present it is filtered off, washed with hot water and the filtrate and washings boiled down to a volume of about 10 ml. If tungsten has separated, 1 g., or a sufficiency, of citric acid is added in order to dissolve it. In either case, the solution is neutralised with a 10 per cent. aqueous solution of sodium hydroxide then 20 ml. of 10N sulphuric acid added, the mixture diluted to 100 ml. with water, 10 ml. of 10 per cent. solution of potassium thiocyanate added, the mixture transferred to a separator and 10 ml. of a solution containing 35 per cent. of stannous chloride in approximately N hydrochloric acid added. After allowing to

stand for 5 minutes the colour due to molybdenum is extracted by shaking, first with 50 ml. then with two further 25-ml. portions of peroxide-free ether. The combined ether extracts are transferred to a 100-ml. Nessler glass and the colour matched against standards. The standards are made by adding an appropriate quantity of a standard solution of molybdenum (prepared in the manner suggested above in the general description of the test) to 25 ml. of an 8 per cent. solution of ferric sulphate, $Fe_2(SO_4)_3.9H_2O$, in 5N sulphuric acid * contained in a separator and, after diluting to 100 ml. with water, proceeding in the same way as for the sample. In matching the colour it is better to prepare a series of standards rather than rely upon the principle of diluting the ether extracts : alternatively, a colorimeter or tintometer may be employed. In preparing the standards it is essential that iron be present since this metal exerts a catalytic effect upon the reaction and intensifies the colour due to molybdenum.

REFERENCES

1. A. D. Braun, *Z. anal. Chem.*, 1867, **6**, 86.
2. W. Bettel, *Chem. News*, 1908, **79**, 40.
3. G. Spurge, *Chem. Eng. Min. Rev.*, 1919, **11**, 258.
4. Clemens Jones, *Trans. Amer. Inst. Min. Engrs.*, 1888–89, **17**, 411.
5. L. C. Hurd and H. O. Allen, *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 396.
6. L. H. James, *Ind. Eng. Chem., Anal. Ed.*, 1932, **4**, 89.
7. W. A. King, *Ind. Eng. Chem.*, 1923, **15**, 350.
8. T. R. Cunningham and H. L. Hamner, *Ind. Eng. Chem., Anal. Ed.*, 1931, **3**, 106.

NICKEL

Among the proposals for the colorimetric determination of this metal may be mentioned that depending upon the production of a reddish colour when potassium thiocarbonate[1, 2] is added to ammoniacal nickel solutions and the method using potassium dithio-oxalate[3, 4] which, in a slightly acid medium, produces a magenta colour in the presence of nickel. Both these methods are subject to the disadvantage that many other metals, notably iron and cobalt, interfere, thus necessitating the application of special procedures. When dimethylglyoxime is added to alkaline solutions containing oxidising agents an intense red coloration is

* Alternatively, a 14 per cent. solution of ferric ammonium sulphate, $Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$, in 5N sulphuric acid may be used.

produced. This effect was investigated by Feigl[5] who showed that the reaction served as a delicate qualitative test for nickel, the sensitivity being 1 part in 1,666,000 as compared with 1 part in 400,000 for the normal reaction of the metal with dimethylglyoxime. Feigl also demonstrated that the colour is due to the formation of a tetravalent nickel compound. The reaction was later applied to quantitative work by Rollet[6], who used bromine as oxidising agent and ammonia as alkali, and this procedure, which has been modified and adapted by other investigators to the determination of small concentrations of nickel in steel[7, 8] and in silicate rocks[9], is probably the best available for colorimetric purposes. The delicate qualitative test for nickel present as impurity in cobalt salts proposed by Middleton and Miller[10] some years before the date of Feigl's investigations utilises the same reaction, the oxidation in this case being effected with hydrogen peroxide. This test is capable of yielding approximately quantitative results and a brief description of it is included below.

Method for Plain Steels[7]. A weighed sample (1 g.) is dissolved in 10 ml. of concentrated hydrochloric acid, 5 ml. of concentrated nitric acid is added to the solution, the liquid boiled for a few minutes then diluted with cold water and transferred to a 200-ml. graduated flask. Diluted ammonia (1 vol. of ammonia s.g. 0·880 and 1 vol. of water) is added from a burette until a slight precipitate of ferric hydroxide is formed which just fails to redissolve, then 2 ml. of a 1 per cent. aqueous solution of potassium cyanide is added, the flask shaken, 10 ml. more of the ammonia solution introduced and the mixture diluted to the mark with warm water, no allowance being made for the volume of the precipitate. The mixture is filtered through a fluted Whatman No. 41 paper.

If copper is known to be absent a suitable aliquot part of the filtrate, expected to contain not more than 0·1 mg. Ni is transferred to a 100-ml. Nessler glass and, if necessary, diluted to the mark with water then 2 ml. of an almost saturated solution of dimethylglyoxime in alcohol added, the mixture stirred, 1 ml. of sodium hypochlorite solution (about 10 per cent. w/v available Cl) added and the liquid again stirred. Another 100-ml. Nessler glass is filled to the mark with water, 6 drops of diluted ammonia added followed by 2 ml. of dimethylglyoxime solution and, after stirring, 1 ml. of sodium hypochlorite solution. Any reddish-brown colour due to nickel which has developed in the test mixture is matched by adding to the second Nessler a standard nickel solution (0·05

mg. Ni per ml.) 3 drops at a time from a narrow 10-ml. burette, several minutes being allowed to elapse between each addition. The standard solution of nickel is prepared by dissolving 0·05 g. of spectroscopically standardised nickel metal in dilute nitric acid and making up the resulting solution to 1 litre with water: alternatively, a solution containing 0·5 mg. Ni per ml. may be made by dissolving 3·368 g. of nickel ammonium sulphate, $NiSO_4.(NH_4)_2SO_4.6H_2O$, in water containing a little sulphuric acid and making up to 1 litre and freshly diluting tenfold as required. If copper is present in the filtrate from the ferric hydroxide a measured portion is acidified with hydrochloric acid, about 5 per cent. of the strong acid being added in excess, and hydrogen sulphide is passed into the warm solution in a rapid stream for 15 minutes. Any copper sulphide (mixed with a little sulphur) is allowed to settle out, the liquid meanwhile being kept warm, and the mixture filtered through paper pulp into a 600-ml. wide-mouthed beaker and the precipitate washed with a 5 per cent. aqueous solution of ammonium chloride. Removal of the whole of the copper by long standing is not necessary since the presence of traces does not influence the determination of nickel. The filtrate is boiled down to low bulk, removed from the source of heat and 50 ml. of concentrated nitric acid added to destroy ammonium salts. Further heat is applied cautiously, the whole being taken to complete dryness, but not baked for any length of time; small extra additions of nitric acid may be necessary to complete the destruction of the ammonium salts. The residue is treated with hot water until dissolved, filtered if necessary, and the colorimetric test applied to the resulting cooled solution.

Application to Manganese Steels[7]. The sample is dissolved in 30 ml. of concentrated nitric acid, the solution evaporated to a small volume and an additional 50 ml. of the strong acid added. The solution is cooled, 5 ml. of chloric acid (s.g. 1·12) added, the mixture boiled for 5 minutes, then cooled, a further 50 ml. of strong nitric acid and 5 ml. of chloric acid added and the solution again boiled for 5 minutes. After cooling, the mixture is filtered by suction through asbestos and the precipitate of manganese peroxide washed with strong nitric acid. The filtrate is boiled down to small volume, 10 ml. of concentrated hydrochloric acid added, the solution again boiled for a few minutes and the colorimetric test applied, copper, if present, being first removed as sulphide.

Application to Cobalt Steels[7]. For every 0·01 g. of cobalt ex-

pected to be present in the sample taken, an additional 5 ml. of 1 per cent. solution of potassium cyanide is added thus producing the yellow cobalticyanide. The test is then continued as described for plain steels until the final filtrate is obtained. Suitable equal parts of the latter are each diluted to 100 ml. with water and

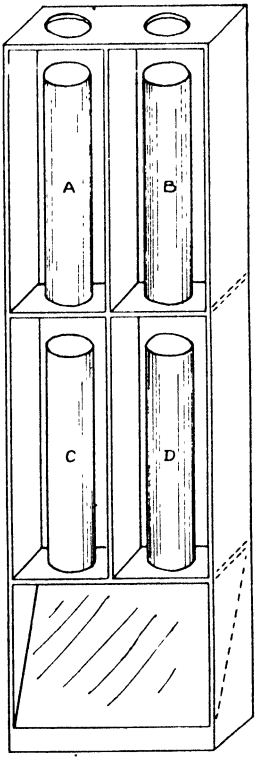


FIG. 8.—THE WALPOLE COMPARATOR

This consists essentially of a box accommodating four Nessler glasses so arranged that light passes vertically upwards through the lower vessels and then into each of the corresponding tubes superimposed above. In this way the intensity of colour reactions conducted in tinted solutions can be measured. (After G. S. Walpole, *Biochem. J.*, 1910, **5**, 207.)

transferred to two Nessler glasses marked A and B which are then placed in the upper compartment of a Walpole comparator[11], Fig. 8, immediately over two other Nessler glasses, C and D, respectively, in the lower compartment, each containing 100 ml. of water, 6 drops of diluted ammonia, 2 ml. of dimethylglyoxime solution and 1 ml. of sodium hypochlorite solution. The same reagents are then added to Nessler glass A and the contents of

Nessler glass D are then titrated with standard nickel solution until the combined colour of the pair BD matches that of AC, the pairs being viewed vertically.

Method for Silicate Rocks[9]. The sample is finely powdered and 0·25 g. of basic rock, or 0·5 g. of an acidic variety, is weighed into a platinum dish and a little water added followed by 0·5 ml. of 70 per cent. perchloric acid and 2·5 ml. of 40 per cent. hydrofluoric acid, these quantities being correspondingly increased for samples greater than 0·25 g. The mixture is evaporated to dryness on a sand-bath, the residue taken up in 0·5 ml. of perchloric acid diluted with 2 or 3 ml. of water and the contents of the dish again evaporated to dryness. The residue is dissolved in about 6 ml. of boiling dilute hydrochloric acid, 5 ml. of a 10 per cent. aqueous solution of sodium citrate added, the cooled mixture cautiously neutralised with strong ammonia solution (s.g. 0·880) and a few drops of the latter added in excess. If there is an appreciable amount of precipitate or insoluble residue in the mixture at this stage it is filtered, the filter washed with small portions of hot water, the paper and its contents ignited, the resulting residue fused with 0·1 g. of anhydrous sodium carbonate, the cooled melt dissolved as far as possible in hot dilute hydrochloric acid, 2 or 3 ml. of a 10 per cent. aqueous solution of sodium citrate added followed by a slight excess of ammonia and the resulting solution reserved for separate treatment.

To the main solution (or the filtrate from any insoluble material) is added 2 ml. of a 1 per cent. alcoholic solution of dimethylglyoxime and the mixture extracted with three portions of chloroform each of about 3 ml. The reserved solution derived from the sodium carbonate melt is similarly treated with dimethylglyoxime and extracted with chloroform. All the chloroform extracts are combined in a separator, vigorously shaken with 10 ml. of approximately 0·3N ammonia, the mixture allowed to separate, the chloroformic layer drawn off, care being taken that drops of the aqueous phase do not accompany it, and the water layer washed with a small additional quantity of chloroform in order to recover any suspended drops of solvent. The chloroformic solution of nickel dimethylglyoxime is shaken with two successive 5 ml. portions of 0·5N hydrochloric acid and the acid extracts transferred to a small tube suitable for colour matching. A series of standards in 10 ml. of approximately 0·5N hydrochloric acid are prepared containing from 0·001 mg. up to not more than 0·05 mg. Ni and to the test solution and each of the standards

5 drops of a freshly prepared saturated aqueous solution of bromine is added. Each mixture is decolorised by the dropwise addition of strong solution of ammonia (s.g. 0·880), 3 drops being added in excess, then 0·5 ml. of a 1 per cent. alcoholic solution of dimethylglyoxime introduced into each tube. Any red colour produced in the test mixture is matched against that of the standards.

Discussion. The above accounts of particular applications will serve as guides to analysts for the employment of this colorimetric method in other connections. In the method given for determining nickel in steels, chromium, molybdenum, aluminium and vanadium are all precipitated, along with the iron, by ammonia. Tungsten may be eliminated in the early stages as tungstic acid, but if the amount is small, filtration is unnecessary as the tungsten is removed quantitatively in the iron precipitate and, in any case, it only interferes by reason of the tendency for separated tungstic acid to obscure the neutralisation point prior to the addition of potassium cyanide. In the special application to cobalt steels copper does not interfere, unless present in large amounts, owing to the excess cyanide in the filtrates. In the procedure for plain steels it is essential that all copper be removed since, otherwise, it gives rise to a pink colour in the final test. The reaction will detect 0·01 mg. Ni in 100 ml. of solution and the colour is stable for some hours but the tint produced in the test liquid may not always precisely match that of the standards owing to the interference of ammonium salts and decomposition products from the excess cyanide added. According to B. Jones[7], the originator of the application of this colorimetric method to steel, the nickel content should not exceed 0·06 per cent. and samples containing a higher concentration should be assayed by gravimetric or volumetric methods. On the other hand, Murray and Ashley[8] claim that by using a Zeiss-Pulfrich step-photometer and maintaining the iron in solution by the addition of citric acid and oxidising with bromine, nickel may be determined with a degree of accuracy amply sufficient for works purposes in steels containing up to 15 per cent. of nickel.

The method given above for the determination of nickel in rocks is not disturbed by copper, cobalt, manganese, chromium or vanadium when these are only present in such amounts as are likely to be encountered in silicate rocks but it may be expected that much copper and cobalt will lead to high results. Manganese in appreciable amounts may cause trouble by oxidising

nickel to the tetravalent state in the ammoniacal solution during shaking, whence low results would be obtained because nickelic dimethylglyoxime is not extracted by chloroform.

Approximate Determination of Nickel in Cobalt Salts[10]. About 0·2 g. of the sample is dissolved in 2 ml. of water, 4 ml. of a 10 per cent. aqueous solution of potassium cyanide, 1 ml. of 6 per cent. hydrogen peroxide and 1 ml. of N sodium hydroxide are added, the mixture warmed on a water-bath for 10 minutes, then 2 ml. of a 10 per cent. aqueous solution of ammonium chloride and 0·2 ml. of a 1 per cent. alcoholic solution of dimethylglyoxime added. The mixture is transferred to a porcelain dish and evaporated to dryness on a water-bath. The presence of 0·004 mg. Ni, or more, is indicated by a pink tint surrounding the margin of the residue.

REFERENCES

1. M. Lucas, *Bull. Soc. chim.*, 1899, **21**, 432.
2. V. Lindt, *Z. anal. Chem.*, 1914, **53**, 165.
3. H. O. Jones and H. S. Tasker, *J. Chem. Soc.*, 1909, **95**, 1904.
4. L. T. Fairhall, *J. Ind. Hyg.*, 1926, **8**, 528.
5. F. Feigl, *Ber.*, 1924, **57**, 758.
6. A. P. Rollet, *Compt. rend.*, 1926, **183**, 212.
7. B. Jones, *Analyst*, 1929, **54**, 582.
8. W. M. Murray, Jr., and S. E. Q. Ashley, *Ind. Eng. Chem., Anal. Ed.*, 1938, **10**, 1.
9. E. B. Sandell and R. W. Perlich, *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 309.
10. A. R. Middleton and H. L. Miller, *J. Amer. Chem. Soc.*, 1916, **38**, 1705.
11. G. S. Walpole, *Biochem. J.*, 1910, **5**, 207.

POTASSIUM

Three methods are described below for the indirect colorimetric determination of this metal. The first, due to Sideris[1], depends upon precipitation of the potassium as sodium potassium cobaltinitrite followed by isolation of the latter and determination of its content of cobalt by means of nitroso-R salt. An alternative procedure for determining the cobalt, suggested by Jacobs and Hoffman[2], depends upon measurement of the emerald green colour produced by the addition of choline hydrochloride in the presence of potassium ferrocyanide and an account of Eden's modification[3] of this method is included here. An alternative and more sensitive reaction depending upon precipitation as silver potassium

cobaltinitrite, originally proposed for qualitative use by Burgess and Kamm[4], has been applied to the determination of potassium in blood serum by Breh and Gaebler[5] : the description given here follows the technique recommended by J. E. Harris[6] which embodies improvements suggested by Robinson and Putnam[7].

Method of C. P. Sideris Using Sodium Cobaltinitrite (*General Application*)[1]. The sample to be tested should be in the form of a solution in dilute hydrochloric acid containing up to 1 mg. of potassium in 5 ml. The solution is rendered slightly alkaline with sodium hydroxide, boiled for 15 minutes in order to remove any ammonia which may be present, filtered and any precipitate washed with hot water. The reaction of the filtrate is adjusted to about *p*H 4·5 with hydrochloric acid, the liquid transferred to a centrifuge tube of 15-ml. capacity and then evaporated to a volume of 0·5 ml. or less. A 12·5 per cent. aqueous solution of sodium cobaltinitrite is diluted with an equal volume of ethyl alcohol (95 per cent.) and 5 ml. of this mixture is added to the centrifuge tube and the latter placed in a refrigerator for not less than 4 hours. After being centrifuged, the supernatant liquor is removed by means of a capillary siphon and the precipitate washed, first with 10 ml. of water and then with 5-ml. portions of acetone until the washings are no longer coloured yellow. The precipitate is dissolved by warming with 5 ml. of 2N sulphuric acid, the solution diluted with water so that the potassium concentration may be expected to lie between 0·002 and 0·04 mg. in 5 ml. and this quantity is then submitted to the colorimetric test for cobalt using the nitroso-R salt method (see p. 44). The factor for converting weight of cobalt found into potassium is 1·327.

Discussion. This is a useful and reasonably reliable method although it will generally be necessary to isolate the potassium, together with sodium, from any other metals which may be present in the sample. The composition of the precipitate of sodium potassium cobaltinitrite has been the subject of many investigations and there is no doubt that it is liable to variation. However, providing the precipitate forms in a solution in which the ratio of the quantity of sodium to that of potassium exceeds 22 the composition of the precipitate may be regarded as constant and closely approximating to the formula $K_2NaCo(NO_2)_6$. The precipitation of less than 0·04 mg. K is not practicable by this procedure and in cases where greater sensitivity is required the method using silver cobaltinitrite should be employed.

Method of A. Eden Using Sodium Cobaltinitrite (*General Application and Application to Serum*)[3]. A solution of sodium cobaltinitrite is made by dissolving 120 g. of sodium nitrite in 180 ml. of water and pouring 210 ml. of this into a solution made by dissolving 25 g. of cobalt nitrate, $Co(NO_3)_2.6H_2O$, in 50 ml. of water. After aspirating air through the mixture until all nitrous fumes have been removed it is set aside in a refrigerator and filtered through a Whatman No. 40 paper immediately before use.

A suitable aliquot part of the solution under examination, expected to contain between 0·1 and 0·3 mg. K (or 1 ml. of serum), is transferred to a 15-ml. centrifuge tube, 0·5 ml. of a 50 per cent. w/v aqueous solution of sodium nitrite added, the mixture diluted to 4 ml. with water and 2 ml. of the above solution of sodium cobaltinitrite added drop by drop from a burette. After stirring, the mixture is left for 1 hour then centrifuged. The clear supernatant fluid is removed with a hand siphon having a fine, slightly upturned tip and the precipitate washed, first, with a saturated aqueous solution of sodium potassium cobaltinitrite then, with two separate portions of 70 per cent. alcohol. After removal of the last washing, 3·5 ml. of water is added, the centrifuge tube immersed in a boiling water-bath until the precipitate has completely dissolved, then, after allowing to cool, 1 ml. of a freshly prepared 1 per cent. aqueous solution of choline hydrochloride is added followed by 1 ml. of a freshly prepared aqueous solution of potassium ferrocyanide and the mixture diluted to 6 ml. with water. The intensity of any green colour produced is compared in a colorimeter with a standard colour prepared by diluting 1 ml. (or other suitable quantity) of a 0·0435 per cent. solution of freshly ignited cobalt sulphate, $CoSO_4$, in 0·005N sulphuric acid to 4 ml. with water and adding 1 ml. each of the solutions of choline hydrochloride and potassium ferrocyanide. The standard cobalt solution contains 0·165 mg. Co per ml. corresponding to 0·20 mg. K.

Discussion. The Lovibond tintometer is not well suited for measuring the green colour produced in this test and it is better to employ either a colorimeter of the Duboscq type or to take measurements in a photometer with suitable light filters against a blank containing a mixture of 1 ml. each of the choline hydrochloride and potassium ferrocyanide solutions diluted to 6 ml. with water. In the above method the factor used for converting the weight of cobalt found into potassium is slightly different from that recommended in other procedures of similar character and is conditioned by the fact that precipitation is conducted in

the presence of a very large excess of sodium nitrite. The solution under examination may be a diluted acid extract of the ash from ignited organic material but it is important that it be free from ammonium salts.

Method Using Silver Cobaltinitrite (*for Blood Serum*)[6]. The following special reagents are required :—

1. Precipitating Reagent. A solution of 120 g. of sodium nitrite in 180 ml. of water is added to a solution made by dissolving 25 g. of cobalt nitrate, $Co(NO_3)_2.6H_2O$ in 60 ml. of 25 per cent. aqueous solution of acetic acid. The mixture is aerated for 2 hours then one-tenth of its volume (usually 28 ml.) of a 40 per cent. aqueous solution of silver nitrate added and the precipitate which first forms is redissolved by shaking vigorously. The reagent which will keep for about a month should be stored away from light in a refrigerator but, prior to use, it is warmed to room temperature and, if necessary, filtered.

2. Washing Fluid. A mixture of 2 vols. of alcohol (95 per cent.), 1 vol. of ether (free from peroxide) and 2 vols. of water.

3. Diazotisation Solution. A freshly prepared mixture of 1 vol. of 0·5 per cent. solution of α-naphthylamine in a 30 per cent. aqueous solution of acetic acid and 2 vols. of 0·5 per cent. sulphanilic acid in the same solvent.

In applying the method to blood serum, or plasma, 1 ml. of the sample is added to 7 ml. of water contained in a centrifuge tube followed by 0·5 ml. of 2/3N sulphuric acid, 0·5 ml. of a 10 per cent. aqueous solution of sodium tungstate and 1 ml. of an aqueous solution of silver nitrate (1·8 per cent.) with thorough mixing after each addition. After standing for 15 minutes the tube is centrifuged, 4 ml. of the clear supernatant fluid is transferred to another 15-ml. centrifuge tube, 1 ml. of alcohol (95 per cent.) added, the tube immersed in a water-bath maintained at 20° C. and, after 5 minutes, 2 ml. of the precipitating reagent is added and the mixture maintained at 20° C. for 30 minutes. The tube is then centrifuged, the bulk of the supernatant fluid removed by means of a capillary siphon and the precipitate washed twice or thrice with the washing fluid, the latter being drained off as completely as possible each time by applying filter paper to the lip of the tube while the latter is held at a slightly tilted angle. The precipitate is dissolved by adding 10 ml. of 0·2N sodium hydroxide and immersing the tube in a boiling water-bath for 10 minutes. The solution is cooled under the tap,

transferred to a 25-ml. graduated flask, diluted to the mark with water, filtered, 5 ml. of the filtrate (representing 0·08 ml. of the original sample) added to 15 ml. of a 10 per cent. aqueous solution of acetic acid contained in a 50-ml. graduated flask, 3 ml. of the diazotisation solution added and, after standing for 5 minutes, the mixture diluted to the mark with 10 per cent. acetic acid and the pink colour matched against that of standards. The latter are prepared by taking amounts of freshly prepared potassium sulphate solution containing from 0·005 mg. K upwards, diluting to 4 ml. with water, adding 1 ml. of alcohol (90 per cent.) adjusting the temperature to 20° C., adding 2 ml. of the precipitating reagent and continuing as above. The standards should be made by appropriately diluting a 0·0223 per cent. solution of potassium sulphate which contains 0·1 mg. K per ml.

(*for Whole Blood*)[6]. Less of the sample should be taken : it is recommended that 0·2 ml. be added to 8·3 ml. of water, followed by 0·5 ml. of 2/3N sulphuric acid, 0·5 ml. of 10 per cent. solution of sodium tungstate and 0·5 ml. of a 1·8 per cent. solution of silver nitrate. After separating the protein precipitate by centrifuging 2 ml. of the clear liquid is mixed with an equal volume of water, 1 ml. of alcohol (95 per cent.) added and the determination continued as described for serum and plasma.

Discussion. This method is more sensitive, although more troublesome to carry out, than the sodium cobaltinitrite procedures. However, it may hardly be accounted so reliable as the latter. According to Harris[6] it is important that precipitation should take place at 20° C. since at temperatures above 25° C. the nitrite to potassium ratio in the precipitate is not constant while, if the mixture is too cool (16° C.), silver nitrite may settle out. Again, there is a tendency for the silver potassium cobaltinitrite to separate in a colloidal form. Fairly accurate control of the amount of silver nitrate added in the preparation of the protein- and chloride-free centrifugate is necessary but a slight excess of either chloride or silver has no effect. Thus, chloride values ranging from 450 to 850 mg. NaCl per 100 ml. are well tolerated under the conditions of the test as described. Beyond these limits it is advisable to increase or decrease the amount of silver nitrate added to the serum (or other sample) as the situation demands. High values result if the silver nitrate concentration is much more than 0·05 per cent., the value theoretically obtained with the lower chloride limit mentioned above. The presence of the ammonium ion will lead to high results but the small amount

of ammonia present in freshly drawn serum or plasma is insufficient to interfere.

REFERENCES

1. C. P. Sideris, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 145.
2. H. R. D. Jacobs and W. S. Hoffman, *J. Biol. Chem.*, 1931, **93**, 685.
3. A. Eden, *Analyst*, 1943, **68**, 167.
4. L. L. Burgess and O. Kamm, *J. Amer. Chem. Soc.*, 1912, **34**, 652.
5. F. Breh and O. H. Gaebler, *J. Biol. Chem.*, 1930, **87**, 81.
6. J. E. Harris, *J. Biol. Chem.*, 1940, **136**, 619.
7. R. J. Robinson and G. L. Putnam, *Ind. Eng. Chem., Anal. Ed.*, 1936, **8**, 211.

SILVER

Traces of silver can often be determined nephelometrically as the chloride and, at present, there is no satisfactory colorimetric procedure available. The method depending upon the reddish colour produced by the interaction of silver ions and *p*-dimethylamino-benzal-rhodanine (*p*-dimethylamino-benzylidine-rhodanine) possesses some limited value but many other metals react to give colours[1, 2]. It has been proposed for control purposes in connection with the sterilisation of water by means of silver[3].

Method[3]. The neutral solution to be examined (containing between 0·06 and 9·0 mg. Ag per litre) is mixed with 1 g. of ammonium acetate and sufficient water to produce 15 ml. To this mixture is added 0·5 ml. of a 0·02 per cent. alcoholic solution of *p*-dimethylamino-benzal-rhodanine and any pink or red colour produced is matched against standards prepared from silver nitrate. A standard solution containing 0·01 mg. Ag per ml. may be prepared by dissolving 1·576 g. of silver nitrate in water slightly acidulated with nitric acid, making up to 1 litre and diluting the resulting solution a hundredfold just prior to use.

Discussion. Soft glass readily adsorbs silver and it is best to employ apparatus made of fused silica but, provided the solutions are not warmed, Pyrex glassware may be used. Gold, platinum, palladium, monovalent copper and mercury also react, while calcium carbonate, sodium nitrate and ammonium nitrate increase the depth of colour due to silver if any one of them is present in amounts greater than 1 mg. per litre. Chlorides may be removed by evaporating the test solution with nitric acid in a silica dish. Nitrites produce a deep yellow colour with the reagent and, if present, should be removed by slightly acidifying

the solution to be tested, adding a little sodium azide and boiling for a few minutes ; the mixture can then be neutralised and the colorimetric test applied.

REFERENCES

1. F. Feigl, *Z. anal. Chem.*, 1928, **74**, 380.
2. K. Heller and P. Krumholz, *Mikrochem.*, 1929, **7**, 214.
3. I. C. Schoonover, *J. Research Natl. Bur. Standards*, 1935, **15**, 377.

SODIUM

Probably the most satisfactory procedure for the colorimetric determination of sodium involves precipitation as uranyl zinc sodium acetate, $(UO_2)_3ZnNa(CH_3COO)_9.6H_2O$, followed by solution of the precipitate, addition of potassium ferrocyanide and measurement of the intensity of the brown colour produced by the uranium present. The use of uranyl zinc acetate for the precipitation of sodium was originally proposed by Kolthoff[1, 2] and the application of the reaction for colorimetric determination was first suggested by Barrenscheen and Messiner[3] but their technique was found to be defective in certain details and it was greatly improved by McCance and Shipp[4]. The method has been employed in biochemical work, particularly for the determination of sodium in blood serum.

Method[4]. The undermentioned reagents are required :—

1. Alcoholic Solution of Uranyl Zinc Acetate. Uranyl acetate (10 g.) is dissolved in 50 ml. of boiling water containing 2 ml. of glacial acetic acid and 30 g. of zinc acetate is dissolved in a separate 50-ml. portion of boiling water containing 1 ml. of acetic acid. The solutions are mixed while boiling, allowed to stand overnight, filtered, an equal volume of absolute alcohol added to the filtrate and the mixture allowed to stand 48 hours at 0° C. then filtered at 0° C.

2. Alcohol Saturated with the Triple Acetate. A sample of uranyl zinc sodium acetate is prepared by adding uranyl zinc acetate to some sodium chloride dissolved in alcohol (50 per cent.), filtering off the precipitate, thoroughly washing it with alcohol (95 per cent.) and suspending it in alcohol of the same strength. If the supernatant liquid is not absolutely clear it should be filtered before use.

3. Alcoholic Solution of Zinc Acetate with Zinc Hydroxide. A slight excess of strong ammonia (s.g. 0·880) is added to a hot strong solution of zinc sulphate, the mixture filtered on a Buchner funnel, the precipitate well washed with hot water and the superficial water then removed by suction. The zinc hydroxide paste is added in small quantities at a time to 12·5 ml. of glacial acetic acid until a slight excess is present, the resulting solution filtered, the filter washed with water, the combined filtrate and washings diluted with more water to 100 ml., 3 ml. of strong ammonia (s.g. 0·880) added and the mixture finally diluted with 300 ml. of alcohol (95 per cent.).

4. Standard Sodium Solution. Exactly 1 g. of sodium chloride is dissolved in water and diluted to 100 ml. For use, 2 ml. of this stock solution is diluted to 100 ml. to make a solution containing 0·2 mg. NaCl, or 0·0786 mg. Na, per ml.

An amount of the solution to be examined, preferably containing between 0·04 and 0·8 mg. Na, is transferred to a centrifuge tube, diluted with water to 2 ml. and 4 ml. of alcoholic zinc acetate added (reagent No. 3 described above). After allowing to stand for 2 hours at room temperature followed by an overnight period at 0° C. the mixture is centrifuged, 3 ml. of the supernatant fluid transferred to another centrifuge tube and 4 ml. of alcoholic solution of uranyl zinc acetate added. After stirring with a thick glass rod until a precipitate commences to form, the mixture is allowed to stand for 1 hour at 0° C. then centrifuged, the supernatant liquid rejected, the tube drained by inverting on filter paper and the precipitate carefully washed once with 5 ml. of alcohol saturated with the triple acetate (reagent No. 2 described above). The washed precipitate is dissolved in water, the solution quantitatively transferred to a 25-ml. graduated flask or, if the precipitate is bulky, indicating that the sample taken contained more than 0·15 mg. Na, a larger size flask, say up to 250 ml. according to discretion. To the aqueous solution in the flask 1 drop of glacial acetic acid is added followed by 0·5 ml. of a 20 per cent. aqueous solution of potassium ferrocyanide and the mixture diluted to 25 ml. (if a larger flask has been used, proportionately larger quantities of acetic acid and potassium ferrocyanide solution should be employed and sufficient water added to bring the liquid to the mark). After standing for 3 minutes, in order to allow the brown colour to develop, it is compared in a

colorimeter with a standard prepared by submitting a known amount of sodium chloride to the same process.

Discussion. This procedure is not disturbed by the presence of calcium, magnesium, iron, barium or strontium. Potassium will not interfere provided the amount present in the solution taken for analysis is not in excess of 0·6 mg. which is about 8 times the quantity of sodium giving a convenient colour. In vegetable materials this ratio is often exceeded and in such cases McCance and Shipp suggest diluting the test solution until 2 ml. contains less than 0·6 mg. K, proceeding as described above until the washing of the precipitate is completed, then evaporating the residual drop of alcohol adding 7·5 ml. of water and 0·5 ml. of a 7 per cent. aqueous solution of potassium ferrocyanide slightly acidified with acetic acid and matching the colour produced against the standard as ordinarily used. In this way sodium may be determined directly in the presence of 30 times its weight of potassium.

Phosphates, if present, are removed by the alcoholic solution of zinc acetate (reagent No. 3) but, if the sample under examination does not contain phosphates or free mineral acid, there is no need to include treatment with this reagent. Arsenates interfere with the method and Caley and Foulk[5], using uranyl magnesium acetate for precipitating the sodium, report that the presence of lithium is inadmissible.

Instead of working through the whole procedure with known amounts of sodium, standards may be prepared directly from a triple acetate solution of known strength. For this purpose, exactly 10 ml. of a 1 per cent. aqueous solution of sodium chloride is mixed with 80 ml. of water and 100 ml. of alcohol (95 per cent.) added, followed by 120 ml. of alcoholic solution of zinc uranyl acetate (reagent No. 1 above). After allowing to stand for at least 1 hour the precipitate is quantitatively collected, washed with ice-cold alcohol (95 per cent.), then dissolved in water and the solution diluted to 1 litre. This constitutes a stable stock solution. In order to prepare a standard colour, exactly 1 ml. is transferred to a 25-ml. graduated flask, 1 drop of glacial acetic acid and 0·5 ml. of a 20 per cent. aqueous solution of potassium ferrocyanide added and the mixture diluted to the mark. The intensity of the resulting colour is close to that obtained when 0·2 mg. NaCl (0·0786 mg. Na) in 2 ml. of water is submitted to all stages of the method and finally made up to 25 ml. However, since the complete procedure involves the addition of alcoholic

solution of zinc acetate to the test solution and a consequent unknown degree of concentration in the volume of the mixed liquids, it will be advisable to check the artificial triple acetate standard against the colour due to natural standards produced by applying the complete method to known quantities of sodium chloride. Since zinc is present in the final solutions used for the colour tests it follows that insoluble zinc ferrocyanide will be produced ; provided, however, that the potassium ferrocyanide is added to the test solution and the standard at the same time and the colours are matched within 10 minutes of their formation no disturbance from this cause need be anticipated.

Scrupulous care is essential when determining small quantities of sodium since the element is ubiquitous and hence there are manifold chances of the apparatus or reagents becoming contaminated. McCance and Shipp[4] recommend taking 0·1 mg. NaCl in 2 ml. and 0·2 mg. NaCl in 2 ml. and submitting the two solutions to every stage of the sodium determination and confirming by means of a colorimeter that one of the final colours is twice the intensity of the other.

Application to Blood Serum. The sample (1 ml.) is mixed in a centrifuge tube with 3 ml. of water and 1 ml. of a 20 per cent. aqueous solution of trichloracetic acid and, after centrifuging, 1 ml. of the clear supernatant fluid (equivalent to 0·2 ml. of the original serum) is transferred to another centrifuge tube and 10 ml. of alcoholic solution of uranyl zinc acetate is added. The mixture is stirred with a thin glass rod until a precipitate begins to form then allowed to stand at 0° C. for 1 hour and centrifuged. The supernatant fluid is discarded, the tube being drained by inverting it over filter paper. The precipitate is carefully washed once with 5 ml. of alcohol saturated with the triple acetate (reagent No. 2 described above) then dissolved in water and the solution quantitatively transferred with more water to a 250-ml. graduated flask. To the aqueous solution in the flask, 0·2 ml. of glacial acetic acid and 2·5 ml. of a 20 per cent. aqueous solution of potassium ferrocyanide is added and the mixture diluted to the mark with water. After mixing and allowing to stand for 3 minutes, in order to permit the colour to develop, it is compared in a colorimeter with a standard prepared by treating 1 ml. of a 0·178 per cent. aqueous solution of sodium chloride (1 ml. contains 0·7 mg. Na) with 10 ml. of uranyl zinc acetate solution and subsequently in the same way as the sample.

Notes on the Application. In the examination of blood serum

for sodium it is not necessary to use the alcoholic solution of zinc acetate since there is no free mineral acid present and the amount of phosphate in serum is not sufficient to interfere with the final colour reaction. The normal content of sodium in serum lies between 325 and 350 mg. per 100 ml. as Na, but is often lowered in Addison's disease, in the acute crises of which it may fall to 250 mg. per 100 ml. Reduction of the sodium content may also occur in other pathological conditions such as intestinal obstruction, diabetic coma and miners' cramp.

REFERENCES

1. I. M. Kolthoff, *Pharm. Weekblad*, 1923, **60**, 1251.
2. I. M. Kolthoff, *Z. anal. Chem.*, 1927, **70**, 397.
3. H. K. Barrenscheen and L. Messiner, *Biochem. Z.*, 1927, **189**, 308.
4. R. A. McCance and H. L. Shipp, *Biochem. J.*, 1931, **25**, 449.
5. E. R. Caley and C. W. Foulk, *J. Amer. Chem. Soc.*, 1929, **51**, 1664.

THALLIUM

Thallous acetate was once tried as a remedy for ringworm but found to be too poisonous[1]. Thallium salts have, however, been successfully employed in rodenticides and insecticides and in the examination of these a colorimetric method of determining the relatively small proportions employed is sometimes useful. An indirect method was proposed by Shaw[2] which depends essentially upon conversion of the thallium to thallic chloride, extraction of the latter with ether, addition of potassium iodide and solution of the liberated iodine in carbon disulphide followed by measurement of the colour so produced. Haddock[3] has rendered the procedure more sensitive by using the starch-iodine colour and proposed the diphenylthiocarbazone extraction method for the preliminary isolation of the thallium from all substances except lead and bismuth.

Method[3]. The following special reagents are required :—

1. Bromine Reagent. To 900 ml. of freshly prepared bromine water 100 g. of disodium hydrogen phosphate is added followed, when it has dissolved, by 100 ml. of concentrated hydrochloric acid.

2. Starch-glycerin Solution. To 50 ml. of water is added 10 g. of soluble starch, the mixture well triturated, the resulting paste poured into 450 ml. of boiling water and 500 ml. of glycerin added. The mixture is stirred, gently boiled for 5

minutes, allowed to cool and the volume adjusted to 1 litre with water. This solution may be stored in amber bottles for several months without deterioration.

To the solution to be examined, which should contain the thallium in the monovalent state (thallic salts are readily reduced by sulphur dioxide) is added 5 g. of citric acid, sufficient ammonia solution to render the mixture alkaline and 0·5 g. of potassium cyanide. The thallium is extracted by shaking this solution with four portions each of 15 ml. of a 0·1 per cent. solution of diphenylthiocarbazone in chloroform. Each extract is washed in succession with the same 20-ml. portion of water contained in a second separator, the bulked chloroformic extracts evaporated to dryness in a suitable flask of Pyrex glass, 1 ml. of concentrated sulphuric acid added to the residue and the organic matter destroyed by heating and adding a few drops of 30 per cent. hydrogen peroxide. The cooled residue is washed into a 250-ml. Phillip's flask with 20 ml. of water, 1 g. of ammonium chloride added followed by 25 ml. of the bromine reagent, the mixture boiled so that it becomes just colourless after the expiration of 3 minutes and then rapidly cooled to 18° C. The solution is transferred to a graduated stoppered 50-ml. cylinder, the volume adjusted to 35 ml., 5 drops of a 25 per cent. solution of phenol in glacial acetic acid added, the liquid shaken and allowed to stand for 3 minutes. At the end of this time, 5 ml. of a freshly prepared 0·2 per cent. aqueous solution of potassium iodide (free from iodate) is added followed by 1 ml. of starch-glycerin solution, the mixture shaken, allowed to stand exactly 5 minutes and the blue colour produced at once measured by means of a Lovibond tintometer employing a 1-cm. cell. The reading in terms of Lovibond blue units is correlated with the amount of thallium in the material originally taken for the wet-oxidation in accordance with the values given in Table V.

TABLE V.—RELATION BETWEEN THE QUANTITY OF THALLIUM AND THE COLOUR PRODUCED (L. A. Haddock, *Analyst*, 1935, **60**, 394)

Quantity of Thallium mg.	Colour observed in 1-cm. cell: Lovibond Blue Units	Quantity of Thallium mg.	Colour observed in 1-cm. cell: Lovibond Blue Units
0·010	0·4	0·090	2·7
0·020	0·7	0·100	3·2
0·030	1·0	0·130	4·0
0·040	1·3	0·150	4·6
0·050	1·6	0·175	5·3
0·070	2·1	0·200	6·0

The starch-iodine colour has almost the same hue as the Lovibond blue glasses and the use of the other colours in the tintometer is hardly necessary.

Discussion. In order to determine the relation between the quantity of thallium and the blue colour produced Haddock made standard solutions of the latter using specially purified thallous nitrate and submitted appropriate amounts to the process of extraction with diphenylthiocarbazone followed by wet combustion of the residues and oxidation of the metal to the trivalent state as described for the examination of samples. Whatever the substance under examination, the full extraction procedure must be carried out in order to avoid disturbance from other sources such as traces of iron : interference from this metal must be carefully avoided and phosphate is included in the bromine reagent as a precautionary measure against trouble from this source. Ammonium chloride is added before the bromine reagent in order to facilitate the subsequent oxidation of the thallium which will not readily take place in sulphuric acid alone.

Monovalent thallium is readily extracted by diphenylthiocarbazone from solutions containing alkali metals, alkaline earth metals, magnesium, aluminium, copper, iron, chromium and silver, while sulphates, chlorides, phosphates and nitrates do not cause any trouble. Nickel, cobalt, mercury and zinc retard the extraction of thallium and the method fails if more than 0·1 g. of any of these is present. Manganese and tin tend to be carried through with the thallium and if either is present a second extraction must be carried out. Thallium cannot be separated from lead and bismuth by means of diphenylthiocarbazone and the method is therefore inapplicable in the presence of either of these metals.

The starch-iodine colour increases in intensity on standing but is sufficiently stable to allow of measurement in a Lovibond tintometer. A control conducted on the reagents should not give any colour. For this test a recording instrument is almost essential since the preparation of natural standards for each determination would be extremely tedious and a Duboscq type colorimeter is inapplicable.

REFERENCES

1. G. Roche Lynch, *Lancet*, 1930, **219**, 1340.
2. P. A. Shaw, *Ind. End. Chem., Anal. Ed.*, 1933, **5**, 33.
3. L. A. Haddock, *Analyst*, 1935, **60**, 394.

TIN

A critical study[1] of the well-known cacotheline test for stannous ions[2] showed it to be quite unsuitable for quantitative work while the method of Hüttig[3], depending upon the reduction of a molybdate reagent, is uncertain in action and undesirably sensitive to variations in the conditions under which the test is applied. The method of choice is undoubtedly that evolved by R. E. D. Clark[4, 5] using 4 - methyl - 1 : 2 - dimercaptobenzene, now usually called " dithiol." This reagent produces a red-coloured compound when warmed with an acid solution of divalent tin and, despite many limitations, the method has proved to be of considerable value.

Method[4, 5]. The reagent solution used for the test should contain 0·2 g. of 4-methyl-1:2-dimercaptobenzene and 0·5 g. of thioglycollic acid in 100 ml. of a 1 per cent. aqueous solution of sodium hydroxide. This mixture is stable for some days at least ; if a milky suspension of the disulphide forms the reagent should be discarded.

For the determination, the acid solution to be tested is treated with a few drops of thioglycollic acid to ensure reduction to the divalent state and is then diluted until it contains not more than 60 p.p.m. Sn. A measured quantity (5 ml.) of this solution is transferred to a graduated test tube and 1 ml. of hydrochloric acid and 1 ml. of a warm jelly of agar are added. This solution is heated to boiling and maintained at that temperature until the agar is in solution. After cooling, 2 ml. of the reagent solution, together with sufficient water to bring the total volume to 10 ml., is added. The tube is immersed in boiling water for 1 minute, then about 2 ml. of the liquid transferred to a porcelain tray (3·3 × 3·3 cm.) such that the depth of the liquid after cooling is almost exactly 2 mm. The colour, which is stable for about 10 minutes, is then measured by means of the Lovibond tintometer using *reflected* light and the red component, expressed in Lovibond units, is correlated with the concentration of tin in accordance with the data presented in Table VI.

Discussion. The reagent produces coloured precipitates with silver, mercury, copper, bismuth, cadmium, arsenic, antimony, nickel, cobalt and lead ; those due to copper, nickel and cobalt are black, while that produced by bismuth is brick-red. The detection of tin is disturbed by the presence of copper, nickel and bismuth, but not so much by cobalt since in this case tin is pre-

TABLE VI.—RELATION BETWEEN THE CONCENTRATION OF TIN AND THE RED COMPONENT OF THE COLOUR PRODUCED (R. E. D. Clark, *Analyst*, 1937, **62**, 661)

Tin : P.p.m. after dilution to 10 ml.	Colour in Lovibond Units		
	Red	Yellow *	Brightness *
2·5	1·0	0·4	—
5·0	2·0	0·7	—
10·0	3·6	0·9	0·1
15·0	5·7	0·9	—
20·0	7·2	0·7	—
25·0	8·7	1·2	0·1
30·0	10·2	0·9	0·1
40·0	12·3	1·0	—
42·5	13·1	0·9	0·1
45·0	14·0	1·0	—
50·0	14·9	1·0	0·2

* These values are subject to slight variation : they are included as guides to accurate matching but only the figures for the red component are considered in the calculation of results.

ferentially precipitated. The compounds produced by the other reacting metals are coloured various shades of yellow so, although the presence of appreciable traces of these will adversely influence the accuracy of colorimetric determinations, approximate results may still be obtainable. Despite its severe limitations this is certainly the best method for the determination of traces of tin so far discovered.

Application to Food and Organic Materials[6]. A suitable quantity of the sample is incinerated in a silica dish, the charred mass transferred to a special distillation flask provided with all-glass joints as illustrated in Fig. 9 and any residue left in the dish washed out with small quantities of concentrated sulphuric acid, the washings being added to the material in the flask. Sufficient sulphuric acid is introduced into the flask to make a total volume of about 30 ml., the distillation apparatus assembled, the side funnel charged with hydrobromic acid (s.g. 1·46 to 1·49) and 5 ml. of water introduced into each of the receiving flasks. The temperature of the oil-bath is raised to 220° C. and hydrobromic acid then allowed to drop on to the contents of the distillation flask at the rate of 1 to 2 drops per second, a steady stream of carbon dioxide being passed through the system in the meanwhile. After distillation has continued for 1 hour the condenser is rinsed, the washing added to the combined distillates, the latter diluted to a known volume (50 or 100 ml.) and the free bromine in an aliquot

part (expected to contain about 0·020 mg. Sn) removed by the addition of just sufficient 25 per cent. w/v solution of phenol in glacial acetic acid and the acidity determined by titration with N sodium hydroxide using methyl orange as indicator. A further aliquot portion is then similarly decolorised by means of phenol and its acidity so adjusted with 30 per cent. aqueous solution of sodium hydroxide that it contains free acid to the extent of 0·5 ml. of concentrated hydrobromic acid (s.g. 1·46 to 1·49 : 46 to 48 per cent. w/w HBr). Then 0·2 ml. of a 0·04 per cent. aqueous solution of thioglycollic acid is added followed by 4 drops of a fairly

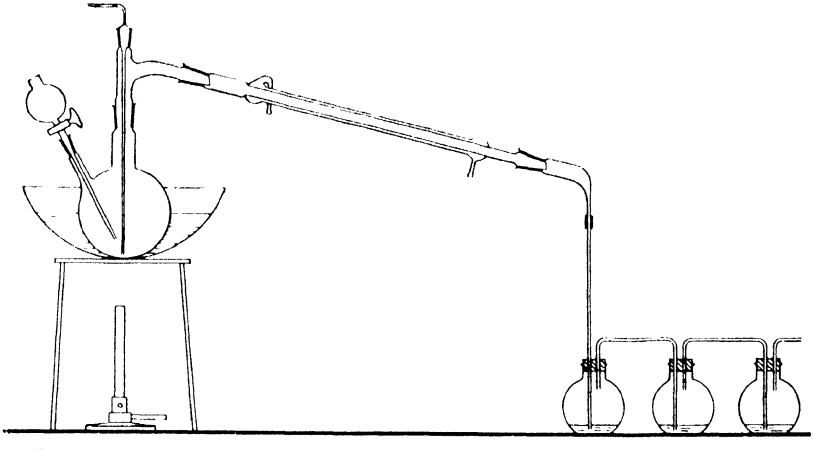


FIG. 9.—APPARATUS FOR THE ISOLATION OF TIN AS STANNIC BROMIDE

Distillation apparatus as supplied by Quickfit and Quartz, Ltd., King's Norton, Birmingham, England, comprising a double-necked flask with dropping funnel, lead-in tube to admit carbon dioxide and exit tube connected to a water-cooled condenser. The distillation flask is heated in an oil-bath provided with a suitable cover so that the dropping funnel is kept cool. (After N. H. Law, *Analyst*, 1942, **67**, 283.)

stiff mucilage of agar and 1 ml. of a freshly prepared 0·1 per cent. solution of 4-methyl-1:2-dimercaptobenzene in 1 per cent. aqueous solution of sodium hydroxide. The mixture is diluted to 10 ml. with water, heated in a boiling water-bath for 1 minute and the resulting colour matched against standards similarly prepared. A stock standard tin solution containing 1 per cent. w/v Sn may be prepared by dissolving pure metallic tin in 5N hydrochloric acid : this solution can be stabilised for some time by adding a few drops of thioglycollic acid but diluted standards should be prepared immediately before use.

Discussion. The above procedure, due to N. H. Law[6], is based upon an observation of Hoffman and Lundell[7] who showed that

stannous and stannic tin could be separated from other metals by distillation from a hydrobromic acid and sulphuric acid mixture at 220° C. Schwaibold, Borchers and Nagel[8] proposed distillation from a mixture consisting of 3 vols. of concentrated hydrochloric acid and 1 vol. of hydrobromic acid (46 to 48 per cent.) but there can be little doubt that it is better to use sulphuric rather than hydrochloric acid. Of the elements capable of quantitative or partial distillation under the conditions described[7] at least twice the amount of arsenic compared with that of tin must be present before interference with the colour test occurs while boron does not occasion any disturbance. Although direct comparison of the test colours against natural standards is recommended by Law it would seem reasonable to assume that the colour produced might be related to the Lovibond scale in the manner given in the general description of the test but a separate calibration would be necessary in order to meet the slightly different conditions.

REFERENCES

1. I. L. Newell, J. B. Ficklen and L. S. Maxfield, *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 26.
2. H. Leuchs and F. Leuchs, *Ber.*, 1910, **43**, 1042.
3. G. F. Hüttig, *Chem.-Ztg.*, 1923, **47**, 341.
4. R. E. D. Clark, *Analyst*, 1936, **61**, 242.
5. R. E. D. Clark, *Analyst*, 1937, **62**, 661.
6. N. H. Law, *Analyst*, 1942, **67**, 283.
7. J. I. Hoffman and G. E. F. Lundell, *J. Research Natl. Bur. Standards*, 1939, **22**, 465.
8. J. Schwaibold, W. Borchers and G. Nagel, *Biochem. Z.*, 1940, **306**, 113.

TITANIUM

Weller's method[1] depending upon the formation of the intensely yellow pertitanic acid when hydrogen peroxide is added to acidified solutions of titanium sulphate is widely employed. This method, with its application to rocks, clays and steel is described below and, in addition, an account is given of an alternative procedure using thymol[2].

Method Using Hydrogen Peroxide[1]. Sulphuric acid is added to the solution under examination until the concentration equals about 10 per cent. and then to the acid liquid is added about one-fifth of its volume of 6 per cent. hydrogen peroxide. If the colour produced is within the working range of the test it is matched

directly against standards similarly prepared but, if the yellow is too deep for comparison, the mixture may be diluted to a known volume, an aliquot part transferred to a Nessler glass, and, after diluting to the graduation mark with water, the paler tint matched and the result calculated accordingly. For most purposes it is sufficiently accurate to prepare a standard solution by fusing 0·500 g. of freshly ignited commercially pure titanium dioxide with about 5 g. of potassium pyrosulphate and, after allowing to cool, dissolving the melt in dilute sulphuric acid : this solution may then be diluted with more acid to make a standard of convenient strength. If 50 ml. Nessler glasses are used for matching, suitable demarcations of tint can be obtained with quantities ranging from 0·25 to 25 mg. TiO_2.

A more precise way of preparing the standard solution consists in heating 0·600 g. of pure, recrystallised potassium fluotitanate, K_2TiF_6, with concentrated sulphuric acid in a platinum dish until most of the fluorine is evolved. More sulphuric acid is added, the mixture evaporated to small volume, but not to dryness, and the process repeated several times until no trace of fluorine remains. The residue is then dissolved in dilute sulphuric acid and the volume made up to 200 ml. producing a standard solution containing 1 mg. TiO_2 per ml.

Discussion. It is important that all trace of fluoride be eliminated from material under test since, if present, it exercises a bleaching effect on the pertitanate colour. Chromium, vanadium and molybdenum interfere since they also give colorations under the conditions of the test. The presence of a small amount of iron is immaterial but large quantities disturb the accuracy of determinations on account of the colour of the iron solution. For approximate work it is sufficiently near to assume that the colouring power of 0·1 g. Fe_2O_3 in 100 ml. of dilute sulphuric acid is about equal to 0·2 mg. TiO_2 in the same volume. However, it is better to add to the standards a quantity of ferric ammonium sulphate equivalent to the amount of iron in the sample. A considerable proportion of phosphoric acid exercises a bleaching effect on the titanium colour and, therefore, if it is employed to compensate the effect of iron it is important that the same amount be added to the standards. A high concentration of alkali sulphate slightly diminishes the intensity of the colour due to titanium and, although this source of error is rarely of consequence, cases may arise which demand that precautions be observed.

Application of the Hydrogen Peroxide Method to Rocks[3]. A suit-

able quantity of the powdered sample, say 1 g., is fused with 10 g. of anhydrous sodium carbonate over a blast burner, the cooled melt extracted with boiling water, the insoluble residue, which contains the hydrolysed sodium titanate, filtered off and thoroughly washed with hot 2 per cent. solution of sodium carbonate. The material on the filter is then dried, fused with potassium bisulphate or potassium pyrosulphate, the cooled melt dissolved in dilute sulphuric acid (10 per cent.), the solution diluted to a convenient volume and, to an aliquot part, 5 ml. of phosphoric acid (s.g. 1·75) added ; the colorimetric determination is then conducted as described above, care being taken to ensure that the standards contain approximately the same amount of alkali sulphate and phosphoric acid as the sample.

Application of the Hydrogen Peroxide Method to Steel (*Total Titanium*). A weighed portion of the sample, say 5 g., is treated with 100 ml. of hot 5N hydrochloric acid until all action ceases, the solution diluted to 300 ml., the mixture rendered just alkaline with ammonia then acidified with 3 or 4 ml. of concentrated hydrochloric acid. The iron is reduced to the ferrous state by adding 5 g. of crystalline sodium thiosulphate previously dissolved in 50 ml. of water and boiling the mixture for 10 minutes.* Boiling is continued for a further 5 minutes after the addition of 3 ml. of phenylhydrazine : paper pulp is added, the mixture filtered, the residue on the filter washed with 25-ml. portions of hot water, ignited in a platinum crucible and the residue fused with 3 g. of anhydrous sodium carbonate to which a little potassium nitrate has been added. The clear melt is allowed to cool, treated with hot water and allowed to stand on a hot plate during several hours until decomposition is complete, the mixture is filtered and, after washing with hot water, the paper and residue is treated with boiling dilute sulphuric acid (10 per cent.) the mixture filtered, the filtrate made up to a suitable known volume and the colorimetric test applied as described above. If there is any colour due

* If the sample of steel contains tungsten, the initial solution in 5N hydrochloric acid is diluted with water to 200 ml., 3 g. of sodium chlorate added, the mixture boiled until the tungsten has been converted into the yellow tungstic acid, the precipitate filtered off and washed with N hydrochloric acid, the combined filtrate and washings being reserved. The tungstic acid is sluiced into a small beaker, dissolved in ammonia and the solution filtered. Any residue on this filter, which may contain titanium, is washed with ammonia, ignited in a porcelain crucible and the ash transferred to the reserved filtrate which is then diluted to 300 ml., rendered just alkaline with ammonia and then acidified with 3 or 4 ml. of concentrated hydrochloric acid. The iron is reduced to the ferrous state by adding 25 g. of crystalline sodium thiosulphate, previously dissolved in 50 ml. of water, and boiling for 10 minutes. The determination of titanium is then continued as described above.

to iron in the final solution used for the colorimetric determination it may be removed by adding a few drops of phosphoric acid, care being taken to ensure that the same amount is also added to the standards.

(*Combined Titanium*). A weighed portion of the sample (5 g.) is treated with 100 ml. of hot dilute hydrochloric acid (10 per cent. HCl). When all action ceases the solution is filtered, insoluble matter being collected on the paper and washed with hot water acidulated with hydrochloric acid until free from iron. The mixed filtrate and washings are set aside for the determination of uncombined titanium (*vide infra*). The insoluble residue is ignited under good oxidising conditions at as low a temperature as possible, fused with 3 g. of sodium carbonate to which a little potassium nitrate has been added, the cooled melt treated with hot water and the mixture filtered. After well washing with hot water, the insoluble residue is ignited until all carbon has been burnt away, fused with about 3 g. of potassium pyrosulphate, the melt dissolved in 10 per cent. sulphuric acid and the colorimetric determination of the titanium conducted as already described.

(*Uncombined Titanium*). The mixed filtrate and washings reserved in the course of the determination for combined titanium are diluted to 300 ml. and treated as described for the determination of total titanium commencing with the neutralisation by ammonia.

Application of the Hydrogen Peroxide Method to Cast Iron. A sample weighing about 5 g. is treated with 100 ml. of hot dilute hydrochloric acid (10 per cent. HCl) and when the reaction has ceased the mixture is filtered, the insoluble matter collected on the paper and well washed with hot water. The paper and residue are dried, ignited in a platinum crucible until all carbon is burnt away then 2 ml. of hydrofluoric acid (40 per cent.) and 1 ml. of 10N sulphuric acid added and the mixture evaporated to dryness. The residue is fused with 2 g. of sodium carbonate, the cooled melt treated with hot water, the insoluble material filtered off, washed with hot water, dried, ignited in a platinum crucible, the carbon-free residue fused with 3 g. of potassium pyrosulphate, allowed to cool and dissolved in 10 per cent. sulphuric acid ; the colorimetric determination of the titanium is then conducted as already described.

Notes on the Applications. The method given for the examination of rocks is recommended by Hillebrand[3]. Alternative procedures have been suggested which include preliminary removal

of silica by treatment with a mixture of hydrofluoric acid and sulphuric acid and in cases of doubt it may be advisable for the analyst to adopt this plan before proceeding to the sodium carbonate fusion.

In steel, titanium is used as a deoxidiser and scavenger rather than as an alloying element. Very little remains in the finished steel, and that which does is probably always present in two conditions: partly as titanium dissolved in the iron, and therefore soluble in dilute hydrochloric acid, and the remainder combined with nitrogen, oxygen, or sulphur and insoluble in dilute acid. In the usual case by far the greater part is present in the combined form. Generally, the amount of titanium present in iron and steel is so small that the colorimetric method is adopted in preference to volumetric or gravimetric procedures. In pig iron, however, the amount may reach 0·5 per cent., the titanium being derived from the original iron ore. The special process given for the determination of titanium in cast iron is necessitated by reason of the high proportion of silicon (about 2 per cent.) which is present.

Application to Clays Using Permanent External Standards. Two discs are available for use with the B.D.H. Lovibond Nessleriser, the one containing standard glasses from 0·25 to 2·5 mg. TiO_2 and the other covering a range from 2·5 to 22·5 mg. TiO_2. A supplementary disc with points from 0·5 to 2 mg. TiO_2 is also issued for use with the stronger disc in order to obtain the necessary small steps.

To apply the test, the group III precipitate obtained from 1 g. of the sample by the usual methods for the analysis of a clay material is fused in a platinum crucible with potassium pyrosulphate. The cooled melt is treated with water, 20 to 30 ml. of concentrated sulphuric acid added and the mixture diluted to 250 ml. To 50 ml. of this solution is added 10 ml. of 6 per cent. hydrogen peroxide, the mixture diluted to 100 ml. and 30 ml. transferred to a Nessler glass and matched against the standards, plain water being employed as a blank. If the original material contains more than 2·5 per cent. of TiO_2, but not more than 4 per cent., only 25 ml. of the acid solution of the melt should be taken.

Method Using Thymol[2]. The reagent is prepared by dissolving 1 g. of thymol in 1 ml. of glacial acetic acid and diluting to 20 ml. with concentrated sulphuric acid. The sample to be tested, which may be the cooled melt from a potassium bisulphate fusion, is dissolved in sulphuric acid and a portion of this solution, expected to contain between 0·01 and 0·1 mg. of TiO_2, transferred

to a stoppered 10 ml. cylinder, 2 ml. of the reagent added and the mixture diluted to 10 ml. with concentrated sulphuric acid. The reddish-yellow colour produced in the presence of titanium may be measured by means of a Lovibond tintometer using a 1-cm. cell and the yellow component, expressed in Lovibond units, correlated with the concentration of titanium in accordance with the data presented in Table VII.

TABLE VII.—RELATION BETWEEN THE QUANTITY OF TITANIUM DIOXIDE AND THE COLOUR PRODUCED

Titanium dioxide (TiO_2) Mg. in 10 ml. of reaction mixture	Colour observed in 1-cm. cell: Lovibond Units		
	Yellow	Red *	Brightness *
0·01	0·8	0·5	—
0·02	1·3 (1·25)	0·6	—
0·03	1·7	0·8	0·1
0·04	2·2	1·1	0·2
0·05	2·7 (2·75)	1·4	0·2
0·06	3·3	1·7	0·2
0·07	3·8	2·0	0·2
0·08	4·4 (4·45)	2·3	0·2
0·09	5·2	2·6	0·2
0·10	6·1	3·1	0·2

* These values are subject to slight variation: they are included as guides to accurate matching but only the figures for the yellow component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

Discussion. Tungsten, molybdenum and chromium interfere since they also yield colours with the reagent but the test is not disturbed by the presence of relatively large amounts of iron, aluminium, calcium, magnesium, vanadium, phosphates or silicates. When conducting the test no special precautions need be taken to prevent slight absorption of moisture from the atmosphere by the strong sulphuric acid but the apparatus employed should be dried before use.

REFERENCES

1. A. Weller, *Ber.*, 1882, **15**, 2592.
2. V. Lehner and W. G. Crawford, *J. Amer. Chem. Soc.*, 1913, **35**, 138.
3. W. F. Hillebrand, *Bull. U.S. Geol. Surv.*, 1919, No. 700, 155.

VANADIUM

The reaction of acidified vanadate solutions with hydrogen peroxide leading to the formation of a reddish-brown colour[1] can be applied to the colorimetric determination of the metal but the method is sensitive to slight variations in the conditions and hence it is necessary to be cautious in the interpretation of the results obtained. The procedure is given here and, in addition, its application to the determination of vanadium in steel is described. An alternative method, not, however, applicable in the presence of iron, has been proposed by Bach and Trelles[2] and depends upon solution of the 8-hydroxyquinoline complex in amyl alcohol. This test has been applied to the examination of natural waters in the Argentine Republic some of which are reported to contain traces of vanadium.

Method Using Hydrogen Peroxide. Sulphuric acid should be added to the solution to be tested until the strength lies between 15 and 20 per cent. ; to this liquid, or an aliquot part of it, 1 drop of 3 per cent. hydrogen peroxide is added and the colour matched against standards prepared by adding appropriate quantities of a solution containing, say, 0·2 mg. V per ml., to sulphuric acid of similar strength followed by 1 drop of hydrogen peroxide to each. The standard solution of vanadium is conveniently made by dissolving 0·459 g. of ammonium vanadate in water containing 15 ml. of concentrated nitric acid and diluting to 1 litre ; this will contain 0·2 mg. V per ml. If the colours are viewed in 50-ml. Nessler glasses the practical range for matching lies between 0·01 and 3·0 mg. V. Samples of minerals and the like should be ignited with fusion mixture to which a little nitrate has been added and the cooled melts dissolved out with 20 per cent. sulphuric acid.

Discussion. The presence of sulphuric acid is necessary for the satisfactory development of the colour although the precise concentration to be employed is not critical. On the other hand, an undue excess of hydrogen peroxide will bleach the colour initially formed. According to Meyer and Pawletta[3] the intensity of the colour depends upon the relative proportions of pentavalent vanadium, hydrogen peroxide and sulphuric acid. They state that the reddish-brown compound is not, as is usually assumed, pervanadic acid, but a peroxidised sulphate, thus :—

$$(VO)_2(SO_4)_3 + H_2O_2 \rightarrow [V(O_2)]_2(SO_4)_3$$

With more peroxide this product is then thought to yield the light-yellow orthoperoxo-vanadic acid, $H_3[V(O_2)O_3]$. Increase in the sulphuric acid concentration restores the reddish-brown colour hence a considerable excess of acid is required, as well as a minimum excess of peroxide over the ratio $V : H_2O_2 = 1$.

Application of the Hydrogen Peroxide Method to Steel[4]. If chromium is absent from the sample under examination, 2 g. of the latter and an equal weight of a plain carbon steel free from vanadium are treated separately with 40 ml. of nitric acid (s.g. 1·2, made by diluting 100 ml. of nitric acid s.g. 1·42 with 155 ml. of water) and the mixtures heated until the specimens completely dissolve. To each is added about 0·1 g. of potassium permanganate, the heating continued for about 5 minutes, then excess of dilute ammonium bisulphite solution added and the mixtures boiled until sulphur dioxide is expelled. The solutions are cooled, transferred to 100-ml. Nessler glasses and a few drops of 3 per cent. hydrogen peroxide solution added to each. A standard solution of vanadium, made in the manner indicated in the description of the general method, is then added to the control from a burette so that, after the volumes of the two solutions have been equalised by appropriately diluting with water, the intensity of colour in each is the same. The proportion of vanadium in the sample under test is then readily calculated.

If the sample contains chromium, an amount of potassium dichromate equivalent to the chromium content is added to the control and the two specimens separately treated by heating with 80 ml. of 40 per cent. w/v sulphuric acid. When most of the iron has dissolved, 25 ml. of concentrated nitric acid should be added to each and the mixtures boiled for some minutes. The solutions are then cooled, transferred to Nessler glasses and the determination finished as before.

Notes on the Application to Steel. The above is the technique originally recommended by McCabe[4]. The utility of the colorimetric method is limited owing to the fact that other alloying metals such as titanium, molybdenum and cerium also give colours with hydrogen peroxide. The influence of the first of these can be readily counteracted by adding a little hydrofluoric acid to the final solutions prior to the addition of hydrogen peroxide but the other metals, and tungsten if present, must be separated before the test for vanadium can be applied. According to McCabe[5] molybdenum, nickel, copper and cobalt may be eliminated by first partially removing the iron from the initial acid

solution of the same sample by extracting once with ether then coprecipitating the remainder of the iron, the titanium, and the vanadium with ammonia, when the latter metal may be determined colorimetrically after solution of the precipitate in dilute sulphuric acid containing sufficient hydrofluoric acid to suppress the colour otherwise produced by titanium. Tungsten is separated by filtration of the original acid solution of the sample.

For routine purposes it is probably simpler to add comparable proportions of the interfering metals to the control as recommended in " Standard Methods of Analysis of Iron, Steel and Ferro-Alloys " (The United Steel Companies Limited, 1933). For steels containing, besides vanadium, nickel, chromium, cobalt and molybdenum, 1 g. of the sample is dissolved in 15 ml. of 8N sulphuric acid, 10 ml. of nitric acid added, the mixture boiled to remove nitrous fumes, diluted to 150 ml. with hot water, excess of potassium permanganate solution added and, after again boiling for 5 minutes manganese dioxide and any tungstic oxide which may be present filtered off through an asbestos pad. Sulphurous acid is added in slight excess, free sulphur dioxide removed by boiling and, after allowing to cool, 5 ml. of phosphoric acid (s.g. 1·75) and 7 ml. of 3 per cent. hydrogen peroxide are added. Any reddish-brown colour produced is matched by adding a standard solution of ammonium vanadate to a control made from vanadium-free steel containing the correct proportions of nickel, chromium and cobalt added as the sulphates, and molybdenum (if the sample contains more than 0·5 per cent.) added as ammonium molybdate.

Method Using 8-Hydroxyquinoline for Natural Waters[6]. A suitable amount of the sample under examination, which should conveniently occupy 50 ml. and may be the original water or a concentrate, is treated in a separator with 0·2 ml. of glacial acetic acid and 0·2 ml. of a 2·5 per cent. solution of 8-hydroxyquinoline in 10 per cent. acetic acid. After mixing, 10 ml. of amyl alcohol is added, the whole well shaken for at least 1 minute and allowed to stand until the alcoholic layer separates. The aqueous portion is rejected and any brownish-red colour imparted to the organic liquid is matched against standards similarly prepared. The most suitable colour for comparison covers a range from 0·01 to 0·08 mg. V. A standard solution is conveniently made by dissolving 2·296 g. of ammonium vanadate in water and diluting to 1 litre ; 1 ml. of this solution will contain 1 mg. V and appropriate dilutions can be made in order to prepare a series of standard colours.

Discussion. This method is based upon a qualitative test pro-

posed by Montequi and Gallego[6] which consists in forming the 8-hydroxyquinoline complex in dilute acetic acid solution followed by extraction with chloroform to which the vanadium compound imparts a violet colour. The iron complex also produces a strong colour in organic solvents and the presence of a trace of this metal will disturb the determination of vanadium. In some cases it may be possible to remove the iron by precipitation with ammonia but the risk of losing vanadium by adsorption should not be overlooked. Fluorides, chlorides, bromides, iodides, sulphates, nitrates, nitrites and sulphites do not interfere with the determination nor does the presence of moderate amounts of calcium, magnesium, sodium, potassium, manganese, lead or zinc. The test is disturbed by the presence of copper; interference by titanium may be obviated by the addition of sodium fluoride which prevents the formation of a colour by this element.

References

1. G. Werther, *J. prakt. Chem.*, 1863, **88**, 195.
2. J. M. Bach and R. A. Trelles, *Anal. Asoc. Quím. Argentina*, 1940, **28**, 111.
3. J. Meyer and A. Pawletta, *Z. anal. Chem.*, 1926, **69**, 15.
4. C. R. McCabe, *J. Ind. Eng. Chem.*, 1913, **5**, 736.
5. C. R. McCabe, *Chem. News*, 1911, **104**, 194 and 202.
6. R. Montequi and M. Gallego, *Anal. Soc. Esp. Fisicoquím.*, 1934, **32**, 134.

ZINC

A satisfactory method for the colorimetric determination of this metal has not yet been discovered. Many years ago Jaffe proposed a test depending upon the production of a green fluorescence by zinc salts in the presence of urobilin[1] and attempts have been made to apply this method quantitatively[2–4] but, apart from the limitation imposed by the necessity for matching a fluorescence, the reaction will not detect less than 0·1 mg. Zn. Isolation as phosphate followed by the colorimetric determination of the anion has been proposed[5] but the method is unreliable owing to the difficulty of removing excess phosphate not combined with zinc[6]. Attention has lately been directed to the application of diphenylthiocarbazone (dithizone) as a colorimetric reagent for zinc[7–13]. Essentially, the method consists in extracting the zinc from a prepared aqueous liquid with chloroformic solution of dithizone

and, either titrating the zinc complex with standard solution of bromine in carbon tetrachloride or matching the red component of the colour produced in the extracts. Difficulties arise due to interference of the green colour of the excess reagent, incomplete extraction of the zinc and interference by other metals and later investigators[11, 12] have stated that the determinations are subject to errors. The method presented below, although complicated, is sensitive and applicable to the determination of traces of zinc occurring in biological material and natural waters ; as it is not likely to be suitable for general use it will only be described within the limits of this application.

Method[14] (*for Biological Material*). Two special reagents are required :—

1. A Solution of Dithizone in Chloroform made and specially purified not more than 2 days before use. About 0·1 g. of dithizone is dissolved in 100 ml. of chloroform and extracted with three 25-ml. portions of 5N hydrochloric acid, the aqueous layers are rejected, 100 ml. of water and 5 ml. of strong ammonia (s.g. 0·880) added, the mixture shaken for 1 minute and, after separation, the chloroform rejected, the alkaline aqueous layer washed with three further separate portions of chloroform, then acidified with hydrochloric acid and the mixture shaken with 100 ml. of chloroform. The latter, after separation, is drawn off and repeatedly washed with water until the washings are neutral in reaction ; the resulting chloroformic solution constitutes the purified reagent.

2. A Solution of Sodium Quinaldinate prepared by adding 0·2 g. of quinaldinic acid (quinoline-2-carboxylic acid) to 3 ml. of water containing 1 drop of phenophthalein indicator solution, neutralising with 0·1N sodium hydroxide and diluting to 10 ml. with water. This reagent will remain in good condition for 2 days if stored in a stoppered bottle.

The initial procedure will vary somewhat according to the nature of the sample but the following description will be found satisfactory for many types of material. A quantity of the sample to be tested expected to contain between 0·02 and 0·10 mg. Zn is incinerated in a silica dish, using a muffle furnace at 500° to 550° C. and the carbon-free ash extracted by boiling with a little 10N hydrochloric acid and then diluting with water and boiling again. The solution is diluted to about 100 ml., or other convenient volume, transferred to a separator, and, for each

100 ml. of liquid, 0·2 g. of tartaric acid and 1 ml. of a 10 per cent. solution of resorcinol in alcohol (95 per cent.) added, the acid being allowed to dissolve before the resorcinol is introduced. A few drops of thymol blue indicator solution are added, then sufficient 5N ammonia to render the colour of the mixture slate blue (approximately *p*H 9) and the liquid shaken with three successive 10-ml. portions of the purified chloroformic solution of dithizone. The chloroform layers are transferred to a second separator and, if the third dithizone extract is red or purple, further extractions are made until the chloroform layer remains green. The combined extracts are washed once with 10 ml. of water and then extracted with four separate 10-ml. portions of 0·1N hydrochloric acid. The mixed acid layers are washed free from any mechanically suspended dithizone reagent by pouring small quantities of chloroform through the aqueous liquid without shaking. The chloroform used for washing is rejected and the acid extract diluted to a convenient volume with 0·1N hydrochloric acid. A portion of this solution is tested for heavy metals with hydrogen sulphide ; if no reaction is obtained, an aliquot part of the solution is transferred to a beaker and the colorimetric test conducted at once commencing with the evaporation of the acid solution to dryness (*vide infra*).

If the slightest trace of heavy metal is present, 1 ml. of a 1 per cent. aqueous solution of cupric chloride is added for each 50 ml. of solution taken. The mixture is boiled to remove dissolved chloroform, the liquid allowed to cool, an equal volume of acetone added the mixture heated to boiling and hydrogen sulphide gas passed in until the precipitated sulphides have coagulated. The liquid is filtered slowly with the aid of suction through a Gooch crucible previously packed with paper pulp * and washed with hydrogen sulphide water. Heavy metals having thus been removed, the filtrate is evaporated to dryness in a beaker of Pyrex glass by gently boiling it over a wire gauze, or if heavy metals were not present originally, an aliquot part of the 0·1N hydrochloric acid extract is similarly evaporated. To the residue is added 0·5 ml. of concentrated nitric acid and about 5 mg. of potassium chlorate, the mixture evaporated to dryness, then a

* *Preparation of Gooch Crucible.* About 0·2 g. of Whatman No. 1 or similar filter-paper is covered with 10N hydrochloric acid and rubbed with a glass rod until thoroughly disintegrated. After dilution with water, the mixture is poured into a Gooch crucible having a base of about 2 cm. in diameter, and the pulp is gently but firmly packed by pressing it with the flattened end of a glass rod and washed with water until free from acid, suction being used.

few drops of 5N ammonia added and the evaporation repeated. To the residue is added 1 ml. of 0·5N acetic acid and the beaker is immersed in a water-bath adjusted to 60° C. When the residue has dissolved, 0·5 ml. of the solution of sodium quinaldinate is added *drop by drop*, the mixture is maintained at 60° C. for 5 minutes then the liquid, together with the precipitate of zinc quinaldinate, is transferred to a 15-ml. centrifuge tube using warm water to wash out the beaker. The suspension is centrifuged, the supernatant liquor rejected, the precipitate washed with three portions of acetone, the solvent decanted and rejected and the residue dried in an oven adjusted to 100° C.

Into a dry Pyrex round-bottomed flask of 50 ml. capacity, fitted with an air condenser by means of a ground-glass joint, is introduced 1 g. of phthalic anhydride, 1·5 g. of naphthalene (recryst.) and 0·5 g. of zinc filings (reagent quality). The dried residue in the centrifuge tube is dissolved by adding 2 ml. of glacial acetic acid (99·5 to 100 per cent.) and boiling the acid for a few moments. The resulting solution is transferred to the Pyrex flask and the tube washed with three separate 1-ml. portions of glacial acetic acid. The condenser is attached to the flask and the contents gently boiled for exactly 20 minutes. The partly cooled solution is decanted into a 25-ml. stoppered measuring cylinder the flask washed several times with a mixed solvent consisting of equal volumes of acetone and ethyl alcohol (95 per cent.), the washings added to the contents of the cylinder and, after allowing to cool, the volume of the liquid adjusted to 25 ml. A portion of the clear yellow liquid is transferred to a 2-inch all-glass cell and the intensity of the colour measured by means of a Lovibond tintometer. The amount of zinc present in the 25 ml. of solution is read from a curve based on the data presented in Table VIII. A control determination is made to correct for minute traces of zinc present as impurity in the reagents employed and the amount so found deducted from that obtained in the primary determination.

If the yellow component of the colour due to the sample is more than 5·4 Lovibond units when measured in a 2-inch cell, the precipitation and colour test should be repeated, employing a smaller aliquot part of the reserved portion of the hydrochloric acid extract.

(*for Natural Waters*). A suitable quantity of the sample (250 or 500 ml.) is acidified with hydrochloric acid, using 1 ml. of 10N acid for each 100 ml. of water, the acidified sample boiled for 5 minutes,

TABLE VIII. RELATION BETWEEN THE QUANTITY OF ZINC AND THE COLOUR PRODUCED

Quantity of Zinc Present in the Actual Test mg.	Colour observed in 2-in. cell : Lovibond Units		
	Yellow	Red *	Brightness *
Nil.	Nil.	—	—
0·01	0·9	0·1	—
0·02	1·8	0·2	—
0·03	2·7	0·4	0·1
0·04	3·5	0·6	0·1
0·05	4·3	0·8	0·1
0·06	4·9	0·9	0·1
0·07	5·4	1·0	0·1

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the yellow component are considered in the calculation of results. As the colour produced is sometimes slightly brighter than the Lovibond glasses it may be necessary to dull it down by means of 0·1 unit of neutral tint.

allowed to cool and 0·2 g. of tartaric acid and 1 ml. of a 10 per cent. alcoholic solution of resorcinol added for each 100 ml. of sample taken. When the tartaric acid has dissolved the solution is transferred to a separator, the reaction adjusted to *p*H 9 with 5N ammonia and the determination continued as described above, beginning with the extraction of the zinc with dithizone.

Discussion. The particular feature in the above procedure for the isolation of zinc is the use of resorcinol for facilitating the extraction with dithizone : in the absence of this substance the removal of zinc is frequently incomplete. Before this method was developed N. D. Sylvester and E. B. Hughes[15] had recommended extraction from an aqueous medium buffered with ammonium acetate at about *p*H 4·5 but, although frequently giving satisfactory results, the procedure is liable to fail when zinc is associated with relatively large proportions of heavy metals and iron. These investigators conduct the final determination volumetrically using potassium ferricyanide and potassium iodide and titrating with 0·002N sodium thiosulphate after the method of Lang[16], hence it is necessary to treat rather large samples in order to detect and estimate traces of zinc. However, for the determination of zinc present in foodstuffs the procedure of Sylvester and Hughes is generally to be preferred to the colorimetric method.

The naphthalene used in the colour test serves as a solvent and prevents the excess of phthalic anhydride from crystallising out of

solution as the mixture cools. A large proportion of the latter reagent is essential to obtain full production of colour. Of the commonly occurring metals, cadmium, nickel and cobalt interfere ; a relatively large quantity of manganese, even if not removed, has no influence on the precipitation of zinc by quinaldinic acid. Phosphates, and other common anions, do not disturb the test and the method is, therefore, suitable for the examination of natural waters, including sea water, particularly as the preliminary evaporation of large volumes is obviated. Great care is necessary to avoid contamination from glass vessels and Pyrex brand should be employed for conducting the test and it is recommended that samples be collected in bottles lined with paraffin wax.

W. L. Lott[17] proposed a method for the colorimetric determination of zinc depending upon precipitation with 5-nitroquinaldinic acid followed by solution of the precipitate in hydrochloric acid containing stannous chloride and measurement of the orange colour produced. It would seem that the method is less sensitive than the one described above as it is stated to be suitable for quantities of zinc ranging from 0·05 to 1·0 mg. The reagent is prepared from quinaldinic acid[18] but as the yield is less than 50 per cent. of the theoretical it is expensive and difficult to obtain.

REFERENCES

1. M. Jaffe, *Virchow's Arch. path. Anat.*, 1869, **47,** 405.
2. R. E. Lutz, *J. Ind. Hyg.*, 1925, **7,** 273.
3. L. T. Fairhall, *J. Ind. Hyg.*, 1926, **8,** 165.
4. V. G. Heller and A. D. Burke, *J. Biol. Chem.*, 1927, **74,** 85.
5. W. R. Todd and C. A. Elvehjem, *J. Biol. Chem.*, 1932, **94,** 609.
6. P. L. Hibbard, *Ind. Eng. Chem., Anal. Ed.*, 1934, **6,** 423.
7. G. Rienäcker and W. Schiff, *Z. anal. Chem.*, 1935, **94,** 409.
8. W. Deckert, *Z. anal. Chem.*, 1935, **100,** 385.
9. H. Fischer and G. Leopoldi, *Wiss. Veroff. Siemens-Konz.*, 1933, **12,** 44.
10. H. Fischer and G. Leopoldi, *Z. anal. Chem.*, 1936, **107,** 241.
11. P. L. Hibbard, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9,** 127.
12. E. B. Sandell, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9,** 464.
13. R. Strohecker, H. Riffart and J. Haberstock, *Z. Unters. Lebensm.*, 1937, **74,** 155.
14. N. L. Allport and C. D. B. Moon, *Analyst*, 1939, **64,** 395.
15. N. D. Sylvester and E. B. Hughes, *Analyst*, 1936, **61,** 734.
16. R. Lang, *Z. anal. Chem.*, 1929, **79,** 161.
17. W. L. Lott, *Ind. Eng. Chem., Anal. Ed.*, 1938, **10,** 335.
18. E. Besthorn and S. Ibele, *Ber.*, 1906, **39,** 2333.

SECTION II

ACID RADICLES

INTRODUCTION

It may be helpful to refer to available colorimetric methods for certain common radicles for which it has not been considered necessary to include monographs in the text. Of these, Steiger's method[1] for fluorides which depends upon their bleaching action on acidified pertitanate solutions is probably the best known. Unfortunately this procedure, as well as all others based upon a similar principle, are subject to interference by so many other substances normally associated with fluorides that their utility is extremely limited. Thus, alkali sulphates, phosphates, vanadates, silicates, nitrates, iron and aluminium all disturb the pertitanate test, whence preliminary isolation of fluorine is almost invariably necessary. When separation has been effected, say as hydrofluosilicic acid by distillation from perchloric acid in the presence of glass beads, it is best to proceed by a volumetric method and to titrate with standard solution of thorium nitrate. This process for the determination of small quantities of fluorine was originally proposed by Willard and Winter[2] who used the zirconium lake of sodium alizarin sulphonate as indicator and it has since been modified by a number of investigators, notably Armstrong[3] who recommended the omission of the zirconium, while a further improvement was effected by Hoskins and Ferris[4] who besides adopting Armstrong's modification, titrated in the presence of a buffer produced by half neutralising monochloracetic acid.

A promising, although somewhat complicated technique, for the colorimetric determination of citrates[5–7] has been evolved on the basis of an observation made by Pucher, Vickery and Leavenworth[8] that when pentabromacetone, produced by the action of potassium permanganate and bromine on citrates, is dissolved in light petroleum and the resulting liquid shaken with a freshly prepared solution of sodium sulphide a transient yellow colour is produced in the aqueous layer which may be stabilised by the

addition of pyridine[5] or dioxan[6]. The method has been applied to biological material of both vegetable and animal origin.

Some attempts have been made to evolve colorimetric methods for the determination of chlorides. Thus M. L. Isaacs[9] recommended adding solid silver chromate to solutions containing soluble chlorides, whence the insoluble silver chloride is precipitated with a corresponding passage into solution of chromate ions, the estimation being completed by measuring the yellow colour produced. A modification of this process has been suggested in which the proportion of dissolved chromate is determined by means of the diphenylcarbazide reaction[10]. Conway has designed an apparatus wherein the chloride to be determined is submitted to wet-combustion and the volatile chlorine passed into potassium iodide solution liberating an equivalent proportion of iodine which is determined by measurement of its colour[11]. In a third method Sendroy[12] adds solid silver iodate to the solution to be examined, thus producing insoluble silver chloride and forming a corresponding quantity of soluble iodate : the amount of the latter is then determined by acidifying with phosphoric acid, adding an alkali iodide and measuring the colour of the liberated iodine. All these procedures have been applied to biochemical investigations and while, no doubt, they are suitable for the specialised purposes for which they were developed, all demand a complicated technique with elaborate attention to detail and, since gravimetric or volumetric methods are more generally applicable, it has not been deemed necessary to describe any one of them in full.

References

1. G. Steiger, *J. Amer. Chem. Soc.*, 1908, **30**, 219.
2. H. H. Willard and O. B. Winter, *Ind. Eng. Chem., Anal. Ed.*, 1933, **5**, 7.
3. W. D. Armstrong, *J. Amer. Chem. Soc.*, 1933, **55**, 1741.
4. W. M. Hoskins and C. A. Ferris, *Ind. Eng. Chem., Anal. Ed.*, 1936, **8**, 6.
5. G. W. Pucher, C. C. Sherman and H. B. Vickery, *J. Biol. Chem.*, 1936, **113**, 235.
6. W. A. Johnson, *Biochem. J.*, 1939, **33**, 1046.
7. F. Dickens, *Biochem. J.*, 1941, **35**, 1011.
8. G. W. Pucher, H. B. Vickery and C. S. Leavenworth, *Ind. Eng. Chem., Anal. Ed.*, 1934, **6**, 190.
9. M. L. Isaacs, *J. Biol. Chem.*, 1922, **53**, 17.
10. B. B. Westfall, T. Findley and A. N. Richards, *J. Biol. Chem.*, 1934, **107**, 661.
11. E. J. Conway, *Biochem. J.*, 1935, **29**, 2221.
12. J. Sendroy, Jr., *J. Biol. Chem.*, 1937, **120**, 419.

BENZOIC ACID

The partial conversion of benzoic acid into salicylic acid by hydrogen peroxide was first noticed by Hanriot[1] and was later the subject of investigations by many observers with a view to its detection, notably by Jonescu[2], under whose name the test is usually known. More recently the reaction has been exhaustively studied by J. R. Nicholls[3] who found that under standardised conditions the proportion of benzoic acid converted closely approximates to 12 per cent. Further modifications of the technique adopted by Nicholls have been made by F. W. Edwards, H. R. Nanji and M. K. Hassan[4] and the procedure recommended by these investigators is described here. It is with reference to its use as a food preservative that particular interest attaches to the determination of small quantities of benzoic acid and the subject is here discussed from this viewpoint.

Another colour test, originated by Mohler[5], involves nitration to *m*-dinitrobenzoic acid followed by reduction to *m*-diaminobenzoic acid and conversion of this to the ammonium salt which forms a deep red solution. Mohler used ammonium sulphide to effect the reduction but Grossfeld[6] improved the method by using hydroxylamine. Based upon this modification a simple technique has been proposed by A. J. Jones[7] for the approximate determination of benzoic acid added as preservative to fruit cordials and the details are included in this monograph.

Method Based Upon Jonescu's Test[4]. The following special reagents are required :—

1. Iron Reagent for the Oxidation made by dissolving 2·7 g. of anhydrous ferric chloride in 13 ml. of N sulphuric acid and diluting to 100 ml. with water.
2. A Standard Solution of Salicylic Acid in 10 per cent. alcohol, containing 0·1 mg. in 1 ml.
3. Iron Reagent for the Colorimetric Determination made by dissolving 0·1 g. of anhydrous ferric chloride in 20 ml. of N hydrochloric acid and diluting to 100 ml. with water.

The benzoic acid, which may have been isolated from a sample of food by extraction with an immiscible solvent or by steam distillation (*vide infra*), is converted into its ammonium salt by adding dilute ammonia, the excess alkali removed by boiling and the solution, which should now be neutral to litmus, diluted to a

convenient known volume. To an aliquot portion of this solution, expected to contain not more than 5 mg. of benzoic acid, is added 5 ml. of 0·1N sulphuric acid, the volume of the mixture adjusted to 15 ml. with water, 1 ml. of the iron reagent for oxidation (No. 1 above) added followed by 1 ml. of a 0·2 per cent. solution of hydrogen peroxide (made by diluting 2 ml. of " 20 vols." solution to 60 ml. with water). After shaking, the mixture is heated in a boiling water-bath for 15 minutes without further agitation. The liquid, which becomes violet in colour, is cooled, about 5 g. of ammonium sulphate is added and the salicylic acid is extracted with three successive quantities of 15 ml. of ether, each ethereal extract being washed with the same 5 ml. of water. The solvent is removed by distillation on a water-bath at 50° C., the residue dissolved in 50 ml. of alcohol (10 per cent.), 1 ml. of iron reagent for the colorimetric determination (No. 3 above) added and the violet colour produced compared with a series of standards made by diluting suitable quantities (say from 0·5 to 5 ml.) of the standard salicylic acid solution to 50 ml. with alcohol (10 per cent.) and adding 1 ml. of the No. 3 iron reagent. The quantity of salicylic acid found is correlated with the amount of benzoic acid originally present by reference to the experimental values quoted in Table IX.

TABLE IX. RELATION BETWEEN THE AMOUNT OF SALICYLIC ACID FOUND AND THE QUANTITY OF BENZOIC ACID ORIGINALLY PRESENT WHEN THE LATTER HAS BEEN PARTIALLY OXIDISED IN JONESCU'S TEST (F. W. Edwards, H. R. Nanji and M. K. Hassan, *Analyst*, 1937, **62**, 172)

Salicylic Acid Found mg.	Benzoic Acid Originally Present mg.	Proportion of Benzoic Acid Converted per cent.
0·1	0·77	13·0
0·15	1·25	12·0
0·2	1·8	11·1
0·25	2·3	10·9
0·3	2·8	10·7
0·35	3·3	10·6
0·4	3·85	10·4
0·45	5·0	9·0

Discussion. This test is capable of detecting 0·5 mg. of benzoic acid and it is specific except for saccharin. If the latter is present it may be separated by extracting the benzoic acid with carbon tetrachloride in which saccharin is almost insoluble. The presence of appreciable amounts of chlorides and sulphates

reduces the proportion of benzoic acid transformed into salicylic acid but nitrates, unless present in large quantity, do not influence the reaction. If salicylic acid is also present in the solution to be tested it must be removed by selective oxidation. The solution is rendered alkaline with 5 ml. of 0·1N potassium hydroxide, the mixture warmed to 50° C. and N potassium permanganate added drop by drop until a persistent pink colour is produced. The liquid is acidified with 5 ml. of 5N sulphuric acid and decolorised by adding an aqueous solution of oxalic acid drop by drop ; after saturating the clear liquid with ammonium sulphate the benzoic acid is extracted with ether, the ethereal extract washed with water and the acid converted into its ammonium salt by shaking with two or three portions of dilute ammonia. The combined ammoniacal extracts are boiled until neutral to litmus and the colorimetric test for benzoic acid applied as described above. The salicylic acid originally present in the sample may be determined directly by the colorimetric ferric salicylate method since benzoic acid does not interfere.

Application to Milk, Cream and Butter[8]. From 25 to 50 ml. of milk (or 10 to 20 g. of cream or butter) is heated under a reflux condenser with an equal weight of concentrated hydrochloric acid until a dark coloured homogeneous mixture is produced. The cooled mixture is extracted three times with 20 ml. of a mixed solvent consisting of equal volumes of ethyl ether and light petroleum (b. pt. 40°–50° C.), the separated solvent layers mixed and a few drops of strong ammonia solution added ; if benzoic acid was present in the original sample, a precipitate of ammonium benzoate will be observed. About 10 ml. of water is added and the mixture shaken ; the aqueous layer is separated and the extraction repeated twice, keeping alkaline with ammonia if necessary. The mixed aqueous extracts are then examined by the method already described above.

Application to Fruit and Vegetable Foods[9]. A convenient weight of the sample (30 to 100 g.) is introduced into a 500-ml. flask and, if a liquid, saturated with salt at the rate of about 40 g. to every 100 g. of water present. If the sample is not liquid it is mixed with water to the required consistency and the mixture saturated with salt. Ordinary kitchen or block salt can be used for the purpose. The mixture is made distinctly acid with phosphoric acid and is then distilled in a rapid current of steam the distillate being collected in a porcelain dish containing about 10 ml. of N sodium hydroxide. This distillation is continued until

approximately 500 ml. of distillate has been collected, the operation generally taking about 90 minutes. During the distillation the flask containing the foodstuff is heated by a separate burner sufficiently to prevent the volume of the liquid being increased by condensation of steam. There should always be a little undissolved salt present. When the distillation is complete the condenser is washed down into the dish with a little 0·1N sodium hydroxide and the combined distillate and washings evaporated over a boiling-water bath until the liquid is reduced to a volume of about 30 ml. The concentrated distillate is allowed to cool to about 40° to 50° C. and treated in the dish with an approximately 5 per cent. aqueous solution of potassium permanganate until a pink colour persisting for some minutes is clearly visible. The mixture is allowed to cool, the excess of permanganate removed by the addition of sodium sulphite or sulphur dioxide gas and a slight excess of sulphuric acid added ; after saturating the clear liquid with ammonium sulphate the benzoic acid is extracted with ether, the ethereal extract washed with water and the acid converted into its ammonium salt by shaking with two or three portions of dilute ammonia. The combined ammoniacal extracts are boiled until neutral to litmus and the colorimetric test for benzoic acid applied.

Notes on the Applications. The method described above for the examination of milk, cream and butter was developed in 1913 by E. Hinks[8] and there is no doubt that it is the best method to adopt when dealing with fatty foods. The presence of boric acid will not interfere with the detection and determination of benzoic acid. Salicylic acid, if present, will be extracted in the same way as benzoic acid and may be determined colorimetrically with ferric chloride (see p. 163).

The procedure given for the extraction of benzoic acid from fruit and vegetable foods was worked out by G. W. Monier-Williams[9] who recommends that the benzoic acid thus removed by steam distillation be extracted from the aqueous solution with a mixed solvent of light petroleum and ethyl ether and finally collected by sublimation and weighed. It is pointed out that both salicylic acid and cinnamic acid may be separated from fruit products by steam distillation and if it is desired to determine either of these substances the oxidation with permanganate should be omitted. Both acids may be isolated by sublimation or, alternatively, the colorimetric method using ferric chloride can be employed for the determination of salicylic acid. There is no

satisfactory colour method for cinnamic acid but its presence may be established by treating the suspected solution with alkaline permanganate when an odour of benzaldehyde will be produced if a cinnamate was present.

Method Based Upon Mohler's Test applied to Fruit Cordials[7]. A measured quantity of the beverage (10 ml.) is transferred to a separator, 5 ml. of 2N sulphuric acid added and the mixture extracted, first with 20 ml. of a mixture of equal volumes of light petroleum and ethyl ether, then with 10 ml. more of the same solvent. The mixed ethereal liquid is extracted with 5 ml. of a 1 per cent. aqueous solution of sodium hydroxide and, after the aqueous layer has been separated, the ethereal liquid is again shaken with 5 ml. of water and the separated aqueous layer added to the sodium hydroxide solution. In making this alkaline extraction, care should be exercised to ensure that the alkali is present in excess and throughout the above procedure the usual extra rinses should be made following each separation in order to eliminate all source of loss.

The alkaline extract and rinsings are evaporated to dryness in a small dish over a boiling water-bath and 5 ml. of nitrating acid (a mixture of 1 vol. of nitric acid, s.g. 1·42, with 9 vols. of sulphuric acid, s.g. 1·84) is added to the residue. The mixture is warmed slightly and when the residue is dissolved the acid solution is transferred to a test tube ($5 \times \frac{7}{8}$ in.) the dish being rinsed, first with 2 ml., then with 1 ml. of the nitrating acid. The acid liquid is quickly heated by means of a free flame to 155° C. and maintained at a temperature between 155° and 160° C. for 3 minutes. After the liquid has cooled down to about 100° C. the tube is immersed in cold water until complete cooling of the acid has been effected when 20 ml. of water is added, the diluted acid is cooled again and transferred to a separator. Any *m*-dinitrobenzoic acid present in the acid liquid is extracted by shaking, first with 15 ml. of ethyl ether, then with a further 10 ml. of the same solvent. The ethereal layers are mixed, the *m*-dinitrobenzoic acid transferred to ammoniacal aqueous solution by extracting the ether with 10 ml. of dilute ammonia (approximately 5 per cent.) and then with two further 5-ml. portions of water. The mixed aqueous ammoniacal extracts are rendered strongly alkaline by the addition of 3 ml. of strong ammonia solution, 1 g. of hydroxylamine hydrochloride added and the mixture warmed to 40° C. If benzoic acid was originally present in the sample a red colour will develop. The mixture is allowed to stand for 20 minutes in order that the colour

may attain its full intensity, then diluted with water to a volume of 50 ml. and the tint compared with that of standards similarly prepared. If 5·7 mg. of benzoic acid be taken as solution of sodium benzoate, evaporated to dryness and nitrated in accordance with the above procedure, the colour produced will be representative of 5 grains to the pint when operating on 10 ml. of the beverage.

Discussion. According to von der Heide and Jacob[10] it is better to nitrate at 130° C. for 10 minutes or, alternatively, at 100° C. for 20 minutes : if the former temperature is exceeded the test becomes less reliable and may fail altogether. Hence it might well be an advantage to apply this modification to the above procedure. The ammoniacal extract of the nitrated acid, separating from the ethers, may vary in colour from almost nothing in a blank to a moderately deep shade of yellow in the test. This is apparently due to extractives from the juice or cordial other than benzoic acid, but no serious disturbance is likely to be experienced from this source. It is important that the colour produced in the assay should be matched against a standard of nearly equal tint since the intensity of tint produced does not vary linearly with the quantity of benzoic acid. In dealing with pulpy preparations A. J. Jones prefers to operate on the moderately clear filtrate without attempting to wash the suspension ; while it may be assumed that the distribution of benzoic acid is uniform throughout the whole mixture, including cellular matter, it is not certain that washing the filter would effect complete displacement from the solid vegetable material.

The test is not specific and it may be expected to respond to any aromatic substance which nitrates in this particular manner ; thus saccharin will react but yields a colour much weaker in intensity than does benzoic acid. Only the merest trace of colour is given by salicylic acid or phenolphthalein.

References

1. M. Hanriot, *Compt. rend.*, 1886, **102,** 1250.
2. A. Jonescu, *J. Pharm. Chim.*, 1909, [vi], **29,** 533.
3. J. R. Nicholls, *Analyst*, 1928, **53,** 19.
4. F. W. Edwards, H. R. Nanji and M. K. Hassan, *Analyst*, 1937, **62,** 172.
5. E. Mohler, *Bull. Soc. chim.*, 1890, [iii], **3,** 414.
6. J. Grossfeld, *Z. Unters. Nahr. Genussm.*, 1915, **30,** 271.
7. A. J. Jones, *Yearb. Pharm.*, 1925, 493.
8. E. Hinks, *Analyst*, 1913, **38,** 555.
9. G. W. Monier-Williams, Reports on Public Health and Medical Subjects No. 39 (1927), "The Determination of Benzoic Acid in Foodstuffs."
10. C. von der Heide and F. Jacob, *Z. Unters. Nahr. Genussm.*, 1910, **19,** 137.

BORATES

Many proposals for the quantitative application of the well-known turmeric test have been published. While some investigators have endeavoured to standardise the colours as developed on strips of paper, there seems to be little doubt that better results can be obtained by working with solutions containing the colouring matter (curcumin) derived from the rhizome of *Curcuma longa*. G. S. Smith has suggested an entirely different method depending upon the change in colour from reddish-violet to blue when a trace of boric acid is added to a solution of quinalizarin in slightly diluted sulphuric acid[1]. The relative simplicity of this test has attracted the attention of workers in agricultural chemistry[2–4]. Dickinson has proposed the use of artificial colour standards prepared from alkaline mixtures of phenolphthalein and thymolphthalein but reports having obtained anomalous results in some instances and recommends using alizarin S in place of quinalizarin and matching against standards made from methyl orange[5]. Although these two latter procedures are somewhat more sensitive and more expeditiously carried out than the turmeric method, the gradation of colour change is not good and both necessitate the use of strong sulphuric acid in the final reaction mixture. In the experience of the present author the turmeric method as developed by K. L. Robinson[6] is the most satisfactory at present available.

Method[6]. The colouring matter from about 20 g. of powdered turmeric is extracted with ether in a Soxhlet apparatus and the ethereal solution diluted to 250 ml. with more ether ; a convenient amount of this is mixed with acid-washed silver sand in the proportion of 4 ml. to 40 g. of sand and the mixture dried on a water-bath. In order to standardise this turmeric-coated sand, convenient quantities of a standard solution of boric acid are evaporated to dryness, each with 0·5 ml. of N sodium hydroxide, in porcelain dishes. The residue in each dish is dissolved in 2 ml. of a 4·5 per cent. aqueous solution of oxalic acid and 40 g. of turmeric-coated sand added, the mixtures well stirred with a glass rod and heated to dryness on the water-bath. A further 2 ml. of the oxalic acid solution is added and, after thoroughly mixing, the contents of the dishes are heated to dryness. The sand is transferred to a sintered glass filter funnel (No. 11 G1) and extracted with 70 per cent. alcohol until the filtrate is colourless, the latter

is then made up to 100 ml. and the intensity of the red component of its colour, which is stable for about 30 minutes, measured by means of a Lovibond tintometer using a 1-cm. cell. In the absence of boron the alcoholic extract of the turmeric-coated sand gives a reading of about 1·0 Lovibond red unit while, with 0·05 mg. of B_2O_3 present, the reading is about 11·0 red units. Since the relation between the intensity of the red component and the quantity of boron is linear the tintometer may be used as a permanent external standard if the value of the blank reading due to the particular batch of turmeric-coated sand in use is deducted from the actual reading. The amount of boron present is then calculated by reference to a straight-line graph passing through the origin and correlating 0·05 mg. of B_2O_3 with 10·0 red units.

Discussion. Owing to the unchanged yellow turmeric in the solution (between 20 and 30 Lovibond yellow units) it is not possible to make satisfactory direct visual comparisons with standards or even to use a Duboscq pattern colorimeter: an instrument such as the Lovibond tintometer or a spectrophotometer which is capable of resolving the colour and measuring the red component is essential. Using the Lovibond instrument it is quite easy to perceive even small differences in the values for the red colour notwithstanding the preponderance of yellow. The above method avoids heating the solution under examination with an alcoholic extract of turmeric, a practice which leads to loss of boron due to volatilisation of ethyl borate. The use of oxalic acid increases the sensitivity of the test. The sand employed should be sifted free from dust, otherwise the final alcoholic extracts may be turbid.

Certain substances, such as phosphates, silicates and salts of the alkali metals, hinder the development of the turmeric red colour and it is therefore generally advisable to remove the boron by distillation as methyl borate prior to attempting its colorimetric determination. The following method proposed by Robinson[6] for dried plant material will be applicable to many other substances.

General Application. An appropriate quantity of the sample is rendered alkaline with a saturated solution of barium hydroxide then dried and ignited and the residue transferred to a distillation apparatus together with 5 ml. of ortho-phosphoric acid (s.g. 1·75) and 15 ml. of methyl alcohol. The flask is heated by immersion in a boiling water-bath until all the alcohol has distilled over, the

distillate being received in a flask containing 0·5 ml. of N sodium hydroxide ; the end of the condenser should dip under the surface of the soda solution. Another flask containing 25 ml. of methyl alcohol is attached in series with the first flask and heated so that a stream of alcohol vapour passes through the latter which is still kept hot in the water-bath ; in this way all the boron is quantitatively removed and the distillate, after evaporation to dryness, is submitted to the colorimetric test as described above. It is necessary to exercise caution in the choice of glassware which must be free from soluble boron.

REFERENCES

1. G. S. Smith, *Analyst*, 1935, **60**, 735.
2. K. C. Berger and E. Truog, *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 540.
3. L. C. Olson and E. E. DeTurk, *Soil Sci.*, 1940, **50**, 257.
4. P. W. Maunsell, *N.Z.J. Sci. Tech.*, 1940, **22**, 100b.
5. D. Dickinson, *Analyst*, 1943, **68**, 106.
6. K. L. Robinson, *Analyst*, 1939, **64**, 324.

BROMIDES

See Section III: Substances of Clinical and Biochemical Significance, p. 196.

CYANIDES

Of the known colour reactions the most suitable for quantitative application are the Prussian blue test in which an alkaline solution of cyanide is warmed in the presence of ferrous sulphate, the ferrocyanide formed being detected by the addition of a ferric salt[1], and Weehuizen's test which depends on the production of a red colour by the oxidation of an alkaline solution of phenolphthalin to phenolphthalein by cupric ions in the presence of cyanide[2]. These two methods have been carefully studied by A. E. Childs and W. C. Ball who describe a procedure for the examination of river waters contaminated with cyanides derived from trade wastes[3]. Other methods which have been recommended include one based upon the formation of a cherry-red coloured alkaline *iso*purpurate by the interaction of cyanides with picric acid[4] and the method involving production of ferric thio-

cyanate by treatment of the sample with ammonium polysulphide and subsequent addition of ferric chloride[5–7]: with regard to the former, other reducing substances yield similar results and the method is limited in application[8] while the latter is complicated and tedious to carry out and is unreliable at extreme limits of dilution.

Prussian Blue Method[3]. To not more than 8 ml. of the solution to be examined, containing not less than 0·2 mg. of cyanide as HCN, is added 0·1 ml. of a 1 per cent. aqueous solution of sodium hydroxide (or just sufficient to render the liquid alkaline) and then 1 ml. of a freshly prepared 0·1 per cent. aqueous solution of ferrous sulphate. After gentle warming the mixture is allowed to stand for about 5 minutes then 0·25 ml. of a 0·1 per cent. aqueous solution of ferric chloride, $FeCl_3$, added followed by 1 ml. of a dilute solution of hydrochloric acid (1 per cent. HCl). The whole is diluted to 10 ml. with water and, after mixing, any blue colour matched against standards similarly prepared. A standard solution may be conveniently made from AnalaR grade potassium cyanide. Assuming that an average sample of this material contains 97 per cent. KCN, then 2·490 g. dissolved in water and diluted to 1 litre will produce a solution containing the equivalent of 1 mg. HCN per ml.

Method of Weehuizen[2]. A reagent is prepared by diluting 10 ml. of a 1 per cent. solution of pure phenolphthalin in 0·2 per cent. aqueous solution of sodium hydroxide to 50 ml. with 2·5 per cent. w/v solution of glycerol and adding 50 ml. of a 0·3 per cent. aqueous solution of cupric acetate and, if the mixture is cloudy, filtering into a stoppered glass bottle. To not more than 8 ml. of the solution to be examined, containing not less than 0·001 mg. of cyanide as HCN, is added 1 ml. of the above reagent, then 0·25 ml. of a 1 per cent. solution of sodium hydroxide (or sufficient to render the mixture alkaline) and the liquid diluted to 10 ml. with water. The temperature of the reaction mixture should at no time exceed 15° C. Any red colour produced is matched against standards made at the same time from an appropriate solution of potassium cyanide.

Application to Water[3]. A known volume of the sample to be tested (500 ml. is a convenient quantity) is filtered free from suspended matter and transferred to a round-bottomed flask fitted with a condenser. Tartaric acid (0·5 g.) is added to the water, which is then gently distilled, a few fragments of porous pot being included to prevent bumping. Should the sample be originally

alkaline, it must first be neutralised with tartaric acid and the 0·5 g. in excess then added. Boiling is continued until 50 ml. has distilled over or, if the concentration of cyanide is less than 1 in 10 million, only 25 ml. of distillate should be collected. If sulphides are present in the original sample a very slight excess of lead nitrate or acetate solution is added to the water before the addition of tartaric acid and the lead sulphide produced filtered off before distillation. An aliquot part of the distillate is submitted to one of the procedures described above, the choice of method being determined by the degree of sensitivity required.

Discussion. In the absence of ferrocyanides, the Prussian blue method is specific for cyanides and this test, rather than any other, is recommended when its relatively low degree of sensitivity permits. It can be used for the examination of crude waters if the concentration of hydrocyanic acid therein is not less than 1 part in 250,000. When so much cyanide is present that a blue precipitate is formed in the test mixture the determination must, of course, be repeated using a smaller aliquot part of the sample.

The Weehuizen test is not specific for cyanides since other substances which are able to oxidise the phenolphthalin will give a positive reaction. Thus ferricyanide and aqueous solutions of the halogens give a similar coloration but ferrocyanides, chromates, nitric acid, ferric chloride and halogen salts give no reaction, and neither does a weak solution of hydrogen peroxide. Sulphides interfere by precipitating the copper in the reagent. This test is applicable to the determination of cyanides in crude waters even when the concentration of the latter is no more than 1 part in 50 million.

The application to the examination of water described above is based upon the fact that hydrocyanic acid is volatile in steam and 95 per cent. or more passes over with the first 5 per cent. of distillate while about 99 per cent. is removed by distilling 10 per cent. of the sample. The distillate should be colourless and free from opalescence, otherwise the presence of steam-volatile impurities such as phenols or fatty acids should be presumed. Since these substances are liable to interfere with the colorimetric determinations it is advisable, if their presence is suspected, to conduct the distillation under reduced pressure and at as low a temperature as practicable in order to minimise the amount passing over into the final test liquid. It should be emphasised that, in carrying out the distillation in this manner, a highly efficient condenser is required. Chlorides, bromides, iodides, ferrocyanides, ferricyanides,

cyanates, thiocyanates, chromates, and non-volatile substances generally, which may be present in the water under examination, will not be found in the distillate. The addition of lead salt for the purpose of withholding hydrogen sulphide will not interfere with the quantitative distillation of cyanide provided that the concentration of the latter in the water does not exceed 1 part in half a million. The presence of a sufficiency of a strong oxidising agent in the water may tend to oxidise cyanide to cyanate thus leading to low results. In the presence of ferrocyanides the results will be high, because distillation of solutions of these compounds with tartaric acid causes the precipitation of ferrous hydrogen ferrocyanide and the liberation of an equivalent quantity of hydrocyanic acid.

REFERENCES

1. E. Berl and M. Delpy, *Ber.*, 1910, **43**, 1430.
2. F. Weehuizen, *Pharm. Weekblad*, 1905, **42**, 271.
3. A. E. Childs and W. C. Ball, *Analyst*, 1935, **60**, 294.
4. E. Durien, *Jahresb. Pharm.*, 1902, 439.
5. G. Lockemann, *Ber.*, 1910, **43**, 2127.
6. C. K. Francis and W. B. Connell, *J. Amer. Chem. Soc.*, 1913, **35**, 1624.
7. M. O. Johnson, *J. Amer. Chem. Soc.*, 1916, **38**, 1230.
8. A. Chaston Chapman, *Analyst*, 1910, **35**, 469.

p-HYDROXYBENZOIC ACID AND ITS DERIVATIVES

The use of *p*-hydroxybenzoic acid (" para-acid "), its sodium salt and its esters as food preservatives is permitted in some parts of the world * but is prohibited in Great Britain by the Public Health (Preservatives, etc., in Food) Regulations (Statutory Rules and Orders 1925, No. 775 as amended by 1926, No. 1557 and 1927, No. 577). Of the esters, the commonest are " Nipagin A," the ethyl ester ; " Nipagin M," also known as " Solbrol," which is the methyl ester ; and " Nipasol M," the propyl ester. F. W. Edwards, H. R. Nanji and M. K. Hassan[1] have proposed a method of colorimetric determination using Millon's reagent with which *p*-hydroxybenzoic acid gives a rose-red colour.

Method[1]. The Millon's reagent should be prepared by dissolving 1 part by weight of mercury in 2 parts by weight of concentrated nitric acid by gentle warming and diluting the resulting solution

* Notably in the United States and Germany.

with twice its volume of water. The freshly prepared reagent is more sensitive than one which has been stored and gives a deeper shade of red in the test.

The *p*-hydroxybenzoic acid, if originally present as one of its esters, must be isolated as the ammonium salt by a technique suited to the work in hand and following on the principles outlined below in the descriptions of the applications of the test. The solution of the free acid in dilute ammonia is boiled until it is neutral to litmus and 20 ml. of the hot liquid, containing not more than 2 mg. of the acid treated in a boiling tube with 2 ml. of Millon's reagent. A series of standards containing 1, 2·5, 5, 7·5 and 10 ml., etc., of an aqueous solution of *p*-hydroxybenzoic acid (0·1 mg. in 1 ml.) diluted to 20 ml. with water, is prepared in boiling tubes, and 2 ml. of Millon's reagent is added to each. The test solution and the standards are heated in a boiling water-bath for not less than 5 minutes, then diluted immediately to 50 ml. in Nessler glasses and the colours compared.

Application to Milk[1]. A suitable quantity of the sample (say 25 ml.) is treated in a 50-ml. graduated flask with 5 ml. of zinc acetate solution (21·9 g. crystallised zinc acetate, $Zn(C_2H_3O_2)_2.2H_2O$, and 3 ml. of glacial acetic acid in 100 ml.) and 5 ml. of potassium ferrocyanide solution (10·6 g. crystallised potassium ferrocyanide, $K_4Fe(CN)_6.3H_2O$, in 100 ml.). The mixture is diluted with water to 50 ml., shaken, allowed to stand for 5 minutes, filtered through a dry paper, 30 ml. of the filtrate saturated with ammonium sulphate, extracted three times with 15-ml. portions of ether, the ethereal extracts collected in a flask and the solvent removed by distillation. To the residue 60 ml. of alcohol and 5 ml. of 0·5N alcoholic potash are added and the mixture boiled under a reflux condenser for 2 hours. The bulk of the alcohol is then removed by evaporation, the residue diluted with water, acidified with dilute sulphuric acid and after saturating the solution with ammonium sulphate extracted with three 15-ml. portions of ether. The mixed ethereal extracts are shaken with three successive 10-ml. portions of dilute ammonia solution, the combined ammoniacal extracts boiled until the solution is neutral to litmus and the colorimetric determination applied as already described.

Application to Wines and Cordials[1]. The sample is saturated with ammonium sulphate (alcohol, if present, being first removed as far as possible by evaporation) then acidified with dilute sulphuric acid and directly extracted with three successive 15-ml.

portions of ether. The solvent is removed by distillation and the hydrolysis with alcoholic potash and conversion of the free acid thus obtained into its ammonium salt carried out by the procedure given above for the application of the method to milk.

Application to Butter[1]. A suitable weight of sample (say 25 g.) is treated with 100 ml. of alcohol (96 per cent.), the mixture boiled under a reflux condenser for 30 minutes, cooled until the fat almost solidifies, then filtered and the residue in the flask washed with two or three successive 20-ml. portions of boiling alcohol and each time cooled again and filtered. To the mixed alcoholic filtrates 20 ml. of 0·5N alcoholic potash is added and the subsequent stages of the process carried out exactly as described for milk.

Application to Sausages, Meat and Fish Pastes and Canned Fish[1]. The sample (25 g.) is treated with 100 ml. of alcohol (96 per cent.), the mixture boiled under a reflux condenser for 30 minutes and filtered while still hot through a Buchner funnel containing a pad of cotton-wool layered over the filter paper. The residue in the flask is washed two or three times with hot alcohol and the filtrate, which may be slightly turbid, treated with 20 ml. of 0·5N alcoholic potash and the determination completed as described for milk.

Discussion. The test with Millon's reagent is not specific for *p*-hydroxybenzoic acid since colorations are obtained with nearly all aromatic substances containing a hydroxyl group attached to the benzene nucleus. No colour is produced when the test is applied to benzoic acid but salicylic acid yields an orange-red which is not sufficiently distinct from the tint given by *p*-hydroxybenzoic acid to ensure certain demarcation between the two compounds. Since *p*-hydroxybenzoic acid gives no appreciable reaction with ferric chloride it may be possible in cases where both preservatives are present to determine the proportion of salicylic acid by application of the ferric chloride test (see p. 163) and to make an approximate determination of the *p*-hydroxybenzoic acid by applying the above method with Millon's reagent using standards containing the correct proportion of salicylic acid. Edwards, Nanji and Hassan[1] recommend the formation of the nearly insoluble copper salt of *p*-hydroxybenzoic acid as a means of establishing its presence: to an aliquot part of the neutral ammonium salt containing not less than the equivalent of 2 mg. of the acid (preferably 5 mg.) is added 1 ml. of a 2 per cent. aqueous solution of copper sulphate, the mixture evaporated on a water-bath to about 1 ml. and the residue allowed to cool, when

characteristic crystals form. Neither benzoic acid nor salicylic acid give this reaction. By applying the tests mentioned above in association with Jonescu's and Mohler's reactions for benzoic acid (see p. 129) and Jorissen's test for salicylic acid (see p. 164) it is possible from the results obtained to establish the identity of any one of this group of preservatives as indicated in Table X.

TABLE X. RESPONSE GIVEN BY BENZOIC, SALICYLIC AND *p*-HYDROXYBENZOIC ACIDS TO IDENTITY TESTS (After F. W. Edwards, H. R. Nanji and M. K. Hassan, *Analyst*, 1937, **62**, 178)

Test	Response		
	Benzoic Acid	Salicylic Acid	*p*-Hydroxybenzoic Acid
Millon's reagent	Nil	Orange-red	Rose-red
Ferric chloride (J. R. Nicholls' technique[2])	Nil	Violet	Nil or almost nil
Copper sulphate	—	No crystals	Characteristic crystals
Jonescu's	Violet	Violet	No colour
Mohler's	Red	Yellow	No colour
Jorissen's	No colour	Red	Yellow

The identification of the different esters of *p*-hydroxybenzoic acid is an intricate and tedious procedure[3, 4]. In Great Britain it is sufficient to return results as " a preparation of *para*-hydroxybenzoic acid equivalent to *x* parts of *para*-hydroxybenzoic acid per million "[1].

The methods given above for the isolation of *p*-hydroxybenzoic acid, or its esters, from various foods may be expected generally to yield from 80 to 90 per cent. of the preservative actually present but, in the case of milk, Edwards, Nanji and Hassan[1] only attained an average recovery of the ethyl ester amounting to 60 per cent.

Stevenson and Resuggan have shown that under certain conditions *p*-hydroxybenzoic acid couples with solutions of benzene diazonium salts to give a dark, reddish-brown mixture of mono-, bis- and tris-azophenols[5]. These substances, which result from decarboxylation of the *p*-hydroxy acid and subsequent coupling, are insoluble in solutions of alkali carbonates, whilst the product obtained by coupling salicylic acid with diazonium salts (namely, 4-hydroxy-azo-benzene-3-carboxylic acid) is readily soluble. Mono- and bis-azo substituted 4-hydroxy-azo-benzene (azo

derivatives of *p*-hydroxybenzoic acid) occur in forms which exhibit a colour reaction that can be utilised for the detection of the para-acid. The diazo solution is prepared by dissolving 5 g. of aniline in a mixture of 13 ml. of concentrated hydrochloric acid and 26 ml. of water, cooling the resulting mixture to 5° C. and adding a solution of sodium nitrite (4·5 g. in 20 ml. of water) until free nitrous acid is present as indicated by starch iodide paper. The diazo solution is kept at a temperature below 5° C. and used within an hour of its preparation. In order to detect *p*-hydroxybenzoic acid alone, a small quantity is dissolved in a little sodium hydroxide solution, the mixture cooled to 5° C., an excess of the diazo solution slowly added and, after standing for a short time, the mixture acidified with hydrochloric acid and extracted with ether; the ethereal layer is then shaken with a little sodium hydroxide solution when the presence of the para-acid is indicated by the development of a deep red colour in the ether, the aqueous layer remaining almost colourless. For a mixture containing the para-acid and salicylic acid, the same procedure for coupling is adopted, the resulting solution acidified and extracted with ether and the ethereal extract then shaken with sodium carbonate solution, which removes all the salicylic acid derivative and leaves the dyestuff due to the para-acid in the ether; the ethereal solution may then be tested with sodium hydroxide and the presence of *p*-hydroxybenzoic acid confirmed. Owing to the fact that the proportions of mono-, bis- and tris-azophenols produced from the para-acid are not constant, the test cannot be applied quantitatively.

REFERENCES

1. F. W. Edwards, H. R. Nanji and M. K. Hassan, *Analyst*, 1937, **62**, 178.
2. J. R. Nicholls, *Analyst*, 1928, **53**, 19.
3. F. Weiss, *Z. Unters. Lebensm.*, 1928, **55**, 24.
4. F. Weiss, *Z. Unters. Lebensm.*, 1930, **59**, 472.
5. S. G. Stevenson and J. C. L. Resuggan, *Analyst*, 1938, **63**, 152.

LACTIC ACID

See Section III: Substances of Clinical and Biochemical Significance, p. 243.

NITRATES

The readiness with which certain phenolic compounds form nitro derivatives capable of giving highly coloured salts constitutes the principle upon which the most successful colorimetric determinations of this radicle are based. Phenoldisulphonic acid was the first reagent of this class to be employed[1] and it remains the most widely favoured. Another phenolic substance which has recently been successfully employed is 2:4-xylen-1-ol[2] (*m*-4-xylenol) the nitro derivative of which is volatile in steam and hence can be readily removed from interfering material. The first method ever proposed for the colorimetric determination of nitrates depended upon the nitration of brucine[3] but the procedure is now rarely employed. Diphenylamine[4–7], diphenylbenzidine[8–14] and diphenylamine sulphonic acid[15] oxidise to blue quinone-imonium derivatives in the presence of sulphuric acid and nitrates and, although useful as delicate qualitative tests, the methods are too much influenced by slight variations in the conditions to admit of their successful application to quantitative work. The use of the zinc-copper couple to effect reduction of nitrates to ammonia followed by determination of the latter by means of Nessler's reagent constitutes a convenient method of determination for the purposes of water analysis[16, 17]. A recent proposal[18] to determine nitrates by treating the dry residue remaining after evaporation of the solution to be tested with a mixture of nitrobenzene and sulphuric acid and measuring the purple colour produced by adding acetone to the mixture and then rendering alkaline with sodium hydroxide failed, in the writer's hands, to yield quantitative results.

Phenoldisulphonic Acid Method. An approximately 25 per cent. w/v solution of phenoldisulphonic acid in concentrated sulphuric acid should be prepared by dissolving 25 g. of crystalline phenol in 158 ml. of concentrated sulphuric acid (nitrogen free), adding 67 ml. of fuming sulphuric acid containing about 20 per cent. of sulphur trioxide and heating the mixture on a boiling water-bath for 2 hours. If prepared in this way the reagent will be free from monosulphonic acids, the presence of which leads to erratic results[19].

A measured quantity of the faintly alkaline or neutral solution to be examined, preferably containing not more than 0·03 mg. N as nitrate, is transferred to a porcelain dish and evaporated to dryness on a boiling water-bath. To the cooled residue is added

1 ml. of the above phenoldisulphonic acid solution, care being taken that the reagent makes contact with the whole of the solid material derived from the sample. After standing for 10 minutes the contents of the dish are transferred to a Nessler glass with the help of 30 ml. of water, the mixture cooled, 10 ml. of an aqueous solution of ammonia (10 per cent. w/w NH_3) added, the mixture again cooled and finally diluted with water to 50 ml. If nitrates were present in the original sample a yellow colour will be produced which may be matched against standards similarly prepared from a solution of potassium nitrate, the effective range being from 0·0025 to 0·03 mg. N. A convenient standard solution can be prepared by dissolving 0·722 g. of potassium nitrate in water, diluting to 1 litre so that 1 ml. contains 0·1 mg. N. as nitrate and making further dilutions from this as necessary.

If the sample contains chlorides in excess of 0·2 part Cl per 100,000 it will be necessary to apply a preliminary treatment by adding 1 ml. of glacial acetic acid to 10 ml. of the sample followed by 0·1 g. of silver sulphate (nitrate free) and, after shaking, filtering through a Whatman No. 32 paper. The test for nitrate is then applied to 5 ml., or other suitable quantity, of the filtrate.

Phenoldisulphonic Acid Method Using Permanent External Standards. A disc, covering the range from 0·0025 to 0·03 mg. N as nitrate, is available for use with the B.D.H. Lovibond Nessleriser and one is also issued for use with the Lovibond Comparator providing standards from 0·005 to 0·1 mg. N as nitrate. The standards for the Nessleriser are based on the technique described above while in the case of the Comparator the final coloured solution is diluted to 25 ml., appropriate modifications being made in the amount of water and dilute ammonia solution taken for the test.

Application to Soil. The sample is dried, powdered, passed through a No. 60 mesh sieve and a suitable quantity (which may be 20 or 50 g.) treated with 250 ml. of a solution containing 0·5 g. of copper sulphate, $CuSO_4.5H_2O$, and 0·1 g. of silver sulphate. After vigorously agitating for about 10 minutes the mixture is allowed to settle, 125 ml. decanted and the copper and silver in this extract removed by adding 0·4 g. of calcium hydroxide and 1 g. of magnesium carbonate, shaking for 5 minutes and filtering. The first 20 ml. or so of the filtrate should be discarded and, if a second filtration is needed, the solution should be passed through the same filter. An aliquot part of the filtrate is then tested for nitrate by the method already described.

An alternative method, suitable for soils giving strongly coloured extracts, consists in mixing the dried and powdered soil with a twentieth of its weight of calcium carbonate and adding four times its weight of water. After shaking for 15 minutes the mixture is allowed to settle, the liquid decanted and the extract clarified by adding alumina cream (*vide infra*) in the proportion of 5 ml. for each 100 ml. After thoroughly shaking, the mixture is filtered through a coarse filter and the nitrate determined on a suitable aliquot part of the filtrate.

Application to Meat Products. The sample is minced and 1 g. transferred to a 100-ml. volumetric flask containing 20 ml. of water and the flask immersed in a boiling water-bath for 15 minutes and periodically agitated. The mixture is allowed to cool, rendered just acid to bromocresol green with dilute sulphuric acid and any nitrites which may be present are oxidised by adding 0·1N potassium permanganate drop by drop until a faint pink colour persists for 1 minute. Chlorides and proteins are then precipitated by adding successively, with shaking after each addition, an excess of a saturated solution of silver sulphate (nitrate free), 5 ml. of a solution of lead subacetate and 5 ml. of alumina cream. The mixture is diluted to 100 ml., again shaken, filtered, and the nitrate determined on a suitable aliquot part by the method given above.

The solution of lead subacetate is made by dissolving 25 g. of lead acetate in 75 ml. of water, adding 17·5 g. of lead monoxide (litharge) and, after having set the mixture aside with occasional agitation for 48 hours, filtering and passing through the filter sufficient water to produce 100 ml. of filtrate.

The alumina cream is prepared by adding a slight excess of ammonia (s.g. 0·880) to a saturated aqueous solution of potassium aluminium sulphate and then cautiously adding more alum solution until the mixture is just acid to litmus.

Discussion. The phenoldisulphonic acid produced by the method of preparation given above is the isomer having the systematic name 1-phenol-2:4-disulphonic acid. The yellow compound formed in the test is triammonium 6-nitrophenol-2:4-disulphonate but, when the amount of nitrates present is high, a minute proportion of picric acid may also be formed[20]. Organic matter interferes with the test and, if present, must be removed by the application of procedures analogous to those given in the above examples. If the concentration of nitrites in the solution under examination exceeds 1 part per million an error will be

introduced and it is therefore important that, when present, they should first be oxidised to nitrates by means of dilute permanganate solution and, after determining the nitrites separately, applying the appropriate correction to the result obtained for the content of nitrates. When interfering substances are removed by precipitation it is important to guard against the possibility of losing traces of nitrates by adsorption. It is for this purpose that acetic acid is directed to be added to samples prior to the removal of chlorides with silver sulphate. Trials conducted by the writer on the method given above for determining nitrates in meat products have yielded promising results and, generally, there was no significant loss of nitrate when known quantities were initially added, although in this respect other methods of clarification as, for example, removal of proteins with phosphotungstic acid, failed completely.

The interference caused by the presence of chlorides constitutes a serious disadvantage of the above method, particularly when it is used for the examination of water supplies and a modification, in which the phenoldisulphonic acid reagent is added to the sample *before* evaporation, has been developed by Frederick[21], who states that the nitrates can be accurately determined on a 25-ml. sample without removal of chlorides even when the concentration of the latter amounts to 100 parts Cl per 100,000. Experiments conducted by the writer have fully confirmed this claim. The phenoldisulphonic acid reagent as ordinarily prepared cannot be used in this method. Frederick has pointed out that the quantity of reagent employed in Sprengel's procedure[1] is enormously in excess of that required to react with an amount of nitrate suitable for colorimetric determination. The modified reagent and the technique as applied to the examination of water are described below. The sensitivity of the test is similar to that of the original phenoldisulphonic acid method and it should be equally applicable to the examination of various materials.

Phenoldisulphonic Acid Method Modified by R. C. Frederick (*for Potable Waters*)[21]. In order to prepare the reagent 4 g. of phenol is mixed with 4 ml. of water (ammonia free) and 100 ml. of concentrated sulphuric acid (nitrogen free) is added ; the whole is heated to 80° to 85° C. for 6 hours, cooled, and diluted to 500 ml. with water ; 300 ml. of concentrated sulphuric acid (nitrogen free) made up to 500 ml. with water (ammonia free) is added to the foregoing solution to give 1 litre of reagent.

The volume of the sample of water, or other solution under

examination should be adjusted, if necessary, so that 25 ml. shall contain not more than about 0·1 mg. N as nitrate. If this quantity is expected to be contained in less than 25 ml. an appropriate quantity of the sample should be diluted to 25 ml. with distilled water. The liquid to be tested is transferred to an evaporating dish of $3\frac{1}{2}$ in. diameter containing a glass rod, 2 ml. of the phenoldisulphonic acid reagent added, the mixture stirred and evaporated over a boiling water-bath until no more water is expelled and the residual liquid darkens. During the process of evaporation the liquid is occasionally stirred and any dark spots on the side of the dish above the surface of the liquid are incorporated in the bulk of the residue by touching with the glass rod. The dish is removed from the water-bath and tilted so that the acid residue makes contact with all parts of the interior wall originally wetted by the sample. The inside of the dish is then washed down with a jet of water from a wash-bottle and the whole again evaporated to "acid dryness." The washing and evaporation is repeated, the residue taken up with water, transferred to a 100-ml. Nessler glass, the dish washed out with small quantities of water, the washings being added to the contents of the Nessler glass, which are then rendered alkaline by the addition of 3 ml. of strong ammonia solution (s.g. 0·880) and the alkaline mixture finally diluted to 100 ml. with more water. Any yellow colour produced is matched against standards similarly prepared from a solution of potassium nitrate.

2:4-Xylen-1-ol Method. The solution under examination, containing not more than 0·025 mg. N as nitrate, is adjusted to a volume of 5 ml. either by evaporation or dilution with water and added to 15 ml. of an 85 per cent. w/w aqueous solution of sulphuric acid. The temperature of the mixture is adjusted to 35° C., 1 ml. of a 1 per cent. w/w solution of 2:4-xylen-1-ol in glacial acetic added, and the temperature maintained at 35° C. for 30 minutes. The liquid is diluted with 100 ml. of water, transferred to a distillation apparatus and 40 ml. distilled into a Nessler glass containing 10 ml. of 2N sodium hydroxide ; any yellow colour produced is matched against standards. A standard distillate is prepared by mixing 1 ml. of an aqueous solution of potassium nitrate containing 3·607 g. per litre (equivalent to 0·5 mg. N per ml. as nitrate) with 5 ml. of water and 15 ml. of 85 per cent. w/w sulphuric acid, cooling to 35° C. and carrying out the nitration and distillation as described above. After dilution to 500 ml., suitable quantities are employed to make a series of standards in Nessler

glasses. It is important that the temperature of the diluted distillate should be the same as that of the standard since the colour intensity of the nitro-compound in alkaline solution increases with the temperature by 0·62 per cent. per 1° C. The convenient range for matching colours lies between 0·002 and 0·025 mg. N as nitrate.

Discussion. The above procedure may well prove to be useful in the examination of soils, meat products and other organic material in cases where it is not possible to secure a high degree of clarification since the 5-nitro-2:4-xylen-1-ol, being volatile in steam, is separated from the solution under examination and hence some coloration in the latter will be immaterial. Further, since evaporation to dryness of the liquid under test is not required and, as it is only necessary to use diluted sulphuric acid to promote the nitration, it may be anticipated that less difficulty will arise from interference due to the presence of dissolved salts and organic matter. Hence, when trouble is experienced in applying the phenoldisulphonic acid method it will be worth while to try the use of 2:4-xylen-1-ol. The clarification of test solutions should be conducted by the procedures already given under the description of the phenoldisulphonic acid method and the same precautions with regard to the presence of chlorides should be observed.

Zinc-Copper Couple Method for Potable Water[16, 17]. A wide-mouthed stoppered bottle of approximately 50 ml. capacity is about three-quarters filled with granulated zinc and the latter covered with an aqueous solution of copper sulphate. After a few minutes, when an appreciable amount of copper has deposited on the zinc, the contents of the bottle are well rinsed with water, the washings drained and the zinc-copper couple then covered with the sample of water under examination. The bottle is stoppered and placed in a warm place (say at about 25° C.) for several hours and, if possible, overnight. When reduction of the nitrates, including nitrites if present, is complete, an appropriate measured quantity is transferred to a Nessler glass, diluted to 50 ml. with water (ammonia free) and the ammonia content determined by means of Nessler's reagent (see p. 382). The quantity to be used for the ammonia determination will vary according to the nitrate content of the sample but for a good quality drinking water 5 or 10 ml. will generally be found convenient. Results are customarily expressed in terms of nitrogen present as nitrate and the value for the content of ammonia found

(after applying any necessary correction for free ammonia originally present in the water) is multiplied by the factor 0·824 in order to convert it to nitrogen.

Discussion. This simple and convenient method is widely used and, provided certain precautions are observed, the results obtained may be regarded as sufficiently accurate for the examination of water supplies. It is important to ensure that complete reduction of nitrates to ammonia has taken place and that no nitrites remain after contact with the zinc-copper couple. Any nitrites originally present in the sample must, of course, be taken into account when calculating the results of the determination of nitrates. If the water possesses colour interfering with the ammonia test or substances precipitated by Nessler's reagent, it will be necessary to distil the sample after reduction of the nitrates before attempting to determine the concentration of ammonia. In general, a low temperature and alkalies retard the reduction while warmth, acids and common salt assist the reaction whence it may sometimes be found advantageous to add a little oxalic acid when applying the test to hard water.

REFERENCES

1. H. Sprengel, *Pogg. Ann.*, 1864, **121,** 188.
2. J. Blom and C. Treschow, *Z. Pflanz. Dungung Bodenk.*, 1929, **13a.** 159.
3. R. Kersting, *Ann.*, 1863, **125,** 254.
4. E. Kopp, *Ber.*, 1872, **5,** 284.
5. J. Tillmans, *Z. Unters. Nahr. Genussm.*, 1910, **20,** 676.
6. J. Tillmans and A. Splittgerber, *Z. Unters. Nahr. Genussm.*, 1911, **22,** 401.
7. J. Tillmans and W. Sutthoff, *Z. anal. Chem.*, 1911, **50,** 473.
8. E. A. Letts and F. W. Rea, *J. Chem. Soc.*, 1914, **105,** 1157.
9. L. Smith, *Z. anal. Chem.*, 1917, **56,** 28.
10. A. F. Lerrigo, *Analyst*, 1930, **55,** 433.
11. G. W. Monier-Williams, *Analyst*, 1931, **56,** 397.
12. W. R. G. Atkins, *J. Marine Biol. Assoc.*, 1932, **18,** 167.
13. D. R. Wood, E. T. Illing and A. E. Fletcher, *Analyst*, 1933, **58,** 149.
14. J. H. Hamence, *Analyst*, 1935, **60,** 532.
15. I. M. Kolthoff and G. E. Noponen, *J. Amer. Chem. Soc.*, 1933, **55,** 1448.
16. J. H. Gladstone and A. Tribe, *J. Chem. Soc.*, 1878, **33,** 139.
17. M. Whitley Williams, *J. Chem. Soc.*, 1881, **39,** 100.
18. M. Pesez, *J. Pharm. Chim.*, 1939, **30,** 112.
19. E. M. Chamot, D. S. Pratt and H. W. Redfield, *J. Amer. Chem. Soc.*, 1911, **33,** 366.
20. E. M. Chamot and D. S. Pratt, *J. Amer. Chem. Soc.*, 1910, **32,** 630.
21. R. C. Frederick, *Analyst*, 1919, **44,** 281.

NITRITES

The well-known Griess test[1], as modified by Ilosvay[2] more than 50 years ago, is still the most favoured method. It depends upon the diazotisation of sulphanilic acid by nitrous acid and the coupling of the compound so formed with α-naphthylamine to produce a red azo dye. Other related compounds have been suggested to replace either the sulphanilic acid[3] or the α-naphthylamine[4] but it is doubtful if any advantage has been achieved. The old method of causing meta-phenylenediamine to couple with itself with the formation of bismarck brown[5] is now little used since the production of colour is inclined to be erratic and is much influenced by the reaction of the solution[6]. In addition to the description of the Ilosvay method, an account is given of a new procedure due to M. B. Shinn[7] in which the nitrous acid is caused to diazotise sulphanilamide (*p*-aminobenzenesulphonamide) and the resulting compound coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride. The writer has confirmed that the advantages claimed for this method are justified.

Method of Ilosvay[2]. Two reagents are required :—

1. Sulphanilic Acid Solution made by dissolving 0·5 g. in 30 ml. of glacial acetic acid, adding 100 ml. of water and filtering. This reagent will remain in good condition for a month or more.
2. α-Naphthylamine Solution. This should not be more than a week old and is made by dissolving 0·1 g. of α-naphthylamine in 100 ml. of boiling water and, after the solution has cooled, adding 30 ml. of glacial acetic acid and filtering.

The test is conducted by adding 2 ml. of each reagent to 50 ml. of the approximately neutral solution under examination and, after standing for 15 minutes, matching any red colour produced against that of standards similarly prepared. The standard solution should be freshly made by dissolving 0·493 g. of sodium nitrite in water, making up to 1 litre and diluting 10 ml. of this to 1 litre to produce a solution containing 0·001 mg. N as nitrite per ml. The useful range of the test lies between 0·0005 and 0·001 mg. nitrite nitrogen.

Method of Ilosvay Using Permanent External Standards. A disc containing nine standard colour glasses, covering a range from 0·00005 to 0·001 mg. N as nitrite, is available for use with the

B.D.H. Lovibond Nessleriser. The standards are designed to match the colours obtained by the technique described above when working at a temperature of 20° C.

Discussion. Should either of the reagents become coloured on keeping they may be improved by shaking with zinc dust and filtering. A high concentration of mineral salts intensifies the depth of colour produced, therefore, in the examination of meat pickling brines, the standards used for comparison should also contain dissolved salts. The red colour due to nitrite attains a maximum intensity in 15 minutes and slowly fades after about half an hour. Samples of water which are highly coloured should be clarified as far as possible with alumina cream while samples of organic material should be treated in accordance with the methods outlined for the determination of nitrates (see p. 147).

Method of Shinn[7]. To the neutral or slightly acid solution under examination, not exceeding 35 ml. in volume and containing not more than 0·05 mg. of nitrite, is added 1 ml. of 5N hydrochloric acid and 5 ml. of a 0·2 per cent. aqueous solution of sulphanilamide. After allowing to stand for 3 minutes, 1 ml. of a 0·5 per cent. aqueous solution of ammonium sulphamate is added and, after 2 minutes, 1 ml. of a 0·1 per cent. aqueous solution of N-(1-naphthyl)-ethylenediamine dihydrochloride is introduced and the mixture diluted with water to 50 ml. Any red colour produced is allowed to develop for 3 minutes then compared with standards. The useful range of the test is the same as that of the Ilosvay method and standards may be prepared from freshly made sodium nitrite solution. Alternatively, by applying the following procedure a 0·2 per cent. sulphanilamide solution may be employed as a standard :—

An approximately 1 per cent. aqueous solution of sodium nitrite is assayed by titration with potassium permanganate, then diluted to contain 0·005 mg. NO_2' per ml. and 5 ml. transferred into each of two 50-ml. graduated flasks. To each flask is added 1 ml. of 5N hydrochloric acid, 5 ml. of a 0·2 per cent. aqueous solution of sulphanilamide and, after 3 minutes, 1 ml. of a 0·5 per cent. aqueous solution of ammonium sulphamate. After standing for a further 2 minutes, 1 ml. of a 0·1 per cent. aqueous solution of N-(1-naphthyl)-ethylenediamine dihydrochloride is added to each and the mixtures are diluted to the mark with water. This pair constitutes sample "*a*" prepared in duplicate. Into each of two other 50-ml. graduated flasks is measured 5 ml. of an accurately prepared 1 to 100 dilution of a 0·2 per cent. aqueous

solution of sulphanilamide followed by 1 ml. of 5N hydrochloric acid, 1 ml. of an approximately 0·1 per cent. aqueous solution of sodium nitrite and 5 ml. of water. After allowing to stand for 3 minutes, 1 ml. of the ammonium sulphamate solution is added to each reaction mixture in order to destroy the excess of nitrite and, after a further 3 minutes, 1 ml. of the coupling reagent is introduced into each flask and the mixtures are diluted to the mark with water. These two mixtures constitute sample "*b*" prepared in duplicate. Samples *a* and *b* are read against each other in a Duboscq-type colorimeter the nitrite equivalent value of the sulphanilamide solution being calculated from the equation :

$$\frac{\text{reading of } a}{\text{reading of } b} \times \text{mg. } NO_2' \text{ in } a \times 20$$
$$= \text{mg. } NO_2' \text{ represented by 1 ml. of 0·2 per cent. sulphanilamide solution.}$$

The same *b* thus constitutes the standard with which unknown test reaction mixtures are compared whence :—

$$\frac{\text{reading of standard}}{\text{reading of unknown}} \times \frac{NO_2' \text{ value of 1 ml. 0·2 per cent. sulphanilamide}}{20}$$
$$= \text{mg. } NO_2' \text{ in sample.}$$

Discussion. This method is based upon the reaction suggested by Bratton, Marshall and their assistants for the colorimetric determination of sulphanilamide[8] (see p. 270). It possesses advantages over the Ilosvay method in that the colour produced is brighter, it reaches its maximum intensity more rapidly and then remains stable for several hours. Since sulphanilamide and nitrite react stoichiometrically in the presence of a suitable excess of either it may often be convenient to prepare a series of standard colours from a sulphanilamide solution the strength of which has been determined in terms of nitrite nitrogen. In this way the use of a highly unstable primary nitrite standard is avoided : a sulphanilamide solution of about 0·2 per cent. concentration will remain unaltered for many weeks if stored in a cool place and screened from strong daylight.

REFERENCES

1. J. P. Griess, *Ber.*, 1879, **12**, 426.
2. L. Ilosvay de Nagy Ilosva, *Bull. Soc. chim.*, 1889, **49**, 388.
3. E. J. Stieglitz and A. E. Palmer, *J. Pharmacol.*, 1934, **51**, 398.
4. F. G. Germuth, *Ind. Eng. Chem., Anal. Ed.*, 1929, **1**, 28.
5. J. P. Griess, *Ber.*, 1878, **11**, 624.
6. R. Uzel, *Coll. Czech. Chem. Comm.*, 1933, **5**, 139.
7. M. B. Shinn, *Ind. Eng. Chem., Anal. Ed.*, 1941, **13**, 33.
8. A. C. Bratton, E. K. Marshall, Jr., D. Babbitt and A. R. Hendrickson, *J. Biol. Chem.*, 1939, **128**, 537.

OXALATES

It has been pointed out by C. A. Mitchell that when oxalic acid is treated with a solution of sodium metavanadate and hydrogen peroxide an orange-red coloration is produced which is apparently due to the formation of a pervanadic complex[1]. Tartaric acid gives a similar reaction but the coloration in this case is only about a quarter as intense and it is suggested that, in the absence of tartrates, the reaction may serve for the approximate colorimetric determination of oxalates.

Method[1]. The solution to be tested is mixed with an equal volume of a 1 per cent. aqueous solution of sodium metavanadate and the resulting mixture then treated with a third of its volume of 3 per cent. hydrogen peroxide solution. Any orange-red colour produced is matched against standards prepared from oxalic acid solutions of known strength.

Discussion. This reaction yields a good colour with a sample containing 0·1 per cent. of oxalic acid while a perceptible tint is given by a 0·03 per cent. solution. When a 1 per cent., or stronger, solution of citric acid is submitted to the test a yellow tint is produced while saturated solutions of succinic or malic acids give transient red colorations. The method is not applicable in the presence of free mineral acids since they produce a pervanadic acid of similar shade, but formic and acetic acids do not interfere.

REFERENCE

1. C. A. Mitchell, *Analyst*, 1933, **58**, 279.

PHOSPHATES

The well-known coeruleomolybdate colorimetric test for phosphates is widely used in biochemical and agricultural research, in the examination of boiler water and in chemical control work applied to various industries. The test consists essentially in adding ammonium molybdate to an acid solution of the phosphate and reducing the resulting phosphomolybdate to a lower state of oxidation with consequent formation of a blue-coloured compound which is said to have the composition $(MoO_2.4MoO_3)_2.H_3PO_4$.

The method was first suggested in 1887 by F. Osmond[1] who used stannous chloride as the reducing agent and during the last

25 years a large number of papers have been published describing various modifications of this method. In 1914 A. E. Taylor and C. W. Miller[2] proposed the use of phenylhydrazine as the reducing agent in place of stannous chloride and since that date several other organic compounds have been employed. Many of these trials have been directed towards minimising the response to silicates which react in the test similarly to phosphates. In this connection Bell and Doisy[3] recommended hydroquinone, which was also adopted by Briggs,[4, 5] but probably the most satisfactory organic reducer for use in the general application of the test is *p*-methylaminophenol sulphate (the " metol " of photography) first proposed by Leiboff[6] and developed by Ernst and Emilio Tschopp[7] and a modification of this test is described below. For the determination of phosphate in blood and urine the procedure due to Fiske and Subbarow[8], who advise 1-amino-2-naphthol-4-sulphonic acid as reducing agent, is generally favoured and E. J. King's[9] modification of this test is included here. The value of these methods is largely determined by the extent to which they respond to silicates and in this particular the method using metol is in advance of all others. Modifications of the original technique using stannous chloride as reducer are still widely employed, largely because of their greater sensitivity, and two useful methods based on this principle are also included. It should also be noted that pyrophosphates and metaphosphates do not react in any of the following tests but they can be determined by including a preliminary hydrolysis.

Method Using *p*-Methylaminophenol Sulphate (*General Application*). Two reagents are required :—

1. Ammonium molybdate . . . . . 10 g.
   Water. . . . . to produce 100 ml.
   When solution of the above is complete it is added slowly to a cooled mixture of
   Sulphuric acid (s.g. 1·84) . . . . 150 ml.
   Water. . . . . 100 ml.
2. Sodium metabisulphite . . . . 40 g.
   Sodium sulphite, $Na_2SO_3.7H_2O$ . . . 1 g.
   p-Methylaminophenol sulphate (" metol ") . 0·2 g.
   Water. . . . . to produce 200 ml.

To apply the test, 5 ml. of reagent No. 1 is added to 25 ml. of the solution under examination (or a suitable quantity diluted to 25 ml.) contained in a Nessler glass and the latter is immersed in a

boiling water-bath for 15 minutes then 5 ml. of reagent No. 2 is introduced and the mixture heated as above for a further 15 minutes. The mixture is cooled by holding the Nessler glass under the tap, then diluted to 50 ml. with distilled water and any blue colour compared with standards similarly prepared. The colour is stable for several hours and the test responds well within the range from 0·005 to 0·1 mg. P_2O_5. A standard solution containing 1 mg. P_2O_5 per ml. can be conveniently made by dissolving 1·916 g. of potassium dihydrogen phosphate in water acidulated with sulphuric acid and diluting to 1 litre: further dilutions of this solution should also be made with water that has been slightly acidified with sulphuric acid. If plain water is employed the diluted solutions quickly lose strength due to adsorption of phosphate by the glass surface of the flask.

Discussion. The above method can be equally well conducted on a smaller or larger scale provided that proportionate amounts of the reagents are used. If necessary the solutions to be examined should be neutralised before applying the test. Under the conditions described, as much as 20 mg. of silica can be present without disturbing the accuracy of the phosphate determination. By heating for 15 minutes with reagent No. 1 before adding the reducer the test is made to tolerate the presence of nitrates, a point of considerable importance in the examination of boiler water. As ordinarily described most of the colorimetric tests for phosphates which employ organic reducers suffer interference from even minute traces of nitrates but in the above procedure the presence of 1 mg. of nitrate nitrogen exercises no effect on the accuracy of the determination. Arsenates react similarly to phosphates but the intensity of the colour produced is less than half that given by the latter. Preliminary reduction with hydrogen sulphide followed by boiling and filtering through paper pulp serves to eliminate interference from this source. Citrates, tartrates, lactates, oxalates, malates, pyruvates, glycollates and certain other organic acids depress the intensity of the colour due to phosphates, while sugars have a similar action; such compounds, if present, should be destroyed by incineration. Iron may be present in a concentration equal to 1000 times that of the phosphate without influencing the accuracy of the results and thus the test offers an advantage over those methods employing stannous chloride as the reducing agent since with these even minute traces of iron alter the shade of colour produced.

Method Using *p*-Methylaminophenol Sulphate with Permanent

External Standards. Discs suitable for the general application of this test are available for use with the Lovibond Comparator and also for the B.D.H. Lovibond Nessleriser. The standards for the former instrument cover the range from 0·005 to 0·1 mg. P_2O_5 and for the latter 0·005 to 0·06 mg. P_2O_5. The technique adopted for use with the Nessleriser is the one given above while the standards for the Comparator fit the same test when it is conducted on 5 ml. of the sample solution, 1 ml. of each reagent being used and the mixture finally diluted to 10 ml.

Method Using 1-Amino-2-naphthol-4-sulphonic Acid (*Blood and Blood Plasma*)[9]. The proteins in the sample of freshly-drawn blood, or oxalated plasma, are precipitated by adding 2 to 8 ml. of a 10 per cent. aqueous solution of trichloracetic acid; the mixture is shaken, filtered and 5 ml. of the filtrate (equivalent to 1 ml. of the sample) mixed with 0·7 ml. of 60 per cent. perchloric acid, 0·7 ml. of a 5 per cent. aqueous solution of ammonium molybdate, 0·5 ml. of amino-naphtholsulphonic acid reagent and sufficient water to produce 10 ml. After standing for 10 minutes the colour is matched against standards similarly prepared. The amino-naphtholsulphonic acid reagent is made by dissolving 0·2 g. of 1:2:4-amino-naphtholsulphonic acid, 12 g. of sodium meta-bisulphite and 2·4 g. of sodium sulphite, $Na_2SO_3.7H_2O$, in 100 ml. of water. A standard solution containing 1 mg. P per ml. can be made by dissolving 4·390 g. of potassium dihydrogen phosphate in water acidulated with sulphuric acid and diluting to 1 litre: suitable dilutions can be made with slightly acidulated water as required. The useful range of the test lies between 0·02 and 0·16 mg. P (approximately 0·08 and 0·63 mg. P_2O_5). The above procedure gives the proportion of inorganic phosphate: in order to determine the total phosphorus as phosphate, 5 ml. of the filtrate from the protein precipitation of a plasma sample, or 0·5 ml. of the corresponding solution derived from a sample of whole blood, is added to 1 ml. of perchloric acid (60 per cent.) and the mixture heated over a small Bunsen flame, a fragment of porous pot being added to ensure steady ebullition. Heating is continued until the fuming liquid becomes colourless, then the cooled residue is diluted to about 5 ml. with water and the colour test conducted as described above, commencing with the addition of 0·7 ml. of ammonium molybdate solution.

(*Urine*)[8]. To determine the amount of inorganic phosphate, 0·2 ml. of the sample is diluted to about 5 ml. with water, 0·7 ml. of perchloric acid (60 per cent.) is added followed by 0·7 ml. of

ammonium molybdate and 0·5 ml. of amino-naphtholsulphonic acid reagent, the mixture being finally diluted to 10 ml. with more water, the colour allowed to develop for 10 minutes and then matched against standards.

Discussion. The simplicity of the amino-naphtholsulphonic acid test is a favourable feature which renders it particularly useful for clinical control work but as it is much more sensitive to silicates than the method using *p*-methylaminophenol sulphate it is not so suitable for general application. An acid extract of blood contains, in addition to the inorganic phosphate, esters of phosphoric acid ; these are present to the extent of less than 1 mg. P per 100 ml. in plasma but whole blood contains from 30 to 80 mg. P per 100 ml. as ester phosphate. It is important that freshly drawn samples should be used for the separate determination of inorganic and ester phosphate since the latter is rapidly hydrolysed by the enzymes of blood. Normal human blood contains from 45 to 115 mg. total P per 100 ml. of which 8 to 18 mg. occurs in the plasma while the amount of inorganic phosphates present in normal plasma varies from 2 to 5 mg. P per 100 ml. In rickets and osteomalacia, determinations of inorganic serum phosphate, observed in conjunction with the serum calcium, assist in diagnosing the existence of the disease, in determining whether it is due to calcium or phosphate deficiency, and in judging the early results of therapy. However, determinations of phosphorus in tissues and body fluids have found most application to physiological studies and at present relatively little data is available for use in clinical diagnosis.

Method Using 1-Amino-2-naphthol-4-sulphonic Acid with Permanent External Standards. The above procedure can be used in conjunction with the Lovibond Comparator, the disc which is issued covering the range 0·02 to 0·16 mg. P.

Method Using Stannous Chloride (*General Application*)[12]. Two reagents are required :—

1. Ammonium molybdate . . . . . 10 g.
   Water. . . . . to produce 100 ml.

   When solution of the above is complete it is added slowly to a cooled mixture of

   Sulphuric acid (s.g. 1·84) . . . . 150 ml.
   Water. . . . . 150 ml.

2. Tin foil . . . . . 0·1 g.
Hydrochloric acid (s.g. 1·18) . . . . 2 ml.
Copper sulphate, 4 per cent. aqueous solution . 1 drop

The above mixture is warmed in a test tube until solution is complete and then diluted to 10 ml. with water. It is important that this reagent be freshly prepared.

A suitable quantity of the solution under test is diluted with distilled water to a volume of about 45 ml., the temperature of the liquid adjusted to 25° C., 1 ml. of reagent No. 1 added, the solution stirred, then 0·15 ml. of reagent No. 2 introduced followed by sufficient water at 25° C. to produce 50 ml. After allowing the mixture to stand for 5 minutes the blue colour due to phosphate is matched against standards similarly prepared. The standard solution is made by dissolving 1·916 g. of potassium dihydrogen phosphate in water acidulated with sulphuric acid, diluting to 1 litre so that 1 ml. contains 1 mg. P_2O_5 and making further appropriate dilutions with slightly acidulated water. This test is suitable for quantities ranging from 0·002 to 0·02 mg. P_2O_5.

Discussion. The above procedure is based upon the recommendation of Denigès[10] as modified by Truog and Meyer[11] and by Holman and Pollard[12]. The depth of the colour produced is, to some extent, dependent on the proportion of reagents added, the temperature, and time of reaction. Silicate may be present in the final solution to the extent of 700 parts per million SiO_2 without influencing the results. The solution under examination should be neutralised before applying the test since alkalis and free mineral acids, apart from that added in the reagents, depress the sensitivity. Certain organic acids, such as citric, oxalic and tartaric, but not acetic acid, interfere with the full development of the colour while, in general, substantial amounts of organic matter will disturb the test. More than 1 part per million of ferric iron will interfere and, if present, it is recommended that it be reduced by means of the Jones reductor[13]. Arsenate responds to the test similarly to phosphates, and should be reduced to arsenite by means of hydrogen sulphide in acid solution.

Method Using Stannous Chloride with Permanent External Standards. A series of glass standards covering the range 0·002 to 0·02 mg. P_2O_5 and based upon the above procedure is available for use with the B.D.H. Lovibond Nessleriser. A corresponding disc for use with the Comparator is also issued : the test is conducted on the same scale and in the same manner as for the

Nessleriser and the glasses embrace a range from 0·02 to 0·22 mg. P_2O_5.

Method Using Solvent Extraction and Stannous Chloride (*General Application*)[14]. To 5 ml. of the neutral solution under examination contained in a small separator is added 1 ml. of 5N sulphuric acid, 1·5 ml. of water, 2·5 ml. of a 5 per cent. aqueous solution of ammonium molybdate and 10 ml. of *iso*-butyl alcohol. The mixture is shaken for 2 minutes, allowed to separate and the aqueous layer discarded. The alcoholic solution is washed by shaking with two successive 5-ml. portions of N sulphuric acid, the latter being rejected. Then 15 ml. of stannous chloride solution (made by freshly diluting a 35 per cent. solution of the salt, $SnCl_2.2H_2O$, in concentrated hydrochloric acid 200 times with N sulphuric acid) is added, the mixture shaken for 30 seconds, allowed to separate and the aqueous layer discarded. The *iso*-butyl alcohol, which will be coloured blue if phosphate was present in the sample taken for test, is transferred to a 10-ml. graduated tube, the separator washed out with ethyl alcohol, the washings being used to dilute the *iso*-butyl alcohol to the mark. The colour is matched against standards similarly prepared. The useful working range lies between 0·001 and 0·1 mg. P_2O_5 but the sensitivity can be easily increased by working on a smaller scale and using less *iso*-butyl alcohol. The standard phosphate solution is made up as described in the preceding method. Proteins, present in biological fluids, may be removed by adding an equal volume of 12 per cent. aqueous solution of trichloracetic acid and centrifuging. Of the supernatant fluid, 5 ml. is measured into a separator and the procedure continued as described above, except that 0·6 to 0·8 ml. of 5N sulphuric acid is added instead of 1 ml.

Discussion. This method was proposed by Berenblum and Chain[14], while a closely similar procedure using either amyl alcohol, ethyl acetate or ether as the organic solvent was published soon afterwards by an independent investigator[15]. The method is particularly useful for the examination of coloured samples. It is claimed that determinations by this method are not disturbed by the presence of sodium fluoride in concentrations up to 0·1M although all other tests are inhibited. Again, it is stated that determinations by this technique are not affected by oxalates to the same extent as other methods. Berenblum and Chain do not make any comment as to the influence of silicates but the present author's trials have shown that, while the presence of amounts

comparable with the phosphate do not disturb the accurate determination of the latter, any appreciable preponderance leads to high results. It was also observed that a small proportion of the phosphomolybdate remained in the aqueous layer after making the single extraction with *iso*-butyl alcohol as prescribed but presumably this is of no moment since the same conditions will apply to the standards. In the writer's hands a mixed solvent consisting of equal volumes of *iso*-butyl alcohol and *iso*-butyl acetate seemed to be more effective.

REFERENCES

1. F. Osmond, *Bull. Soc. chim.*, 1887, **47**, 745.
2. A. E. Taylor and C. W. Miller, *J. Biol. Chem.*, 1914, **18**, 215.
3. R. D. Bell and E. A. Doisy, *J. Biol. Chem.*, 1920, **44**, 55.
4. A. P. Briggs, *J. Biol. Chem.*, 1922, **53**, 13.
5. A. P. Briggs, *J. Biol. Chem.*, 1924, **59**, 255.
6. S. L. Leiboff, *J. Lab. Clin. Med.*, 1931, **16**, 495.
7. Ernst Tschopp and Emilio Tschopp, *Helv. Chim. Acta*, 1932, **15**, 793.
8. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 1925, **66**, 375.
9. E. J. King, *Biochem. J.*, 1932, **26**, 292.
10. G. Denigès, *Compt. rend.*, 1920, **171**, 802.
11. E. Truog and A. H. Meyer, *Ind. Eng. Chem., Anal. Ed.*, 1929, **1**, 136.
12. W. M. Holman and A. G. Pollard, *J. Soc. Chem. Ind.*, 1937, **56**, 339T.
13. Clemens Jones, *Trans. Amer. Inst. Min. Engrs.*, 1888–89, **17**, 411.
14. I. Berenblum and E. Chain, *Biochem. J.*, 1938, **32**, 295.
15. K. Stoll, *Z. anal. Chem.*, 1938, **112**, 81.

SALICYLIC ACID

The well-known test for salicylic acid using ferric chloride can be applied for quantitative work but, for the latter purpose, it is important to conduct the determination under carefully controlled conditions. The quantitative aspect of the test was the subject of a careful study by J. R. Nicholls[1] while the procedure described below follows the recommendation of F. W. Edwards, H. R. Nanji and M. K. Hassan[2] who have slightly modified the technique suggested by Nicholls. The method is identical with that employed for measuring the amount of salicylic acid produced by the oxidation of benzoic acid when making colorimetric determinations of the latter substance (see p. 129). Another test, known as Jorissen's[3], depends upon the production of a red colour when salicylic acid is treated with an alkali nitrite and copper

sulphate in the presence of acetic acid. This reaction has also been studied by Nicholls[1] and the technique advocated by this investigator is given here.

Method Using Ferric Chloride[2]. A quantity of the ether extract, or other residue, from the sample under examination, expected to contain between 0·05 and 0·5 mg. of salicylic acid, is dissolved in 50 ml. of alcohol (10 per cent.), 1 ml. of the iron reagent as described in the monograph on Benzoic Acid (reagent No. 3 on p. 129) added and the violet colour produced compared with a series of standards made by diluting suitable quantities (say from 0·5 to 5 ml.) of a standard salicylic acid containing 0·1 mg. per ml. in 10 per cent. alcohol to 50 ml. with the same solvent and adding 1 ml. of the No. 3 iron reagent.

Discussion. Nicholls[1] found that the optimum intensity of the violet colour was developed in 0·005N mineral acid solution and that in less acid media the shade of tint produced was uncertain and difficult to match while in the presence of stronger acid the intensity of the ferric salicylate colour weakened. The difficulty of making so careful an adjustment of the reaction was overcome by Edwards, Nanji and Hassan[2] by conducting the colour test in 10 per cent. alcohol. The isolation of salicylic acid from foods may be effected by the methods already described for the separation of benzoic acid (see p. 131). A test is included in the British Pharmacopœia, 1932, for the determination of free salicylic acid in acetylsalicylic acid which is based upon a procedure recommended by A. J. Jones[4].

Method for Free Salicylic Acid in Acetylsalicylic Acid. A weighed quantity of the sample (0·6 g.) is transferred to a measuring cylinder and dissolved in 9 ml. of alcohol (90 per cent.), the solution diluted to 90 ml. with water and 60 ml. poured into a Nessler glass. To the remaining 30 ml. is added 3 ml. of alcohol (90 per cent.), the mixture diluted to 60 ml. with water and transferred to a second Nessler glass. To the contents of each glass is added 1 ml. of a freshly prepared 0·2 per cent. aqueous solution of ferric ammonium sulphate and any violet colour produced in the first Nessler glass is matched by adding to the second Nessler from a burette a freshly prepared 0·01 per cent. aqueous solution of salicylic acid. Since there is a difference of 0·2 g. in the amount of acetylsalicylic acid in the two solutions it follows that 1 ml. of the standard 0·01 per cent. solution of salicylic acid is equivalent to 0·05 per cent. of free salicylic acid in the sample under examination.

Discussion. The British Pharmacopœia stipulates that the official material shall not contain more than 0·05 per cent. of free salicylic acid. The substance is less liable to undergo deterioration in the pure state than when made up into tablets. Nutter Smith[5] has pointed out that certain substances, notably tartaric acid and citric acid, interfere with the development of the ferric salicylate colour by forming non-ionised salts with the iron and 1 per cent. of citric acid will mask the presence of about 0·2 per cent. of free salicylic acid.

Method Based Upon Jorissen's Test[1]. To between 10 and 40 ml. of a neutral solution containing salicylate equivalent to not more than 1 mg. of salicylic acid is added 1 ml. of a 2 per cent. aqueous solution of sodium nitrite and 1 ml. of a 0·3 per cent. solution of copper sulphate, $CuSO_4.5H_2O$, in 10 per cent. acetic acid. The vessel containing this mixture is immersed in a boiling water-bath for 15 minutes, and the liquid is cooled and diluted with water to 50 ml. The red colour produced is matched by adding a standard colour solution to a blank consisting of 49 ml. of water to which has been added 1 ml. of the acid copper solution used in the test. The standard colour is prepared by carrying out the above test on 5 ml. of a 0·1 per cent. salicylic acid solution diluted to 40 ml., 5 ml. of each of the reagents being used and the final solution being diluted to 100 ml. (1 ml. of this standard colour is equivalent to 0·05 mg. salicylic acid).

Discussion. This method gives good results and may sometimes be useful in helping to establish the identity of a preservative since benzoic acid does not respond and *p*-hydroxybenzoic acid gives a yellow colour easily distinguished from the red given by salicylic acid (see p. 143). It should be noted that phenolphthalein gives a strong reaction with Jorrisen's test.

References

1. J. R. Nicholls, *Analyst*, 1928, **53**, 19.
2. F. W. Edwards, H. R. Nanji and M. K. Hassan, *Analyst*, 1937, **62**, 172.
3. A. Jorissen, *Bull. Acad. roy. Sci. Lettres Beaux-arts Belg.*, 1882, [iii], **3**, 256.
4. A. J. Jones, *Chem. & Drugg.*, 1919, **91**, 402.
5. A. Nutter Smith, *Analyst*, 1920, **45**, 412.

SILICATES

The determination of traces of silicates in water by measurement of the yellow colour produced by the addition of ammonium molybdate and a mineral acid appears to have been first reported by Jolles and Neurath[1]. Phosphates give a similar reaction but, under the same conditions, the time taken for the colour to reach its maximum intensity is different[2] and Schreiner developed a method for the separate determination of silicates and phosphates based upon the observation that if ammonium molybdate and nitric acid are added to a silicate solution an hour apart the colour of the silicomolybdate possesses half the intensity of the colour produced by adding the reagents simultaneously, whereas no such difference occurs with phosphates[3]. Schreiner pointed out that when the concentration of the phosphates is low by comparison with the silicates a relatively large error in the determination of the latter may result and Lincoln and Barker suggested that this difficulty might be overcome by adding a known amount of a soluble phosphate prior to making the colorimetric estimations[4]. In the experience of later investigators these methods have not proved to be reliable and it has been demonstrated by Thayer[5] that, in any given case, there is a certain concentration of phosphate capable of exerting a maximum disturbance on the determination of silicate whence, within limits, values above or below exercise lesser disturbances while relatively large amounts of phosphate inhibit the formation of colour. A more promising approach to the problem was suggested by the observation of Denis and von Meysenbug in 1922 that the presence of oxalic or citric acid tends to prevent the formation of colour due to phosphates[6]. This fact was confirmed by other investigators[7–9] and was found to apply to a large number of hydroxy acids[10] while Parri and Scotti[11] noticed that the presence of alkali citrates interferes with the phosphate reaction in much greater degree than with the formation of colour due to silicates. Isaacs[12] introduced the use of sodium sulphite as a reducing agent in order to form a blue-coloured complex and Rodillon suggested a mixture of hydroxylamine hydrochloride and sodium hydrosulphite for the same purpose[13]. In a proposed modification of Isaacs' method E. J. King employs a mixture of sodium sulphite and hydroquinone to effect reduction of the silicomolybdate[14]. The simple method applied without reduction is described below together with its application to aluminium and

aluminium alloys and, in addition, King's method as used by A. J. Amor[15] for the examination of lung tissue in the chemical study of silicosis. Finally, a procedure involving reduction and taken from the present author's unpublished data is included.

Method Without Reduction. A suitable quantity of the sample to be examined, containing between 0·05 and 1·0 mg. SiO_2, is transferred to a 50-ml. Nessler glass and diluted to the mark with water. The temperature is adjusted to between 25° and 35° C., 2 ml. of a freshly prepared 10 per cent. aqueous solution of ammonium molybdate is added followed by 4 ml. of 2N sulphuric acid and, after stirring, the mixture is set aside for 10 minutes and any yellow colour produced matched against standards. The latter are best prepared from a silicate solution of known strength made by fusing pure precipitated silica with anhydrous sodium carbonate, leaching the melt with water, filtering, diluting to a convenient volume, conducting a gravimetric determination on an aliquot portion and then suitably diluting the remainder after neutralisation with dilute sulphuric acid.

Method Without Reduction Using Permanent External Standards. Owing to the trouble entailed in preparing natural standards for this test the B.D.H. Lovibond Nessleriser disc, covering a range from 0·05 to 1·0 mg. SiO_2 is particularly useful: the nine glass standards are employed in conjunction with the technique described above.

Discussion. In this test, the concentration of the sulphuric acid present in the final reaction mixture is important and variations in either direction may result in diminution of the intensity of colour due to silica. Apart from the interference caused by phosphates, iron, if present in greater concentration than 20 parts per million, disturbs the colorimetric determination of silicates owing to the formation of iron silicomolybdate which is more intensely coloured than the ammonium salt; if phosphate is also present, errors due to iron are further magnified by the production of the still more deeply coloured iron phosphomolybdate. These limitations to the method have been the subject of an exhaustive study by Thayer[5] who has described a procedure whereby iron is removed by precipitation as ferric phosphate after acidification with acetic acid, the excess phosphate removed from the filtered mixture by addition of calcium chloride and a slight excess of ammonia and the silicate finally determined after filtering off the precipitated calcium phosphate.

Application to Aluminium and its Alloys of the Method Without

Reduction[16]. (*a. Silicon Content up to* 0·03 *per cent.*) To 1 g. of millings contained in a 200-ml. nickel crucible is added 30 ml. of sodium hydroxide solution (10 g. per 100 ml. of water, freshly prepared in a nickel dish). When the reaction is complete the liquid is boiled for 2 minutes, allowed to cool and transferred to a beaker containing 38·5 ml. of diluted nitric acid (made by mixing 1 vol. of acid s.g. 1·42 with 2 vols. of water) and the mixture heated, but not boiled, until the aluminium hydroxide has dissolved. After cooling, the acid solution is transferred to a 150-ml. Nessler glass, diluted with water to 140 ml. and 10 ml. of a freshly prepared 10 per cent. aqueous solution of ammonium molybdate added. After allowing the yellow colour to develop for 10 minutes it is matched against standards prepared by adding standardised picric acid solution to water contained in Nessler glasses. A blank determination on the reagents employed should be made using 30 ml. of the sodium hydroxide solution and 22 ml. of the diluted nitric acid. The standard picric acid solution, initially made by dissolving 0·148 g. of the dry solid in 1 litre of water, is standardised by matching the colour of appropriate dilutions against tests performed on a silicate solution of known strength. The latter is prepared by fusing 0·022 g. of pure precipitated and dehydrated silica with anhydrous sodium carbonate, leaching out with water, neutralising with nitric acid, boiling off carbon dioxide and diluting the cooled, filtered solution to 1 litre. The picric acid solution is conveniently adjusted so that 1 ml. is equivalent to 0·01 mg. Si.

(*b. Silicon Content up to* 0·3 *per cent.: Manganese less than* 0·5 *per cent.*) To 0·1 g. of millings contained in a small nickel crucible is added 10 ml. of sodium hydroxide solution (10 g. per 100 ml. of water, freshly prepared in a nickel dish). When the reaction is complete the liquid is boiled for 5 minutes, allowed to cool and transferred to a beaker containing 10 ml. of diluted nitric acid (made by mixing 1 vol. of acid s.g. 1·42 with 2 vols. of water) and the mixture heated, but not boiled, until the aluminium hydroxide has dissolved. After cooling, the acid solution is transferred to a 100-ml. Nessler glass, diluted with water to 90 ml. and 10 ml. of a freshly prepared 10 per cent. aqueous solution of ammonium molybdate added. After allowing the yellow colour to develop for 10 minutes it is matched against standards prepared after the manner already indicated. A blank determination on the reagents employed should be made using 10 ml. of the sodium hydroxide solution and 7·5 ml. of the diluted nitric acid. If

the sample has a high iron content, as in the case of certain hardener alloys (which may contain also copper and chromium) the initial solution in alkali should be filtered through a 7-cm. Whatman paper No. 541 into the diluted nitric acid and the insoluble material on the paper washed with cold water. This filtration of the alkaline solution removes all elements that would give rise to a coloured or turbid acid solution. According to W. H. Hadley[16], the originator of these procedures, in the case of samples " having a high iron content associated with a high magnesium content it is preferable to proceed in the normal manner but to filter the acid solution through a small Whatman No. 44 paper into the Nessler glass in order to remove any turbidity due to the iron. This alternative procedure reduces any error which otherwise might be introduced by filtration of a soda solution containing a small proportion of undecomposed and undissolved magnesium-silicon compound."

(*c. Silicon Content up to* 0·3 *per cent.: Manganese more than* 0·5 *per cent.*) To 0·1 g. of millings contained in a small nickel crucible is added 10 ml. of sodium hydroxide solution (10 g. per 100 ml. of water, freshly prepared in a nickel dish). When the reaction is complete the liquid is boiled for 5 minutes, allowed to cool and transferred to beaker containing 10 ml. of diluted nitric acid (made by mixing 1 vol. of acid s.g. 1·42 with 2 vols. of water) and the mixture heated, but not boiled, until the aluminium hydroxide has dissolved. About 5 drops of sulphurous acid solution (6 per cent. H_2SO_3) is added with stirring, the heating continued for a few minutes until all manganese dioxide has dissolved, then 0·1N potassium permanganate added dropwise until a permanent pink colour is obtained. The liquid is decolorised by the addition of a 0·5 per cent. aqueous solution of oxalic acid and, after cooling, the liquid transferred to a 100-ml. Nessler glass, diluted with water to 90 ml. and 10 ml. of a freshly prepared 10 per cent. aqueous solution of ammonium molybdate added. After allowing the yellow colour to develop for 10 minutes it is matched against standards prepared after the manner already indicated. A blank determination on the reagents employed should be made using 10 ml. of the sodium hydroxide solution and 7·5 ml. of the diluted nitric acid.

Notes on the Application. In developing the above procedures Hadley has aimed to adjust the acidity of the final reaction mixtures within a range equivalent to *p*H 1·1 to 2·0 since his preliminary work showed that only within this degree of acidity could

reproducibile results be obtained[16]. The second of the above methods (*b*, using 0·1 g. of sample) is applicable to alloys containing less than 0·5 per cent. of manganese, 12·5 per cent. of copper, 0·35 per cent. of chromium, 30 per cent. nickel and/or 0·75 per cent. of iron, while the indicated modification permits of its application to special alloys of high iron content. The procedure *c* is suitable for hardener alloys containing up to 8 per cent. of manganese.

So far as the examination of aluminium and its alloys is concerned disturbance of the test by reason of its reaction to phosphates is immaterial since this radicle is rarely formed in association with these products. The silicon content of these alloys is often a matter of considerable importance owing to its profound effect on the character of the metal. Thus, if the amount of silicon in aluminium itself exceeds 0·5 per cent. undue ductility results while in the case of aluminium-magnesium alloys small concentrations of silicon help to toughen the metal and when the proportion exceeds a value which is frequently quite critical for any given alloy the latter becomes brittle. There are many alloys, now extensively employed in engineering construction, which are required to contain 10 per cent. or more of silicon and which exhibit significant changes in characteristics even when only slight deviations from the optimum concentrations occur ; thus accurate determinations of high silicon contents in aluminium alloys are frequently needed. This necessity is not satisfactorily met by the standard gravimetric procedures because, apart from the excessive time occupied by their performance, difficulty is often encountered in achieving complete oxidation of the silicon to silica. Colorimetric methods are not, in general, well suited for determining constituents present in large proportions but for this particular case Hadley has attempted to meet the difficulty by applying modifications of the above procedures and measuring the extinction value $\left(\text{i.e. } \log_{10} \frac{\text{initial light intensity}}{\text{emergent light intensity}}\right)$ by means of a Zeiss-Pulfrich photometer[17]. It was found that when working with a sample weighing 0·1 g., a final solution volume of 100-ml. and a 50-mm. cell for the extinction measurement, the maximum silicon content capable of determination is approximately 1·5 per cent., since above this value the light absorption is nearly complete. For higher concentrations of silicon the solution volume has to be increased and there is a consequent diminution in precision. In view of the importance attaching to the determination

of silicon in these products Hadley's methods are reproduced below.

Application to Aluminium and its Alloys of the Method Without Reduction[17]. (*d. Silicon Content* 0·3 *to* 1·5 *per cent.: With Alloying Metals not exceeding* 0·5 *per cent. Mn*, 0·75 *per cent. Fe*, 12·5 *per cent. Cu*, 30 *per cent. Ni and* 0·35 *per cent. Cr*) To 0·1 g. of millings contained in a small nickel crucible is added 10 ml. of sodium hydroxide solution (10 g. per 100 ml. of water, freshly prepared in a nickel dish). When the reaction is complete the liquid is boiled for 5 minutes, allowed to cool and transferred to a beaker containing 10 ml. of diluted nitric acid (made by mixing 1 vol. of acid s.g. 1·42 with 2 vols. of water) and the mixture heated, but not boiled, until the aluminium hydroxide has dissolved. After cooling, the solution is filtered through a Jena 3G1 sintered glass funnel and, if necessary, further filtered through a Whatman No. 44 paper, then transferred to a 100-ml. graduated flask, diluted with water to 90 ml. and 10 ml. of a freshly prepared 10 per cent. aqueous solution of ammonium molybdate added. After allowing the colour to develop for 10 minutes the extinction value is measured with a Pulfrich photometer in conjunction with a mercury lamp using a 50-mm. cell and light filter transmitting the mercury line 4358Å. A blank determination on the reagents should be made using 10 ml. of the sodium hydroxide solution and 7 ml. of the diluted nitric acid. The observed extinction value is correlated with the proportion of silicon in the sample by reference to a graph constructed from the data given in Table XI.

TABLE XI. RELATION BETWEEN THE EXTINCTION VALUE AND THE PROPORTION OF SILICON IN ALUMINIUM AND ALUMINIUM ALLOYS (After W. H. Hadley, *Analyst*, 1942, **67**, 5)

Extinction Value for the Mercury Line 4358Å Observed with Zeiss-Pulfrich Photometer Using 50-mm. Cell	Silicon Content Using 0·1 g. of Sample and a Solution Volume of 100 ml. per cent.
0·244	0·20
0·312	0·26
0·503	0·41
0·674	0·56
0·777	0·67
0·909	0·79
0·974	0·85
1·163	1·02
1·49	1·33
1·72	1·55

(*e. Silicon Content* 0·3 *to* 1·5 *per cent.: Manganese more than* 0·5 *per cent. and other Elements as Specified in d*) The yellow solution for colour measurement is prepared precisely in the manner already described under *c* above. A blank determination is conducted using 10 ml. of the sodium hydroxide solution and 7 ml. of the diluted nitric acid and the determination completed using the photometer as described under method *d*.

(*f. Silicon Content* 0·3 *to* 1·5 *per cent.: Alloys Containing Fe, Cu, Ni and Cr above limits specified in d and Manganese*) To 0·1 g. of millings contained in a small nickel crucible is added 10 ml. of sodium hydroxide solution (10 g. per 100 ml. of water, freshly prepared in a nickel dish). When the reaction is complete the liquid is boiled for 5 minutes, allowed to cool and filtered through a 7-cm. Whatman No. 541 paper into a small beaker containing 10 ml. of diluted nitric acid (made by mixing 1 vol. of acid s.g. 1·42 with 2 vols. of water) the filter paper being rinsed through with cold water. The determination is then completed as described under procedure *d*, a blank being made with the appropriate modification using 7 ml. of the diluted nitric acid.

(*g. Silicon Content* 3·0 *to* 14·0 *per cent.*) To 0·1 g. of millings contained in a small nickel crucible is added 10 ml. of sodium hydroxide solution (40 g. per 100 ml. of water, freshly prepared in a nickel dish). When the reaction is complete the liquid is boiled for 5 minutes, allowed to cool, transferred to a beaker containing 60 ml. of diluted nitric acid (made by mixing 1 vol. of acid s.g. 1·42 with 2 vols. of water) and the mixture heated, but not boiled, until the aluminium hydroxide has dissolved. After cooling, the solution is filtered through a Jena 3G1 sintered glass funnel into a 200-ml. graduated flask, the filtrate diluted to the mark with water then 20 ml. transferred to a 100-ml. graduated flask. This aliquot portion is then diluted with water to 90 ml. and 10 ml. of a freshly prepared 10 per cent. aqueous solution of ammonium molybdate added. After allowing the colour to develop for 10 minutes the extinction value is determined using a 50-mm. cell as defined in procedure *d*. A blank determination on the reagents is made using 45 ml. of the diluted nitric acid. For alloys of high silicon content the only common interfering element is manganese and if this metal is present the modification described under method *e* should be applied.

Additional Note on Hadley's Procedure. Following the publication of the above methods, A. Staples reported that low results had been obtained in the case of certain hiduminium RR. alloys

although in general the colorimetric process had given satisfaction[18]. However, it is pointed out by B. A. Scott[19] that the difficulty encountered with samples containing alloying amounts of magnesium and silicon may be overcome by attacking in a nickel crucible 0·1 g. of millings with 10 ml. of a 10 per cent. aqueous solution of sodium hydroxide mixed with 1 ml. of a 3 per cent. solution of hydrogen peroxide (AnalaR grade) and, after the initial reaction has subsided, evaporating to a pasty mass and pouring into diluted nitric acid (made by mixing 1 vol. of acid s.g. 1·42 with 2 vols. of water). The assay is then continued as already described excepting that any residual hydrogen peroxide is removed from the warm acid mixture by means of saturated aqueous solution of potassium permanganate followed by oxalic acid.

Method of E. J. King, With Reduction[14]. To the neutral solution to be tested, containing between 0·05 and 0·8 mg. SiO_2, is added 3 ml. of a 10 per cent. aqueous solution of acetic acid, 3 ml. of a 10 per cent. aqueous solution of ammonium molybdate and 2 ml. of a 0·5 per cent. solution of hydroquinone in 20 per cent. aqueous solution of sodium sulphite, $Na_2SO_3,7H_2O$, and the volume of the mixture made up to 25 ml. with water. At the same time a series of standards is prepared using a silica solution of known strength (see p. 166) and, after allowing all the tests to stand at room temperature for 30 minutes, the intensity of the blue colour developed in the test mixture is compared with that of the standards, either by direct matching or by means of a colorimeter.

Application of E. J. King's Method to Animal Tissue[14, 15]. The sample is ashed, 0·5 g. of the finely powdered residue weighed into a platinum crucible and 1 ml. each of a saturated aqueous solution of boric acid and a 10 per cent. aqueous solution of magnesium nitrate, $Mg(NO_3)_2.6H_2O$, added followed by 3 ml. of concentrated nitric acid. The mixture is warmed on a boiling water-bath until a clear solution is obtained, when it is further heated on an electric hot plate until just dry, then ignited over a Bunsen flame until a white ash is obtained. Several drops of concentrated nitric acid are then added to reconvert the magnesium oxide to nitrate, the mixture evaporated just to dryness and the residue dissolved in a little water which has been slightly acidified with acetic acid. After adding 2·5 ml. of sodium hydroxide solution (made by dissolving 2·3 g. of metallic sodium in water contained in a nickel crucible and diluting to 100 ml.), the mixture is warmed in a

water-bath in order to effect solution of the silica and 5 ml. of N ammonium chloride added. After allowing to stand for several minutes the precipitate of magnesium ammonium phosphate is filtered off, the filtrate neutralised with a 10 per cent. aqueous solution of acetic acid and the silicate determined by the method described above.

Discussion. In this test the presence of an amount of phosphate commensurate with the quantity of silicate exercises little influence upon the colour produced, but with ten times as much phosphate as silicate the intensity of the colour due to the latter is slightly diminished. Iron, when present even in minute traces, vitiates the test but nitrates do not interfere with the development of colour. From the foregoing, it follows that the method is well suited to the chemical investigation of silicosis since, in the solutions as prepared for the colorimetric determination, iron and phosphates will have been eliminated. The silica content of the ash of normal human lungs may lie between 1 and 3 per cent. while the ash of the fibrotic areas of silicotic lungs may contain anything to upwards of 40 per cent.

Method With Reduction. To not more than 34 ml. of the neutralised solution to be tested, contained in a Nessler glass and expected to contain between 0·01 and 0·08 mg. SiO_2, is added 1 ml. of exactly N sulphuric acid and 5 ml. of a freshly prepared 10 per cent. aqueous solution of ammonium molybdate. After allowing to stand for precisely 5 minutes, 5 ml. of an 8 per cent. aqueous solution of sodium citrate, $Na_3C_6H_5O_7.2H_2O$, is added followed by 5 ml. of a 20 per cent. aqueous solution of potassium metabisulphite containing 0·1 per cent. of hydroquinone. The Nessler glass containing the mixture is immersed in a boiling water-bath for 5 minutes, the liquid then cooled and, if necessary, diluted to 50 ml. with water. Any blue colour produced is compared with standards made from a solution prepared in the manner described on p. 166.

Discussion. In carrying out the above procedure it is important to ensure that the potassium metabisulphite is free from all trace of iron. It is better to use pure sodium metabisulphite since it leads to the production of brighter blue tints but high grades of this reagent may not be readily available. This method is about a hundred times less sensitive to phosphates than it is to silicates hence it follows that phosphate equivalent to 1 mg. P_2O_5 may be expected to give a just perceptible colour. A distinct blue is produced by the presence of phosphate equal to 5 mg. P_2O_5.

Arsenates react in precisely the same manner as phosphates. Iron and nitrates, even when present only in traces, seriously disturb the formation of colour due to silicates.

REFERENCES

1. A. Jolles and F. Neurath, *Z. angew. Chem.*, 1898, **11**, 315.
2. F. P. Veitch, *J. Amer. Chem. Soc.*, 1903, **25**, 169.
3. O. Schreiner, *J. Amer. Chem. Soc.*, 1903, **25**, 1056.
4. A. T. Lincoln and P. Barker, *J. Amer. Chem. Soc.*, 1904, **26**, 975.
5. L. A. Thayer, *Ind. Eng. Chem., Anal. Ed.*, 1930, **2**, 276.
6. W. Denis and L. von Meysenbug, *J. Biol. Chem.*, 1922, **52**, 1.
7. C. F. Bordeianu, *Ann. Sci. Univ. Jassy*, 1927, **14**, 353.
8. H. Riehm, *Z. Pflanz. Düngung Bodenk.*, 1932, **25A**, 323.
9. Ernst Tschopp and Emilio Tschopp, *Helv. Chim. Acta*, 1932, **15**, 793.
10. D. R. Davies and W. C. Davies, *Biochem. J.*, 1932, **26**, 2046.
11. W. Parri and G. Scotti, *J. Pharm. Chim.*, 1933, **18**, 513.
12. L. Isaacs, *Bull. Soc. Chim. biol.*, 1924, **6**, 157.
13. G. Rodillon, *Bull. Biol. Pharm.*, 1934, **1**, 33.
14. E. J. King, *Bull. Soc. Chim. biol.*, 1930, **12**, 903.
15. A. J. Amor, "The Chemical Aspects of Silicosis." London: H. K. Lewis & Co., Ltd., 1942.
16. W. H. Hadley, *Analyst*, 1941, **66**, 486.
17. W. H. Hadley, *Analyst*, 1942, **67**, 5.
18. A. Staples, *Analyst*, 1942, **67**, 287.
19. B. A. Scott, *Analyst*, 1942, **67**, 389.

SULPHATES

See Section III: Substances of Clinical and Biochemical Significance, p. 273.

SULPHIDES

In the presence of hydrochloric acid and an oxidising agent hydrogen sulphide reacts with *p*-aminodimethylaniline (*asym.* dimethyl-*p*-phenylenediamine) to form methylene blue. So long ago as 1883 Emil Fischer called attention to the possibility of applying the reaction for the detection of hydrogen sulphide[1] and it was first used for analytical purposes by Lindsay[2] for the determination of sulphides in pig iron. Later, the method was studied by Mecklenburg and Rosenkränzer[3] who effected improvements in the colour test while Almy[4] developed a procedure for the preliminary isolation of hydrogen sulphide from the sample to be

tested. The complete technique employed by Almy was particularly designed for the assessment of spoilage in meat and fish, but it should be readily applicable to a wide variety of products with but slight and obvious modifications.

Method[4]. The following reagents are required :—

1. A freshly prepared 0·04 per cent. Solution of *p*-Aminodimethylaniline Hydrochloride in 5N hydrochloric acid.
2. A 0·1M Acid Solution of Ferric Chloride made by dissolving 27 g. of the hydrated salt, $FeCl_3.6H_2O$, in 500 ml. of concentrated hydrochloric acid and diluting to 1 litre with water: from this a 0·02M Solution is made as required by dilution with water.
3. An approximately 20 per cent. Stock Solution of Zinc Acetate made by treating 135 g. of glacial acetic acid with a slight excess of an aqueous suspension of zinc oxide and dilution with water to 1 litre : this is filtered and diluted to make 2 per cent., or 0·6 per cent. Solution as required.

A series of standards is first prepared. Into each of fifteen graduated flasks of 500 ml. capacity is introduced 75 ml. of 2 per cent. zinc acetate solution. From 315 to 375 ml. of cool, recently boiled, distilled water (the exact quantity being dependent upon the amount of hydrogen sulphide solution to be added subsequently) is added to the contents of each flask, the requisite amount of hydrogen sulphide solution B (*vide infra*) is then introduced, promptly followed, in order, by 25 ml. of the diamine reagent and 5 ml. of 0·02M ferric chloride. The volume of the standard hydrogen sulphide solution in each flask before addition of the diamine reagent should be about 450 ml. The temperature of the solutions in the flasks at the time of adding these reagents should be uniform, preferably not varying more than 0·5° C. from the mean in any flask and, of course, equal to that of the prepared test solutions. After standing for 2 hours the several standards are diluted to the mark with water. The hydrogen sulphide solution B is made by diluting a stronger solution A. To prepare the latter, hydrogen sulphide, evolved from ferrous sulphide and hydrochloric acid, is washed with cold water and passed slowly for about 2 minutes into 300 ml. of cool, boiled, distilled water. An aliquot portion of the resulting solution is added to an excess of a known amount of 0·01N iodine and the mixture titrated with 0·01N sodium thiosulphate solution. An amount of solution A corresponding to about 30 ml. of 0·01N solution is added to

approximately 450 ml. of diluted zinc acetate solution (about 15 ml. of the 2 per cent. solution with 435 ml. of water) in a 500-ml. graduated flask and the mixture diluted to the mark with water. This solution B, containing approximately 0·01mg. H_2S per ml., is then used *immediately* for the preparation of the standards, a

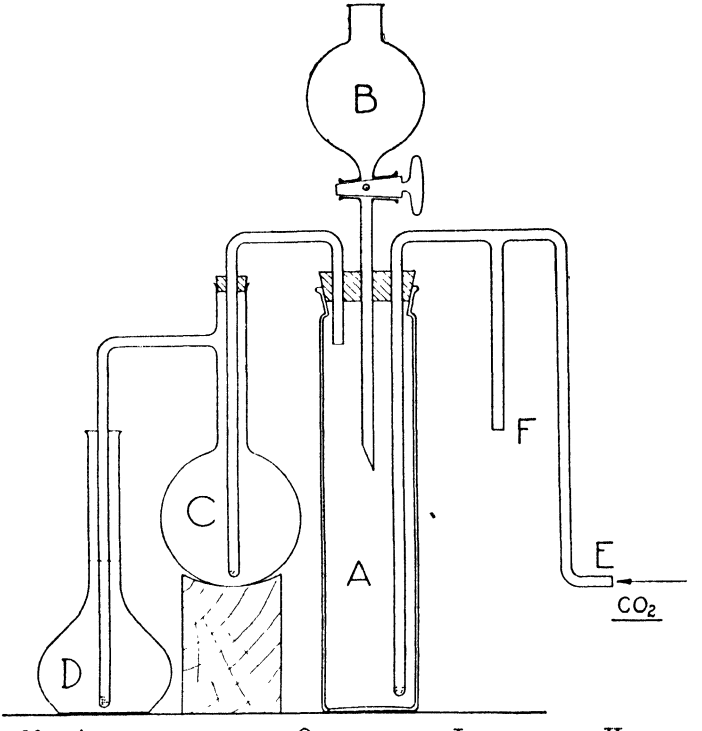


FIG. 10. APPARATUS FOR THE QUANTITATIVE ISOLATION OF HYDROGEN SULPHIDE

Carbon dioxide is supplied through the tube E which ends in a perforated bulb in the cylinder A. A manometer, conveniently charged with aqueous solution of zinc chloride of s.g. 2, is attached at F. Hydrogen sulphide is liberated from the sample by hydrochloric acid added from the funnel B and swept by the carbon dioxide into the flasks C and D where it is trapped by an aqueous solution of zinc acetate. (After L. H. Almy, *J. Amer. Chem. Soc.*, 1925, **47**, 1381.)

convenient set being obtained by using 1, 2, 3, 4, 5, 7, 10, 12·5, 15, 20, 25, 30, 40, 50 and 60 ml.

A suitable quantity of the finely divided sample to be examined is introduced into a tall cylinder as shown at A in Fig. 10 and 50 ml. of water added. The three-hole stopper is fitted to the cylinder, 50 ml. of 5N hydrochloric acid introduced into the dropping funnel B, 30 ml. of the 0·6 per cent. zinc acetate solution

placed in the distilling flask C, 20 ml. of the same liquid introduced into the 100-ml. volumetric flask, D, and the apparatus assembled as indicated in the drawing. The stopcock in the funnel is turned to allow the acid to run into the mixture, the flow being stopped in time to prevent the last few drops from leaving the bulb. Carbon dioxide is then passed through the train at a pressure equivalent to about 40 mm. of water, the aëration being continued at this pressure for 15 minutes. The carbon dioxide is shut off, the two receiving vessels are disconnected and the solution in the distilling flask is washed into the volumetric flask, using 40 ml. of water altogether. To the 90 ml. of liquid thus produced is added 5 ml. of the diamine reagent, followed by 1 ml. of 0·02M ferric chloride, the solution being gently agitated after each addition. After 2 hours the volume is brought to 100 ml. by the addition of water and the intensity of any blue colour produced is compared in a colorimeter with that of the standard which most nearly agrees in depth of tint.

Discussion. If the zinc acetate solution after the aëration is markedly turbid, due to precipitated zinc sulphide, it probably contains a concentration of sulphide greater than that represented by the highest standard and must, therefore, be diluted before the test reagents are added. For this purpose the liquid is diluted to the mark with or without the previous addition of a small amount of dilute hydrochloric acid (to dissolve zinc sulphide should it be in the form of a flocculent precipitate), an appropriate aliquot portion is removed to another 100-ml. volumetric flask containing 15 ml. of a 2 per cent. aqueous solution of zinc acetate and the resulting mixture diluted and treated as described above. Foaming may be minimised by the addition of a few drops of diphenyl ether or *sec.*-octyl alcohol (capryl alcohol). If this treatment proves to be inadequate, as may happen in the case of albuminous materials such as egg products, the addition of about 2 ml. of a 40 per cent. aqueous solution of sodium tungstate may be efficacious. As a precaution, rubber fittings should be thoroughly washed before use although generally no measurable blank attributable to contamination either from this source or from any impurity in the carbon dioxide need be feared.

If the standards are stored in a cool, dark place they will remain unaltered for some weeks, apart from the pale colours which develop a greenish tint. The aëration with carbon dioxide, which constitutes the only significant difference in treatment received

by the test solutions as distinct from that applied to the standards, does not influence the colour produced on addition of the reagents except in the case of solutions representing less than 0·07 mg. per litre of hydrogen sulphide ; with these pale colours there may be a deficiency of tint equal to about 10 per cent. Methyl and ethyl sulphides and ethyl mercaptan do not interfere, but when the hydrogen sulphide reagents are added directly to pure solutions of these substances pink or yellow colours result. The lack of interference of these related sulphur compounds in the test must be due to the fact that they are not absorbed by the zinc acetate solution.

The method is applicable to mineral waters and to sewage while St. Lorant and Reimann have employed it for the determination of sulphides in fæces[5]. For the latter purpose a weighed portion of the sample is ground in a ball mill with a 5 per cent. aqueous solution of borax, a suitable aliquot part of the resulting suspension is transferred to the cylinder of the evolution apparatus and 50 per cent. phosphoric acid is used for the liberation of the hydrogen sulphide. For the determination of labile sulphur in gelatin[6] 5 g. of the sample, cut into small pieces (0·5 cm. square), is added to 25 ml. of a 1 per cent. solution of silver chloride in concentrated ammonium hydroxide, the mixture warmed at 50° C. for 2 or 3 hours then evaporated by stronger heating to a volume of about 10 ml. and the blackened material transferred to the evolution apparatus. In this case hydrochloric acid is used to liberate the hydrogen sulphide but the aëration should be continued for 1 hour as the silver sulphide is slow to decompose.

References

1. E. Fischer, *Ber.*, 1883, **16**, 2234.
2. W. G. Lindsay, *Columbia School Mines Quart.*, 1902, **23**, 24.
3. W. Mecklenburg and F. Rosenkränzer, *Z. anorg. Chem.*, 1914, **86**, 143.
4. L. H. Almy, *J. Amer. Chem. Soc.*, 1925, **47**, 1381.
5. I. St. Lorant and F. Reimann, *Biochem. Z.*, 1930, **228**, 300.
6. S. E. Sheppard and J. H. Hudson, *Ind. Eng. Chem., Anal. Ed.*, 1930, **4**, 73.

TARTRATES

Proposals for the colorimetric determination of tartrates include the use of sodium metavanadate[1] and a quantitative application[2] of Fenton's well-known test using ferrous sulphate and hydrogen peroxide[3, 4]. It has been shown by C. A. Mitchell[5] that sodium

metavanadate is better suited to the colorimetric determination of oxalates and since Fenton's more characteristic test is capable of giving approximately quantitative results it is described here in connection with the determination of tartaric acid in baking powder.

Method[2]. A suitable quantity of the sample, 2 g. if the baking powder is expected to contain 40 per cent. of total tartaric acid, is transferred to a small beaker, water added drop by drop until carbon dioxide ceases to be evolved then a further 45 ml. of water added and the liquid stirred until all the tartrates present are dissolved. The mixture is filtered in order to remove starch and the insoluble matter washed with several small portions of water. The reaction of the combined filtrate and washings is adjusted, if necessary, so that it lies between *p*H 5·7 and 6·7 as shown by testing with chloro-phenol red or bromo-cresol purple, the solution then diluted to 100 ml. and 10 ml. transferred to a 25-ml. flask. To this is added 0·2 ml. of a freshly prepared 1 per cent. aqueous solution of ferrous sulphate, $FeSO_4.7H_2O$, and 0·2 ml. of a 3 per cent. aqueous solution of hydrogen peroxide. The mixture is allowed to stand until the yellow colour first produced changes to brown and then the beaker is immersed in an ice-bath. When the colour of the solution has changed to lavender 5 ml. of N sodium hydroxide is immediately added, the flask stoppered, the contents well mixed, the flask replaced in ice for 10 minutes and then the intensity of the lavender colour matched against standards similarly prepared. The standard solution is prepared by adding 10·7 ml. of N sodium hydroxide to 5 ml. of a 16 per cent. w/v solution of *d*-tartaric acid and diluting to 100 ml. with water ; 10 ml. of this solution (*p*H 6·2) will give a colour of suitable intensity for making comparisons by means of a Duboscq type colorimeter.

Discussion. This reaction is not given by sugars or by citric, succinic, malic or oxalic acids. According to Anderson *et al.*[2] *l*-tartaric acid, ammonium *l*-tartrate and meso-tartaric acid produce a colour equal to that due to *d*-tartaric acid (allowance being made in the latter case for one molecule of water of crystallisation) but racemic tartaric acid only yields a tint of approximately half the intensity. It is important to ensure that the reaction of the sample and the standard are the same and preferably equivalent to *p*H 6·2. Aluminium does not interfere but the colour test is not applicable in the presence of calcium or phosphate.

REFERENCES

1. F. P. Underhill, F. I. Peterman and A. G. Krause, *J. Pharmacol.*, 1931, **43**, 351.
2. A. K. Anderson, A. H. Rouse and T. V. Letonoff, *Ind. Eng. Chem., Anal. Ed.*, 1933, **5**, 19.
3. H. J. H. Fenton, *Chem. News*, 1876, **33**, 190.
4. H. J. H. Fenton, *Chem. News*, 1881, **43**, 110.
5. C. A. Mitchell, *Analyst*, 1933, **58**, 279.

SECTION III

SUBSTANCES OF CLINICAL AND BIOCHEMICAL SIGNIFICANCE

INTRODUCTION

Blood Analyses. A number of the procedures described in this Section are micro-chemical in character and have been developed for the quantitative examination of small samples of capillary blood. These are particularly useful in investigations requiring many determinations, since the need for performing numerous venipunctures and the addition of an anti-coagulant substance to the sample is obviated, while the degree of accuracy attainable is fairly satisfactory and their wide acceptance is at least tentative evidence of their utility. The concentration of some substances in arterial blood is different from that in venous blood and is often of greater clinical significance, notably in the case of glucose, which is best determined in capillary blood, the value being the same as that obtained by using arterial blood. All methods described for use with capillary blood are equally applicable to samples of venous blood. Capillary blood may be taken from a puncture on the ear, but the most convenient place is probably over the bed of the thumb-nail, which should be wiped clean with ether or spirit and a stab about 2 mm. deep made with a Hagedorn needle, a piece of soft rubber tubing wrapped tightly above the knuckle of the thumb and the latter flexed. If blood does not flow easily, the rubber is released and the hand shaken in a downward direction in order to ensure an adequate extrusion of blood when the tourniquet is replaced. A special capillary pipette is held horizontally with its point in the drop of blood, which is allowed to run in exactly to the mark (0·1 or 0·2 ml.), the pipette wiped, the blood then transferred to water or isotonic sodium sulphate solution, and, by alternate blowing and sucking, the pipette washed several times with the liquid.

Isotonicity of Sodium Sulphate Solution. According to a deter-

mination made many years ago by R. R. Bennett and F. W. Gamble[1], using the cryoscopic method, a solution of sodium sulphate containing 4·07 per cent. w/v of the hydrated salt is isotonic with human blood serum. This figure is in close accord with the result (3·90 per cent. w/v) recently obtained by the author, but in the majority of original papers presenting methods of blood analysis it appears to be customary to describe a solution containing 3·00 per cent. w/v of the hydrated salt as isotonic. Since such a preparation seems to be satisfactory in practice it is specified in the descriptions of appropriate procedures which follow.

Urine Analyses. For most quantitative investigations it is advisable to work with a 24-hour sample and the total volume excreted should be known. At a given hour the patient should be instructed to empty the bladder, the urine being discarded; thence all subsequent specimens, including the one passed at the same given hour next day, are collected, the patient being instructed to micturate before defæcating during the period of collection so that no urine is lost. For routine qualitative tests it is customary to sample the urine first passed in the morning but when investigating cases of glycosuria it is wiser to collect urine after meals, since mild cases, or early stages of the disorder, may be overlooked if only night specimens are examined for glucose. Again, G. A. Harrison observes[2] that " in certain cases of intermittent proteinurea the night's urine may be free from protein, whilst the sample obtained a few hours after rising may contain an abundance." Excepting when determinations of uric acid are to be made, the addition of 1 per cent. by volume of concentrated hydrochloric acid is probably the most satisfactory method of preserving samples between the time of collection and examination. Preservation by the addition of a little toluene or chloroform is often practiced but if the latter is employed care must be taken to remove it by boiling prior to starting the analyses since it is liable to interfere with the determinations, particularly those for glucose. Storage in a refrigerator is convenient but, while retarding the growth of organisms, it does not inhibit changes due to their action, and it should never be prolonged.

Clinical Notes. The information relating to the clinical significance of biochemical tests which is embodied in the monographs belonging to this Section is only intended as a rough guide for analytical work and to indicate the purpose of the determination. The clinical interpretation of the results of chemical

analyses is, of course, a complex subject and it is not intended that the incomplete comments included here should ever be considered from the clinician's viewpoint.

Protein Units. In general, the application of colorimetric methods to the determination of substances belonging to this group is still unsatisfactory and consequently only a few procedures actually tried by the author and which appeared to depend upon reasonably characteristic reactions have been included. So far as can be ascertained, the existing colorimetric methods for such important units as tyrosine, tyramine, histamine and glutathione are based on general reactions and are subject to interference from large numbers of substances of allied constitution from which they can only be separated by the application of complicated processes.

Amino-acids. Apart from the monograph on the determination of amino-acid nitrogen in blood and urine using Folin's general reagent, no proposals for the colorimetric determination of this group have been included. As in the case of protein units, most of the suggested colorimetric methods rely upon reactions which are not characteristic and, therefore, it has not been considered necessary to discuss them, particularly in view of the difficulties attendant upon analytical separation of individual amino-acids. However, it may not be inappropriate to mention a proposal[3] to determine serine by distilling off the formaldehyde formed by the action of periodic acid[4–6] in the presence of potassium arsenite and applying to the distillate a quantitative modification of Eegriwe's colour test using 1:8-dihydroxynaphthalene-3:6-disulphonic acid (chromotropic acid)[7].

REFERENCES

1. R. R. Bennett and F. W. Gamble, *Pharm. J.*, 1912, **88**, 354.
2. G. A. Harrison, "Chemical Methods in Clinical Medicine," 2nd Ed., London: J. & A. Churchill, Ltd., 1937, p. 292.
3. M. J. Boyd and M. A. Logan, *J. Biol. Chem.*, 1942, **146**, 279.
4. B. H. Nicolet and L. A. Shinn, *J. Amer. Chem. Soc.*, 1939, **61**, 1615.
5. L. A. Shinn and B. H. Nicolet, *J. Biol. Chem.*, 1941, **138**, 91.
6. B. H. Nicolet and L. A. Shinn, *J. Biol. Chem.*, 1941, **139**, 687.
7. E. Eegriwe, *Z. anal. Chem.*, 1937, **110**, 22.

AMINO-ACID NITROGEN (IN URINE AND BLOOD)

A general colorimetric reaction for amino-acids using sodium β-naphthoquinone-4-sulphonate was evolved by Folin[1, 2] and the latter substance is now frequently termed Folin's amino-acid reagent. Although amino-acids presumably result from digestion of food proteins and are the units essential to the synthesis of body proteins as well as being the first products of disintegrated tissues, their concentration and distribution in the body remains extraordinarily constant in health and disease and neither excessive intake of protein food or prolonged fasting appears to greatly disturb the amount present in the blood.

Method (*for Urine*)[1]. A standard solution of amino-acetic acid is required. This is made by adding 0·536 g. of the solid amino-acid and 2 g. of sodium benzoate to a litre flask containing 0·1N hydrochloric acid, and, when the solids have dissolved, diluting to 1 litre with more 0·1N hydrochloric acid. This solution will contain 0·1 mg. of amino-acid nitrogen per ml.

A sufficient quantity of the sample, say, from 5 to 25 ml. and expected to contain from 0·3 to 2 mg. of amino-acid nitrogen, is diluted to 25 ml. with water and transferred to an Erlenmeyer flask containing 3 g. of permutit powder (between No. 60 and 80 mesh). The mixture is agitated gently for 5 minutes, the supernatant liquid decanted into another flask containing a further 3 g. of permutit powder, and after shaking for another period of 5 minutes the liquid, now freed from ammonia, is poured off. To three test-tubes graduated at 25 ml. is added 1, 2 and 3 ml. respectively of the standard solution of amino-acetic acid and 1, 2 and 3 ml. of a 1·25 per cent. aqueous solution of sodium carbonate, Na_2CO_3. To another similar tube is added 5 ml. of the ammonia-free urine followed by 1 ml. of 0·1N hydrochloric acid and 1 ml. of the 1·25 per cent. sodium carbonate solution. The contents of the four tubes are each diluted to 10 ml., and 5 ml. of a freshly prepared 0·5 per cent. aqueous solution of sodium β-naphthoquinone-4-sulphonate is added. After the liquids have been mixed the tubes are set aside in the dark for about 15 minutes, then inspected. If the mixture in the tube containing the sample appears much darker than the strongest standard the test should be repeated, using a smaller volume of the original urine. After the tests have stood in the dark overnight they are rendered acid to phenolphthalein by the addition of

2·5 per cent. solution of sodium acetate, $NaC_2H_3O_2.3H_2O$, in a 25 per cent. aqueous solution of acetic acid, and then to each tube is added 5 ml. of a 4 per cent. aqueous solution of sodium thiosulphate, $Na_2S_2O_3.5H_2O$, with sufficient water to bring the volume of each test to 25 ml. The intensity of the yellowish-brown colour of the test solution is compared with that of the most suitable standard by means of a colorimeter.

(*for Blood*)[2]. A protein free blood filtrate is prepared by precipitation with tungstic acid as described on p. 220 and 5 ml. transferred to a test tube with a graduation mark at 15 ml. To a test tube with a graduation mark at 30 ml. is transferred 1 ml. of a standard solution of amino-acetic acid (made by diluting 70 ml. of the standard prescribed for the examination of urine to 100 ml. with 0·1N hydrochloric acid and hence containing 0·07 mg. of amino-acid nitrogen per ml.), and to each of the tubes is added 1 drop of phenolphthalein indicator solution. To the standard is added 1 ml. of the 1·25 per cent. solution of sodium carbonate and to the test mixture sufficient of the same alkali to produce a pink tint equal in intensity to that formed in the standard. An additional 5 ml. of water is then added to the standard mixture, followed by 2 ml. of a freshly prepared 0·5 per cent. aqueous solution of sodium β-naphthoquinone-4-sulphonate, and at the same time 1 ml. of the same reagent is added to the test liquid. After shaking and setting aside in a dark cupboard overnight, 2 ml. of the solution of sodium acetate in dilute acetic acid is introduced into the standard and 1 ml. into the test mixture, then 2 ml. of the sodium thiosulphate solution is added to the standard and 1 ml. to the test. Finally, the latter is diluted with water to 15 ml. and the former to 30 ml. and the relative intensity of the colours is compared in the usual manner.

Discussion. The sodium thiosulphate is included in order to decolorise the excess of sodium β-naphthoquinone-4-sulphonate. The concentration of amino-acid nitrogen in human blood lies between 5 and 8 mg. per 100 ml., while the tissues contain five or ten times this amount; between 1 and 2 per cent. of the total nitrogen of the urine is combined as amino-acids. As has already been observed, it might be expected that the concentration and distribution of such important substances would be readily affected by disease, but in fact acute yellow atrophy of the liver is the only disorder in which any notable alteration (values of 15 to 25 mg. per 100 ml. of blood) has been recorded. Thus it seems that the proper handling of these substances is of such vital

importance to the body that the capacities of the regulatory mechanisms, particularly the liver, are provided with a wide margin of safety. The importance of the analytical determinations lies in their application to physiological and pathological studies rather than to clinical diagnosis.

REFERENCES

1. O. Folin, *J. Biol. Chem.*, 1922, **51**, 393.
2. O. Folin, *J. Biol. Chem.*, 1922, **51**, 377.

BARBITURATES

A colorimetric method capable of giving approximately quantitative results has been developed on the basis of a test originated by Zwikker depending upon the production of a violet colour when an acid of the barbituric series is treated with a solution of anhydrous cobalt chloride in methyl alcohol followed by a solution of barium oxide in the same solvent[1]. Koppanyi and his co-workers recommend the use of cobalt acetate in place of cobalt chloride and an alkali consisting of a chloroformic solution of *iso*-propylamine[2, 3] and this method has been adapted by Levvy[4] to the determination of barbiturates in blood. In view of the toxicological significance of these compounds an application to muscular tissue is included below.

Method (*for Blood*)[4]. In a mortar of 250 ml. capacity, 20 ml. of the sample under examination (coagulated or citrated) is mixed with 2 g. of sodium dihydrogen phosphate ; then, in small portions at a time with constant grinding, is added 40 g. of anhydrous sodium sulphate. After thorough mixing of blood and sulphate the mortar is placed in a vacuum desiccator for 15 minutes in order to remove superficial moisture. The resulting dry cake is powdered, transferred to a Soxhlet thimble, covered with a plug of cotton wool and extracted for 3 hours with about 80 ml. of a mixed solvent consisting of equal volumes of peroxide-free ether and light petroleum (b. pt. below 40° C.), a few glass beads being introduced into the flask to facilitate steady boiling. When extraction is complete about 0·25 g. of a mixture consisting of 3 parts of powdered decolorising charcoal and 1 part of magnesium oxide is added to the resulting ether-petroleum solution

and then removed by filtering through a Jena sintered glass funnel directly into a distilling flask and washing with small quantities of the same mixed solvent. The combined filtrate and washings are distilled and the crystals of barbiturate which remain in the flask dissolved in chloroform, the solution transferred to a small stoppered measuring cylinder and diluted to 10 ml. with more chloroform. To 2 ml. of this solution is added 0·1 ml. of a 1 per cent. solution of cobalt acetate in methyl alcohol (AnalaR grade) followed by 0·6 ml. of a 5 per cent. v/v solution of *iso*-propylamine in methyl alcohol and the resulting violet colour is matched against a series of six standards prepared by applying the same test to chloroformic solutions containing from 0·4 to 2·4 mg. of the corresponding barbituric acid (not the sodium salt) in 2 ml.

(*for Urine*)[5]. It is generally sufficient to acidify the sample with dilute sulphuric acid and extract the barbituric acid by shaking with several portions of chloroform and to apply the colorimetric determination to the resulting chloroformic solution. Highly coloured and concentrated urine should be cleared by shaking an appropriate quantity with an equal volume of a 10 per cent. aqueous solution of copper sulphate after sufficient sodium or potassium hydroxide has been added to produce complete precipitation of the copper. The mixture is filtered, acidified with dilute sulphuric acid and the liberated barbituric acid extracted with chloroform.

(*for Tissues*)[5]. The weighed organs are ground with sufficient 5 per cent. sodium or potassium hydroxide solution to effect a good degree of liquefaction within 24 hours. An equal volume of a 10 per cent. aqueous solution of copper sulphate is added, the mixture well stirred, filtered, the filtrate acidified with dilute sulphuric acid, shaken with several successive portions of chloroform and the colour test applied to the resulting chloroformic extract.

As an alternative preliminary treatment, pepsin and a 3 per cent. aqueous solution of hydrochloric acid may be added to the finely divided organs, the mixture allowed to stand for 24 hours, then rendered alkaline with soda or potash, an equal volume of copper sulphate solution added and the determination completed as before.

A third method consists in acidifying a weighed sample by mixing with sodium dihydrogen phosphate, adding liquid air until the tissue is frozen then pulverising and extracting a con-

venient amount directly with chloroform using 10 ml. per g. of tissue. This procedure is satisfactory for any organ with the exception of the brain and spinal cord which, preferably, should be disintegrated with alkali. However, the latter method is not applicable to the determination of either hexobarbitone (Evipan) or ethyl-methyl-butyl-thiobarbituric acid (Pentothal) which are unstable in strongly alkaline solutions.

Discussion. In the examination of blood, and of tissues by the freezing method, the sodium dihydrogen phosphate is added in order to liberate the barbituric acid from combination as the sodium salt. Owing to the relative insolubility of the free acids, determinations cannot be conducted upon the filtrates from tungstic or trichloracetic acid protein precipitations. The peroxide-free ether, if not available by purchase, may be prepared from material of ordinary quality by washing with aqueous ferrous sulphate solution and water, then distilling into a bottle containing clean copper wire. The treatment of the extract with the charcoal-magnesium oxide mixture removes phospholipins which otherwise disturb the colour test: slight adsorption of the barbituric acid occurs during this process but no alternative procedure is available. To minimise this effect, the charcoal may be washed, first with peroxide-free ether containing a little glacial acetic acid, then with ether until free from acid. Standard solutions of barbituric acids may be stored for quite long periods of time in dark, glass-stoppered bottles. Theobromine, theophylline and thymine give colours under the conditions of the test but will not generally be present in specimens being examined for barbiturates.* In patients who had been receiving a daily dose for years Levvy found about 5 mg. per 100 ml. of blood[4].

The large number of alkyl derivatives of barbituric acid variously employed as sedatives, hypnotics or as anæsthetics and mostly marketed under proprietary names is apt to lead to confusion. In this connection Jespersen and Larsen have collected data concerning the melting point and solubility in water appertaining to many of the acids and suggest, as further means of identification, the melting point of the xanthydrol and the *p*-nitrobenzyl derivatives[6].

* In this connection the analyst should bear in mind that certain barbiturates employed as sedatives, phenobarbitone in particular, are administered in association with theobromine. A tablet of phenobarbitone and theobromine is included in the British Pharmaceutical Codex, 1934, and similar preparations are on the market under proprietary names, including Tab. Xantharbin, Theoba, Theogardenal Tablets, Theotone Tablets and Theominal Tablets.

The former is prepared by dissolving 1/400 mol. of the barbituric acid in 10 ml. of glacial acetic acid, adding 1/200 mol. of xanthydrol and boiling the mixture for a short time when the derivative usually settles out readily although, in some cases, it may be necessary to set aside for a while or to scratch the sides of the vessel with a glass rod.

To make the *p*-nitrobenzyl derivative 1/400 mol. of the barbituric acid is dissolved in 5 ml. of water with the addition of 1/800 mol. of sodium carbonate for every replaceable hydrogen atom. (If the compound is unknown, 0·5 g. of the acid and 1·08 g. of sodium carbonate, $Na_2CO_3.10H_2O$, are taken.) To the resulting solution 1/400 mol. of *p*-nitrobenzyl chloride is added for every replaceable hydrogen atom, the mixture is heated under a reflux condenser for 15 minutes using a boiling water-bath, then allowed to cool and filtered. The precipitate thus separated is washed with a little alcohol and water, shaken with 10 ml. of N sodium hydroxide, washed with water and finally purified by dissolving in chloroform and precipitating with alcohol. It should be observed that by this method sufficient *p*-nitrobenzyl chloride is used to permit of substitution of the two imido hydrogen atoms and also of the two methylene hydrogen atoms and that any mono-substituted compound containing free imido hydrogen is removed by treatment with sodium hydroxide.

The data relevant to these derivatives, as given by Jesperson and Larsen, is presented in Table XII which also contains the trade names and chemical identity of a selection of barbiturates which have found application in medical practice.

References

1. J. J. L. Zwikker, *Pharm. Weekblad*, 1931, **68**, 975.
2. T. Koppanyi, W. S. Murphy and S. Krop, *Proc. Expt. Biol. Med.*, 1933, **31**, 373.
3. J. M. Dille and T. Koppanyi, *J. Amer. Pharm. Assoc.*, 1934, **23**, 1079.
4. G. A. Levvy, *Biochem. J.*, 1940, **34**, 73.
5. T. Koppanyi, J. M. Dille, W. S. Murphy and S. Krop, *J. Amer. Pharm. Assoc.*, 1934, **23**, 1074.
6. J. C. Jesperson and K. T. Larsen, *Arch. Pharm.*, 1937, **275**, 28.

TABLE XII. TRADE NAMES OF BARBITURIC ACIDS: TOGETHER WITH PHYSICAL DATA AFTER J. C. JESPERSON AND K. T. LARSEN (*Arch. Pharm.*, 1937, **275**, 28)

Proprietary Name	Chemical Name	Melting Point, °C.			Solubility of Free Acid in Water, g. per kg.	
		Acid	Xanthydrol derivative	*p*-Nitrobenzyl derivative	At 20° C.	At 37° C.
Alurate	5-allyl-5-*iso*propyl-barbituric acid	139·5	226·5	192·0	3·40	5·60
Amytal	5-ethyl-5-*iso*amyl-barbituric acid	141·0	251·0	145·5	0·64	0·99
*Barbital	5:5-diethyl-barbituric acid	188·5	246·5	193·5	6·29	9·49
Butisol	5-ethyl-5-*sec*butyl-barbituric acid	—	—	—	—	—
Butobarbital	5-ethyl-5-butyl-barbituric acid	—	—	—	—	—
Cyclonal	Same as Evipan					
Cyclopal	Same as Cyclopen					
Cyclopen	5-allyl-5-*cyclo*pentenyl-barbituric acid	—	—	—	—	—
Delvinal	5-ethyl-5-(1-methyl-1-butenyl)-barbituric acid	—	—	—	—	—
†Dial	5,5-diallyl-barbituric acid	171·5	242·5	192·5	1·25	2·53
Dormin	5-ethyl-5-allyl-barbituric acid	159·4	242·0	196·3	4·02	6·55
Eldoral	5-ethyl-5-(1-piperidyl)-barbituric acid	—	—	—	—	—
Etoval	Same as Butobarbital					
Eunarcon	5-(2-bromallyl)-5-*iso*propyl-1-methyl-barbituric acid	—	—	—	—	—
Evipal	Same as Evipan					
‡Evipan	1:5-dimethyl-5-(1-*cyclo*hexenyl)-barbituric acid	143·9	—	114·5	0·29	0·64
Gardenal	Same as Luminal					

Heberal	Same as Ortal					
Hexanastab	Same as sodium salt of Evipan					
Idobutal	5-butyl-5-allyl-barbituric acid	126·1	240·0	127·5	1·51	2·01
Ipral	5-ethyl-5-*iso*propyl-barbituric acid	—	—	—	—	—
Isonal	Same as Alurate					
§Luminal	5-ethyl-5-phenyl-barbituric acid	174·9	219·0	183·5	0·88	1·84
Malonal	Same as Barbital					
Mebaral	Same as Prominal					
Medinal	Same as sodium salt of Barbital					
Methexenyl sodium	Same as sodium salt of Evipan					
Narconumal	5-allyl-5-*iso*propyl-1-methyl-barbituric acid (sodium salt)	—	—	—	—	—
Nembutal	Same as sodium salt of Pentobarbital					
Neonal	Same as Butobarbital					
Noctal	5-*iso*propyl-5-(2-bromallyl)-barbituric acid	—	—	200·5	—	—
Nostal	Same as Noctal					
Ortal	5-ethyl-5-hexyl-barbituric acid	—	—	—	—	—
Pentenal	5-ethyl-5-*cyclo*pentenyl-barbituric acid	—	—	—	—	—
‖Pentobarbital	5-ethyl-5-(1-methylbutyl)-barbituric acid	—	—	—	—	—
Pentothal	5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid	—	—	—	—	—
Pernocton	5-*sec*butyl-5-(2-bromallyl)-barbituric acid	—	—	191·5	—	—
Pernoston	Same as Pernocton					
Phanodorm	5-ethyl-5-(1-*cyclo*hexenyl)-barbituric acid	176·4	257·0	196·0	1·64	2·25
Phanodorn	Same as Phanodorm					
Phenobarbital	Same as Luminal					
¶Prominal	1-methyl-5-phenyl-5-ethyl-barbituric acid	173·2	—	114·5	0·15	0·12
Proponal	5;5-dipropyl-barbituric acid	146·5	269·0	182·3	0·63	1·08
Rectidon	5-(2-bromallyl)-5-(1-methylbutyl)-barbituric acid	—	—	—	—	—
Rutonal	5-methyl-5-phenyl-barbituric acid	233·7	282·0	197·0	0·76	1·34
Sandoptal	5-allyl-5-*iso*butyl-barbituric acid	135·9	—	—	—	—
Seconal	5-allyl-5-(1-methylbutyl) barbituric acid (sodium salt)	—	—	—	—	—
Sigmodal	5-amyl-5-(2-bromallyl)-barbituric acid	—	—	—	—	—
Somonal	Same as Luminal					

TABLE XII. TRADE NAMES OF BARBITURIC ACIDS: TOGETHER WITH PHYSICAL DATA AFTER J. C. JESPERSON AND K. T. LARSEN (*Arch. Pharm.*, 1937, **275**, 28)—*Continued*

Proprietary Name	Chemical Name	Melting Point, °C.			Solubility of Free Acid in Water, g. per kg.	
		Acid	Xanthydrol derivative	*p*-Nitrobenzyl derivative	At 20° C.	At 37° C.
Soneryl	Same as Butobarbital					
Veronal	Same as Barbital					
—	5-methyl-barbituric acid	206·6	—	208·0	—	—
—	5-ethyl-barbituric acid	191·5	—	213·5	—	—
—	5-*iso*propyl-barbituric acid	216·7	207·5	189·0	5·96	10·15
—	5-ethyl-5-butyl-barbituric acid	122·5	250·0	148·5	3·15	4·88
—	5-allyl-5-phenyl-barbituric acid	154·9	222·5	152·0	1·10	1·90

Notes. Most of the above proprietary names refer to the sparingly soluble acids: in the majority of cases the water-soluble salts, when issued, are indicated by adding the word " Sodium " either before or after the registered name.

Official names have been given to members of the series marked, as under :—

* Barbitonum (Barbitone) British Pharmacopœia, 1932. Sodium salt is called Barbitonum Solubile.

† Allobarbitonum (Allobarbitone) British Pharmaceutical Codex, 1934.

‡ Hexobarbitonum (Hexobarbitone) B.P., 1932, Third Addendum, 1941. Sodium salt is called Hexobarbitonum Solubile.

§ Phenobarbitonum (Phenobarbitone) British Pharmacopœia, 1932. Sodium salt is called Phenobarbitonum Solubile.

‖ Pentobarbitalum Sodicum (Pentobarbital Sodium), United States Pharmacopœia XII (1942). This is the sodium salt of 5-ethyl-5-(1-methylbutyl)-barbituric acid.

¶ Phemitonum (Phemitone) B.P., 1932, Third Addendum, 1941.

BILIRUBIN (IN BLOOD PLASMA OR SERUM)

In 1914 A. A. Hijmans van den Bergh and his collaborators[1, 2] commenced an investigation on a method for assessing the efficiency of hepatic function depending on the measurement of the colour produced by the addition of *p*-sulphophenyldiazonium chloride (Ehrlich's Diazo Reagent) to the blood plasma. The test is applied in two ways which are referred to as the " direct qualitative " and the " indirect quantitative " reactions. That the significance of the double technique may be understood it is necessary to allude briefly to the conceptions now entertained as to the causes underlying the various types of jaundice. The bile pigments, bilirubin and biliverdin, are formed from hæmoglobin derived from disintegrated blood cells and are converted by bacteria in the intestine into urobilin and its colourless precursor, urobilinogen. It is considered that hæmoglobin in the blood stream is converted into bile in the Kupffer cells of the liver whence it is normally passed on through the polygonal cells to the lumen of the bile capillaries. According to this theory there are three kinds of jaundice, caused, in the one case, by defects in the capillary ducts which prevent the natural escape of the bile (obstructive), or, secondly, failure of the polygonal cells to transmit the pigment from the Kupffer cells to the capillaries with the result that it passes directly into the blood stream (hæmolytic) and, finally, by a combination of obstruction and direct absorption (infective hepatic). The complete van den Bergh test is capable of differentiating between bilirubin which has passed through the polygonal cells and pigment absorbed directly into the blood stream from the Kupffer cells.

Method[3]. The following special solutions are required :—

1. Ehrlich's Diazo Reagent, made by dissolving 1 g. of sulphanilic acid in 15 ml. of concentrated hydrochloric acid, diluting with water to 1 litre and, just before use, mixing 10 ml. of the resulting solution with 0·3 ml. of a freshly prepared 0·5 per cent. aqueous solution of sodium nitrite.

2. An Artificial Colour Standard consisting of a 2·161 per cent. aqueous solution of cobaltous sulphate, $CoSO_4$. The colour of this solution corresponds with that due to 1 in 200,000 bilirubin (0·5 mg. per 100 ml. or 1 van den Bergh unit). If stored in the dark the solution is stable for several weeks.

Plasma is prepared by collecting about 10 ml. of blood in an

" oxalated " tube (made by introducing 0·2 ml. of a 10 per cent. aqueous solution of potassium oxalate and evaporating to dryness in the course of sterilisation), centrifuging and pipetting off the clear supernatant fluid.

Direct Qualitative Test. Into each of three small tubes 0·25 ml. of the plasma is introduced and to one of them, the control, 0·2 ml. of water is added. To another of the tubes is added a small flake of caffeine sodium salicylate and after the salt has dissolved 0·2 ml. of Ehrlich's diazo reagent is mixed in when, owing to the presence of the caffeine salt, a prompt and definite reaction takes place as shown by the production of a purple tint. When this colour has attained its maximum, 0·2 ml. of the diazo reagent is added to the actual test sample in the third tube and any alteration in colour is compared with the fully developed reaction in the tube containing caffeine. In the test sample one of three possible phenomena may take place :—

An immediate direct reaction occurs in which the development of colour begins at once and attains maximum intensity in 10 to 30 seconds : the depth of the bluish-violet colour varies with the concentration of bilirubin. Alternatively, a direct delayed reaction takes place in which a reddish colour, deepening to violet, begins to appear in about 15 minutes. Finally, the response may correspond with a direct biphasic reaction in which a reddish colour appears immediately and takes much longer to deepen into a violet hue.

Indirect Quantitative Test. To 1 ml. of the plasma contained in a centrifuge tube is added 0·5 ml. of the diazo reagent and, after mixing and standing for 1 minute, 2·5 ml. of alcohol (absolute or 96 per cent.) and 1 ml. of a saturated aqueous solution of ammonium sulphate are added. After allowing the precipitated protein material to flocculate, the mixture is centrifuged and the colour of the supernatant fluid compared with that of standards prepared from the cobaltous sulphate solution and ranging from 0·1 to 1·0 unit. The colour of the cobalt solution prepared as directed above is equivalent to the latter value and the weaker standards are set up by making the appropriate dilutions. If the supernatant fluid in the test is deeper in colour than the 1·0 unit cobalt standard it should be suitably diluted with alcohol (67 per cent., i.e. 2 vol. of absolute alcohol mixed with 1 vol. of water) until a suitable match can be obtained. The dilution of the plasma in the preparation of the supernatant fluid is 1 to 4, and not 1 to 5, because the saturated aqueous solution of ammonium

sulphate remains as a separate layer (1 ml.) and does not contain azobilirubin.

Method Using Permanent External Standards : *Indirect Quantitative Test.* A disc containing nine glass standard colours ranging from 0·2 to 2·0 mg. bilirubin per 100 ml. (0·4 to 4·0 van den Bergh units) is available for use with the Lovibond Comparator. The technique used in connection with this permanent standard is that recommended by McNee and Keefer[3] and is identical with that described above.

Discussion. The test may equally well be performed on serum but whether plasma or serum is used it is most important that there should be no visible hæmolysis and, in addition, the test should be conducted within about 2 hours of taking the blood. According to Vaughan and Haslewood[4] the normal concentration of bilirubin in the plasma lies between 0·2 and 1·7 mg. per 100 ml.

An immediate direct reaction indicates obstructive jaundice, whilst a delayed direct reaction points to the hæmolytic variety. The biphasic reaction is found in cases of infective jaundice, where both obstructive and non-obstructive processes are at work. All samples which yield a positive direct reaction also give a positive indirect reaction but the reverse does not necessarily hold true. In a marked case of non-obstructive jaundice there may be as much as 12 units of bilirubin present in the plasma. The renal threshold value of bilirubin has also been the subject of investigations and it is considered by some authorities that bile does not appear in the urine until at least 4 units are present in the blood but in hæmolytic jaundice it is quite possible to have between 5 and 18 units present in the blood with no bile in the urine[5]. The recognition of latent jaundice in which there is slight icterus but not sufficient bile to be apparent in the urine constitutes one of the most important developments of this test ; cases of pernicious anæmia are usually associated with this group of disorders.

An alternative procedure for conducting the indirect quantitative test has been recommended by King, Haslewood and Delory[6] which may be followed by observing the undermentioned modifications of the technique already given. The sulphanilic acid solution is prepared by dissolving 1 g. in 250 ml. of N hydrochloric acid and diluting to 1 litre with water. The artificial standard is made by mixing exactly 1 ml. of a 0·29 per cent. solution of methyl red in glacial acetic acid with 5 ml. of the same solvent, mixing with 14·4 g. of hydrated sodium acetate and

diluting to 1 litre with water. This standard solution has a reaction equivalent to *p*H 4·63, contains 2·9 mg. of methyl red per litre and matches the colour obtained when 0·1 mg. of bilirubin is treated with diazo reagent in a final volume of 25 ml. The actual test is applied by treating 1 ml. of plasma or serum with 0·5 ml. of diazo reagent, 0·5 ml. of a saturated aqueous solution of ammonium sulphate and finally 3 ml. of absolute alcohol. After shaking and allowing to stand a few minutes the mixture is filtered and the colour of the filtrate matched against the standards. Under these conditions the dilution of the plasma closely approximates to 1 to 4, allowance being made for the volume of the precipitate and for the change in volume when alcohol is added to water.

It is frequently found that brownish or purple tints produced in the reaction make colorimetric comparison with the artificial standards difficult. These extraneous colours (probably due to traces of substances other than bilirubin which react with the diazo reagent) may be largely eliminated by the use of a green light filter.

REFERENCES

1. A. A. Hijmans van den Bergh and J. J. de la Fontaine Schluiter, *Proc. K. Akad. Wetensch. Amsterdam*, 1914, **17**, 807.
2. A. A. Hijmans van den Bergh and P. Muller, *Biochem. Z.*, 1916, **77**, 90.
3. J. W. McNee and C. S. Keefer, *Brit. Med. J.*, 1925, ii, 52.
4. J. Vaughan and G. A. D. Haslewood, *Lancet*, 1938, **234**, 133.
5. G. E. Beaumont and E. C. Dodds, "Recent Advances in Medicine," 10th Ed., London: J. & A. Churchill, Ltd., 1941, p. 154.
6. E. J. King, G. A. D. Haslewood and G. E. Delory, *Lancet*, 1937, **232**, 886.

BROMIDES (IN CEREBROSPINAL FLUID AND BLOOD)

Proposals for the colorimetric determination of bromides include measurement of the colour of bromine in aqueous solution after liberation with chlorine water[1], or a modification in which the element is extracted with carbon tetrachloride[2], while other procedures are based upon the action of bromine in restoring the colour of a solution of basic fuchsin in sulphuric acid[3–5] or in sulphurous acid[6]. Where a high degree of sensitivity is required the method originally proposed by Baubigny[7] in which the liberated bromine is allowed to react with an excess of fluorescein to form eosin is more satisfactory and has been discussed by several

investigators[8–14] while the less sensitive, but extremely simple, procedure depending upon the addition of gold chloride to the solution containing bromide and measurement of the brownish-yellow colour produced is also of value[15–18]. Both procedures have been applied to the examination of body fluids for the purpose of detecting bromide intoxication and it is in this connection that the latter two methods are described. In general practice, volumetric methods are to be preferred to any of the known colorimetric procedures.

Method Using Fluorescein (*Cerebrospinal Fluid*)[14]. A suitable quantity of the sample (1 or 2 ml.) is transferred to a stout glass tube of approximately 7 ml. capacity, 2 ml. of N sodium ethoxide (prepared from sodium and alcohol which has been redistilled from sodium hydroxide) added, the tube sealed with a minimum of air space above the surface of the liquid and immersed in a boiling water-bath for 1 hour. After allowing to cool, the tube is opened and the contents transferred to a nickel dish, 10 ml. of water being used for rinsing. To this liquid is added 3 ml. of N potassium hydroxide, 0·1 ml. of a 20 per cent. aqueous solution of sucrose and the mixture well stirred with a glass rod, the rod being rinsed with a little water and removed. The solution is evaporated to dryness on a boiling water-bath, the dish and its contents placed in an oven at 150° to 160° C. for an hour and then ignited in a muffle furnace at 480° to 500° C. for a further period of 1 hour. To the residue is added 20 ml. of water, the carbon broken with a glass rod and after digestion on a boiling water-bath for a few minutes the liquid is filtered through a freshly washed 9-cm. Whatman No. 5 paper. The charred residue is extracted with an additional 20 ml. of hot water the filtrate being combined with that from the initial extractions. The filter paper is returned to the nickel dish, the contents of the latter treated with 1 ml. of N potassium hydroxide, the mixture evaporated on a water-bath, dried at 150° to 160° C. then ignited in a muffle furnace as before and finally again extracted with hot water, the filtrate being combined with that from the previous extraction. The filtered liquid is transferred to a platinum dish, evaporated to dryness, the residue dried at 150° to 160° C. then ignited in a muffle furnace at 480° to 500° C. The residue is then moistened with a little water, the mixture evaporated, dried and again ignited. The alkaline residue is dissolved in a very little water, the solution neutralised with 2N sulphuric acid and diluted to 10 ml. with water. Portions of this solution, varying from 0·5 to

2 ml., are transferred to test tubes each containing 0·5 ml. of a *p*H 5·5 buffer solution (prepared by mixing 100 ml. of N sodium acetate with 15 ml. of N acetic acid) and to each is added 0·1 ml. of a 0·1 per cent. solution of fluorescein in 0·025N sodium hydroxide followed by sufficient water to produce 3 ml. After stirring, 0·05 ml. of a 1 per cent. aqueous solution of chloramine T is added, the test liquids well agitated and, after 1 minute, the reaction stopped by adding 0·1 ml. of a 5 per cent. aqueous solution of sodium hydroxide containing 0·5 per cent. of sodium thiosulphate. The pink colours so produced are matched against a series of standards prepared at the same time from 0·1 to 2 ml. of a standard solution containing 0·01 mg. Br per ml. (0·0149 g. KBr per litre).

Method Using Gold Chloride (*Blood*)[17]. A suitable quantity of venous blood is drawn, allowed to coagulate and to 2 ml. of the serum is added 4 ml. of water and 1·2 ml. of a 20 per cent. aqueous solution of trichloracetic acid. After shaking and allowing to stand for 10 minutes the mixture is filtered through a 7-cm. Whatman No. 1 paper, 2 ml. of the filtrate transferred to a test tube and 0·4 ml. of a 0·5 per cent. aqueous solution of gold sodium chloride, $AuCl_3.NaCl.2H_2O$, added. Any yellowish-brown colour produced is matched against standards. The latter are prepared by diluting appropriate quantities of a 0·145 per cent. aqueous solution of sodium bromide to 10 ml. with water within a range from 0·5 ml. (equivalent to 25 mg. NaBr per 100 ml. of blood) to 7·5 ml. (equivalent to 375 mg. NaBr per 100 ml. of blood) in steps of 0·5 ml. To 5 ml. of each of these standard dilutions is added 1 ml. of 20 per cent. trichloracetic acid and 1 ml. of the gold sodium chloride solution. These standards are so arranged that direct comparison gives mg. NaBr per cent. in the serum treated as described. The concentration of the sodium bromide solution from which they are made is corrected in order to eliminate the error due to absorption of bromide in the precipitation of protein from serum.

Discussion. The technique given above for the fluorescein method is due to P. J. Hardwick[14]. The somewhat complicated procedure used for the destruction of the organic matter of the sample is adopted as a precaution against loss of volatile bromine compounds whence the method is suitable for the investigation of suspected poisoning by substances such as methyl bromide. The colour test itself is quite simple and since it is not disturbed by the presence of chlorides it should prove useful in a variety of

connections. The gold chloride test is less precise but is generally regarded as sufficiently accurate for clinical purposes. The normal level of blood bromide is 0·5 to 2·5 mg. per 100 ml. but where bromides are being freely administered as medicine the value may rise to 300 mg. or more and if high levels are maintained for long periods of time toxic symptoms are liable to ensue. The procedure described here was adopted by Barbour, Pilkington and Sargant who give a detailed report of an extensive clinical study which suggests that bromide intoxication may well be of common occurrence[19].

REFERENCES

1. W. J. Dibdin and L. H. Cooper, *Analyst*, 1910, **35**, 159.
2. O. R. Sweeney and J. R. Withrow, *J. Ind. Eng. Chem.*, 1917, **9**, 674.
3. J. Frezouls, *Ann. Falsificat.*, 1934, **27** 351.
4. R. Indovina, *Biochem. Z.*, 1935, **275**, 286.
5. R. C. Lopez, *Farm. moderna*, 1935, **46**, 55.
6. E. Oppenheimer, *Arch. Exptl. Path. Pharmacol.*, 1921, **89**, 17.
7. H. Baubigny, *Compt. rend.*, 1897, **125**, 654.
8. D. Ganassini, *Boll. Chim. Farm.*, 1904, **43**, 153.
9. A. Labat, *Bull. Soc. chim.*, 1911, **9**, 393.
10. R. Lorenz, E. Grau and E. Bergheimer, *Z. anorg. Chem.*, 1924, **136**, 90.
11. H. Zondek and A. Bier, *Klin. Wochenschr.*, 1932, **11**, 633.
12. F. Hahn, *Compt. rend.*, 1933, **197**, 245.
13. H. Ucko, *Biochem. J.*, 1936, **30**, 992.
14. P. J. Hardwick, *Analyst*, 1942, **67**, 223.
15. F. K. Walter, *Z. Ges. Neurol.*, 1919, **47**, 380.
16. A. Hauptmann, *Z. Neurol. Psychiat.*, 1926, **100**, 332.
17. O. Wuth, *J. Amer. Med. Assoc.*, 1927, **88**, 2013.
18. W. A. Taylor, *J. Lab. Clin. Med.*, 1927, **13**, 495.
19. R. F. Barbour, F. Pilkington and W. Sargant, *Brit. Med. J.*, 1936, ii, 957.

CHOLESTEROL (IN BLOOD)

The most successful method for the colorimetric determination of cholesterol is based upon the Liebermann-Burchard reaction in which a green colour is produced on the addition of acetic anhydride and sulphuric acid to a chloroformic solution. In the procedure recommended by Myers and Wardell[1] the sample of whole blood, plasma or serum is mixed with calcium sulphate (hemihydrate), the cholesterol removed by continuous extraction for 90 minutes with chloroform and the colour test applied to a suitable aliquot portion of the resulting solution. Reinhold[2] used anhydrous sodium sulphate in place of calcium sulphate and described a neat all-glass extraction apparatus. The method given below, which is a simplification of Bloor's[3] procedure as

recommended by G. E. Sackett[4], avoids the somewhat troublesome continuous extraction process, the cholesterol being removed directly from the blood by mixing the sample with alcohol and ether.

Method[4]. Exactly 9 ml. of alcohol (95 per cent.) and 3 ml. of ether are introduced into a centrifuge tube and 0·2 ml. of the sample of whole blood, or plasma, added. The tube is stoppered and, after being well shaken, is allowed to lie in a horizontal position for 30 minutes. The mixture is then centrifuged for about 3 minutes, the clear supernatant liquid decanted into a small beaker, and the latter placed on a hot water-bath until the solvent has just evaporated. The dry residue is treated with four 1-ml. portions of chloroform, each extract being transferred to a tube graduated at 5 ml. The mixed extracts are diluted to 5 ml. with more chloroform, 2 ml. of acetic anhydride added and, after mixing, 0·1 ml. of concentrated sulphuric acid is introduced. The tube is stoppered, the contents mixed and allowed to stand in the dark for 10 minutes. The intensity of the colour produced is at once compared in a colorimeter with a standard prepared by similarly treating 5 ml. of chloroform containing 0·4 mg. of cholesterol. The standard solution of cholesterol is conveniently made by diluting 8 ml. of a 0·1 per cent. chloroformic stock solution of anhydrous cholesterol to 100 ml. with the same solvent. It should be noted that cholesterol in the form of notched plates (crystallised from 90 per cent. alcohol) is the monohydrate and if this product is employed 0·105 g. should be taken in place of 0·1 g. of the anhydrous variety.

Method Using Permanent External Standards. A disc containing nine glasses and covering a range from 80 to 500 mg. cholesterol per 100 ml. of blood is available for use with the Lovibond Comparator. The standards are designed for use in conjunction with the above technique. If these permanent standards are used, a blank consisting of 5 ml. of chloroform, 2 ml. of acetic anhydride and 0·1 ml. of concentrated sulphuric acid should be introduced into the left-hand tube of the instrument.

Discussion. The blood of normal subjects contains between 110 and 240 mg. of total cholesterol per 100 ml., about one-third being present as free cholesterol while the remainder, mainly in the plasma, is combined as esters. Total cholesterol is determined by the ordinary colorimetric procedures and for most purposes this provides adequate information. A microchemical method for differentiating the two forms has been worked out by

Schoenheimer and Sperry[5] depending upon a special application of the colour test together with a gravimetric determination as cholesteryl digitonide. High cholesterol values are encountered in nephritis (particularly the nephrotic type), diabetes, biliary obstruction and xanthomatosis, while hypocholesterolæmia occurs in severe anæmia (including some cases of the pernicious variety), cachexias and serious forms of sepsis. Determinations of cholesterol may be of considerable interest in special investigations but, in the present state of our knowledge, the results do not possess important clinical significance since, usually, abnormal values are only obtained in disorders which have advanced sufficiently to exhibit more obvious evidence of their identity.

REFERENCES

1. V. C. Myers and E. L. Wardell, *J. Biol. Chem.*, 1918, **36**, 147.
2. J. G. Reinhold, *Amer. J. Clin. Path.*, 1936, **6**, 22.
3. W. R. Bloor, *J. Biol. Chem.*, 1916, **24**, 227.
4. G. E. Sackett, *J. Biol. Chem.*, 1925, **64**, 203.
5. R. Schoenheimer and W. P. Sperry, *J. Biol. Chem.*, 1934, **106**, 745.

COLLOIDAL GOLD REACTION (ON CEREBROSPINAL FLUID)

Carl Lange[1] observed that, whereas normal cerebrospinal fluid protects a solution of colloidal gold from precipitation with an electrolyte the fluid derived from patients suffering from certain diseases of the central nervous system lacks this protective property. By noting the effect of adding gold sol to serial dilutions of cerebrospinal fluid in which sodium chloride has been incorporated it is possible to differentiate between general paralysis, general syphilis of the nervous system and acute meningitis.

Method. It is most important that the distilled water used for preparing the reagents listed below should be freshly redistilled from an all-glass apparatus free from rubber connections. The first three solutions will keep well but the gold sol should be made on the same day that the test is conducted.

1. Saline Solution containing 0·4 per cent. of sodium chloride.
2. A 1 per cent. aqueous Solution of Potassium Oxalate (neutral).
3. A 1 per cent. aqueous Solution of Gold Sodium Chloride, $AuCl_3.NaCl.2H_2O$.

4. Gold Sol prepared by adding to 100 ml. of redistilled water contained in a chemically clean Pyrex glass flask 1 ml. of the potassium oxalate solution, heating the liquid to the boiling point, and, after maintaining at this temperature for 2 minutes adding 1 ml. of the gold sodium chloride solution, at first drop by drop until the colour appears, then the remainder at once, and finally allowing the preparation to cool. The liquid should be bright cherry red in colour with only a very slight fluorescence.

A series of eleven test tubes, which have been cleaned in boiling dilute hydrochloric acid, rinsed in distilled water and dried in an oven at about 150° C., are set out in a rack and 0·9 ml. of saline solution introduced into the first and 0·5 ml. of the same substance added to the other tubes. Using a clean dry pipette, 0·1 ml. of the cerebrospinal fluid under examination is mixed with the saline in the first tube, 0·5 ml. of this mixture is transferred to the second and, after mixing, 0·5 ml. of this is transferred to the third tube, and so on until the tenth tube is reached from which 0·5 ml. of fluid is taken and discarded. Nothing is added to the eleventh tube the contents of which constitute the control. In this way dilutions of cerebrospinal fluid are made in a geometrical series in which the first term is 1 in 10 and the last 1 in 5120. To every tube, including the control, is added 2·5 ml. of the gold sol, the contents mixed and the tubes set aside for 12 to 24 hours. Starting with the first tube, the colour of the series is expressed by a succession of cyphers: 0 indicating that no change from the bright cherry red has occurred; 1, that a slight change to deeper red has taken place; 2, that the colour is lilac to purple; 3, signifying deep blue; 4, corresponding with a light blue liquid containing a purplish precipitate; and 5, implying that a heavy bluish precipitate has formed and that the supernatant fluid has become colourless. These changes simply represent increasing precipitation of the gold from the colloidal solution and the results are interpreted in accordance with the following average findings:—

Normal response	00000000000
Paretic response (general paralysis)	55554331000
Luetic response (tabes dorsalis and cerebrospinal syphilis)	01231000000
Meningitic response (acute meningitis)	00001344300

Method Using Permanent External Standards. A disc for use with the Lovibond Comparator is available which is fitted with four glasses to check the colour of the gold sol before commencing the test and five to determine the degree of precipitation. The technique recommended for use with this disc is precisely the same as described above.

Discussion. Occasionally it is found that a deposit of metallic gold occurs in tubes preceding a 5 or 4 and there is a resultant loss of colour in the supernatant fluid which is reddish or purplish. Such tubes may be assessed by considering the degree of colour lost and the amount of precipitate rather than the actual hue of the upper fluid. The only difficulty in the application of this test lies in the preparation of the gold sol : if it is too acid it may be precipitated even by normal cerebrospinal fluid while a slight excess of alkali will depress the sensitivity of its response to pathological specimens. It is important to avoid the inadvertent use of the compound normally sold as " gold chloride " which is $AuCl_3.HCl.3H_2O$ and produces a strongly acid solution. If there is doubt as to the sensitivity of the sol it is advisable to check it against normal and known syphilitic cerebrospinal fluids. The method of preparation given above is due to Mellanby and Anwyl-Davies[2] and, if the instructions are carefully carried out, no difficulty should be encountered.

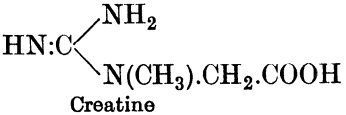
Lange has claimed that the colloidal gold reaction is more sensitive than the Wassermann test, a view which has been confirmed by some investigators. The value of the method for the diagnosis of syphilitic lesions of the central nervous system is generally acknowledged, but its utility in cases of meningitis is not so certain[3]. According to Greenfield and Carmichael[4] if the Wassermann reaction is negative in both blood and cerebrospinal fluid a paretic response in the colloidal gold test is indicative of disseminated sclerosis, while the persistence of the same response during periods of extended antisyphilitic treatment strongly suggests a diagnosis of general paralysis.

REFERENCES

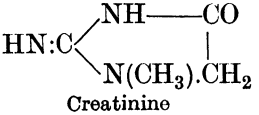
1. C. Lange, *Z. Chemotherap.*, 1912, **1**, 44.
2. J. Mellanby and T. Anwyl-Davies, *Brit. J. Expt. Path.*, 1923, **4**, 132.
3. J. Cruickshank, *Brit. J. Expt. Path.*, 1920, **1**, 71.
4. J. G. Greenfield and E. A. Carmichael, *J. Neurol. Psychopath.*, 1927, **7**, 220.

CREATINE AND CREATININE
(IN TISSUES, URINE, BLOOD AND MEAT EXTRACTS)

Creatine is methyl guanidine acetic acid, creatinine being its anhydride.



Creatine



Creatinine

Creatine and creatinine occur in the muscular tissues and to a lesser extent in most of the organs of vertebrates and both substances are also found in blood and urine. In the laboratory creatine is readily converted into creatinine by heating with dilute mineral acid and a similar change occurs in the living organism. The question of the origin and significance of these two substances in the biological economy has been the subject of extensive biochemical research but the mode of their formation and their function still remains obscure. The analytical determination of these two substances is of some importance and is generally effected colorimetrically by measuring the yellowish-red colour produced by the action of creatinine on alkaline picrate solution. This test was originated for qualitative use by M. Jaffe[1] and first adapted quantitatively by Folin[2]. The colour produced is due to reduction of alkaline picrate by creatinine : creatine does not react and it is determined by taking the difference between the creatinine originally present and that formed after treatment of samples with hot dilute hydrochloric acid. According to A. Chaston Chapman[3] reduction of picric acid results in the formation of a mixture of dinitromonoaminophenol, mononitrodiaminophenol (picramic acid) and triaminophenol but, latterly, some doubt has been cast upon these conclusions although the identity of the products of the reaction have not been certainly established[4–7]. In any case, it should be emphasised that the reaction is not specific for creatinine since a similar coloration is produced more or less readily by most reducing agents including dextrose, lævulose, maltose, acetone and hydroxylamine, all of which react in the cold, while formaldehyde and urea give the colour when the reaction mixture is warmed[3]. Folin originally employed a solution of potassium dichromate as an artificial standard but subsequently recommended the use of creatinine solutions of known concentration[8–9] and this practice is now

generally adopted. Procedures are given below for the determination of creatine in animal tissues, in urine, in blood and in meat extracts.

Method for Animal Tissues[10]. An alkaline picrate reagent and standard creatinine solution are required :—

1. The Alkaline Picrate Solution is made by mixing 5 vols. of a saturated aqueous solution of pure picric acid (about 1·2 per cent. w/v) with 1 vol. of a 10 per cent. w/v aqueous solution of sodium hydroxide. The solutions of acid and alkali should be stocked separately and the mixture freshly made just before use.

2. A Creatinine Stock Standard, containing 1 mg. creatinine per ml., is prepared by dissolving 1·602 g. of creatinine zinc chloride in 0·1 N hydrochloric acid and diluting with the same solvent to 1 litre.

3. A Diluted Standard, containing 0·005 mg. per ml., made by treating 5 ml. of the stock standard with 10 ml. of 0·1N hydrochloric acid and diluting with water to 1 litre.

Tissues, or organs, should be removed as promptly as possible after the death of the animal. If abundant material is available it should be run through a meat chopper in order to produce a uniform sample but in the case of small organs it will generally be sufficient to cut into small pieces with scissors. Portions weighing approximately 1 g. (or if the creatinine content is high it may be necessary to operate on smaller quantities) are transferred to tared 50-ml. glass-stoppered Erlenmeyer flasks which are at once closed to prevent evaporation of moisture and again weighed. The material in each flask is treated with 20 ml. of 2N sulphuric acid, the vessels are covered with tin foil and heated for 45 minutes in an autoclave at 15 lb. pressure. After cooling, each solution is transferred to a 100-ml. graduated flask with the aid of 40 to 50 ml. of water and 18 ml. of 2N sodium hydroxide added, followed by 5 ml. of a 10 per cent. aqueous solution of sodium tungstate, $Na_2WO_4.2H_2O$. The mixture, which should now be faintly acid to Congo red indicator, is diluted to the mark with water and well shaken. After allowing to stand for 5 minutes the solution is filtered and 10 ml. of the filtrate treated with 5 ml. of alkaline picrate solution. Alternatively, if the colour is too deep for satisfactory matching, suitable quantities of the filtrate should be diluted to 10 ml. with water and treated with 5 ml. of alkaline picrate solution. As nearly as possible at the same

time, suitable quantities of the diluted standard solution (say from 1 ml. upwards) are diluted to 10 ml. with water and mixed with 5 ml. of the alkaline picrate solution. After standing for 10 minutes in order to permit the colours to develop fully the sample is matched against a series of standards by direct visual observation or, alternatively, is compared with a standard of closely similar intensity by means of a colorimeter.

Discussion. The colorimetric comparisons should be completed, especially in the analysis of liver tissue which may be rich in glycogen, within 12 minutes from the time the colour is developed. The sodium tungstate serves primarily as a clarifying and decolorising agent rather than as a precipitant. Usually, the amount of precipitable material is small after autoclave hydrolysis; indeed, in analyses of muscle tissue the tungstate treatment might be omitted without seriously disturbing the accuracy of the creatinine determinations. Beef muscle tissue contains about 0·4 per cent. total creatinine (i.e. creatine plus creatinine) while the concentration in the organs is much less, being approximately 0·03 per cent. in the liver and about half this value in the kidneys and pancreas. There appears to be a tendency for the concentration of total creatinine to assume a uniform value for any particular tissue of any one species of animal. In this connection some interesting results obtained by Rose, Helmer and Chanutin[10] are presented in Table XIII.

TABLE XIII. TOTAL CREATININE (CREATINE PLUS CREATININE) CONTENT OF RABBIT TISSUE (After W. C. Rose, O. M. Helmer and A. Chanutin, *J. Biol. Chem.*, 1927, **75**, 543)

Rabbit No.	Creatinine Concentration, mg. per 100 g.						
	Muscles of Leg	Heart	Brain	Liver	Spleen	Kidneys	Testes
1	515	223	121	27	27	19	188
2	517	217	106	24	—	20	194
3	492	193	114	24	27	19	182

Method for Urine[11] (*Free Creatinine*). A convenient quantity of the sample, say 2 ml., is transferred to a 100-ml. graduated flask and 1 ml. of the stock standard solution (1 mg. creatinine per ml.) is introduced into another similar flask and 20 ml. of a saturated aqueous solution of picric acid is added to each followed by

precisely 1·5 ml. of a 10 per cent. aqueous solution of sodium hydroxide. After standing for 10 minutes in order to allow the colours to develop, each mixture is diluted to the mark with water and the relative intensities of the colours are compared in a colorimeter. If the readings of standard and unknown differ by more than 50 per cent. the determination should be repeated with a more suitable amount of the sample. Alternatively, a series of standards may be prepared and the colour due to the sample matched by direct visual observation.

(*Total Creatinine.*) To 1 ml. of the sample contained in a 300-ml. flask is added 20 ml. of a saturated aqueous solution of picric acid and the flask and its contents weighed to within 0·1 g. About 150 ml. of water is added and the liquid boiled gently for 45 minutes and then more rapidly until the volume is reduced to about 20 ml. After cooling, sufficient water is added to restore the flask and contents to the original weight. To 1 ml. of stock standard creatinine solution contained in another flask is added 20 ml. of the saturated aqueous solution of picric acid and then to both the sample and the standard 1·5 ml. of a 10 per cent. aqueous solution of sodium hydroxide is added and, after standing for 10 minutes, the resulting colours are compared as before.

(*Creatine.*) This is taken as the difference between the figure representing the concentration of total creatinine and the value found for the free creatinine.

Discussion. As an alternative to the boiling operation described above, Folin[11] advocated introducing 1 ml. of urine and 20 ml. of saturated picric acid solution into a 100-ml. graduated flask, covering the mouth of the latter with tin foil and heating the mixture in an autoclave at 115° to 120° C. for 20 minutes, cooling, adding 1·5 ml. of 10 per cent. solution of sodium hydroxide, allowing to stand 10 minutes and diluting to the mark with water. The colour thus produced is compared with a standard prepared by mixing 1 ml. of stock creatinine solution (1 mg. per ml.) with 20 ml. of picric acid solution and 1·5 ml. of 10 per cent. solution of sodium hydroxide and, after allowing to stand 10 minutes, diluting to 100 ml. with water. The procedure is convenient when many analyses have to be conducted at one time but it is inapplicable in the presence of dextrose since the sugar causes darkening of the mixture; by employing the simple boiling technique it is possible to obtain reasonably accurate determinations of creatinine even when 5 per cent. of dextrose is present in the sample.

It is most important that the picric acid employed for creatinine determinations should be pure[12]. Methods for the purification of the commercial product have been proposed[13] but the AnalaR grade is satisfactory and will generally be more convenient.

The normal human subject excretes in the urine from 0·6 to 1·2 g. of total creatinine in 24 hours[14].

Method for Blood[15]. Exactly 0·2 ml. of whole blood is transferred to a centrifuge tube containing 1·4 ml. of a 3·0 per cent. aqueous solution of sodium sulphate, $Na_2SO_4.10H_2O$, and 0·2 ml. of a 10·0 per cent. aqueous solution of zinc sulphate, $ZnSO_4.7H_2O$, and 0·2 ml. of 0·5N sodium hydroxide are added. The mixture is well agitated, then centrifuged, and to 1 ml. of the clear supernatant fluid (equivalent to 0·1 ml. of the original sample) is added 0·5 ml. of the alkaline picrate solution as employed for the determination of creatinine in animal tissues (see p. 205). After allowing the mixture to stand for 10 minutes so that the yellowish-red colour may develop to its full intensity it is matched against standards made by diluting appropriate quantities (say 0·5 to 3·0 ml.) of the diluted standard (0·005 mg. creatinine per ml.) to 5 ml. with water and mixing 1 ml. of each of the solutions so produced with 0·5 ml. of alkaline picrate reagent and allowing the colour to develop.

Discussion. The above is the procedure recommended by King, Haslewood and Delory[15] and is based upon the work of Somogyi[16–18]. The accuracy of the method is severely limited since the colour produced with the normal range of creatinine concentration is little greater than that of the alkaline picrate itself. According to Delory and Jacklin[19] the precision of the determination may be augmented by introducing into the calculation the value of a blank mixture as observed with a Duboscq type colorimeter and employing the expression

$$C_t = \frac{R_b - R_t}{R_b - R_s} \times \frac{R_s}{R_t} \times C_s$$

where C_s and C_t are the concentrations of the standard and test respectively, R_s and R_t the corresponding readings and R_b the reading of the blank. Matching of the colours may be facilitated by the use of an Ilford blue-green spectral light filter[20]. There is reason to suppose that in blood and plasma filtrates there are substances other than creatinine which respond to the alkaline picrate test. Calculated as "creatinine" the concentration in normal blood lies between 0·7 and 2 mg. per 100 ml., although it

has been observed to rise to 3·5 mg. per 100 ml. Definite retention of creatinine in the blood only occurs in cases where the kidney damage is well advanced and hence the results obtained do not possess the diagnostic value which attaches to determinations of blood urea. According to Behre and Benedict[21], only a small part, if any, of the colour ordinarily formed with alkaline picrate in blood filtrates can be attributed to creatinine even when the chromogenic substance is greatly increased by renal retention. These investigators showed that whereas kaolin quantitatively adsorbs creatinine in amounts such as, from the colour reaction, would be estimated to be present in blood filtrates, it does not remove any of the chromogenic substance. Similarly, animal charcoal does not affect the material in blood which gives rise to the colour with the alkaline picrate reaction, although it readily removes creatinine. Again, methods of isolation which yielded creatinine from solutions of that substance failed when applied to blood. Behre and Benedict concluded that there might be some creatine present in blood, but practically no creatinine, and that the creatinine in urine is presumably formed in the kidneys from some precursor. However, at present, the question cannot be regarded as having been finally settled.

Method for Meat Extract. A 10 per cent. w/v aqueous solution, or suspension, of the sample is prepared and to 10 ml. of this, contained in a 100-ml. graduated flask, is added 30 ml. of N hydrochloric acid and 60 ml. of water ; this mixture is heated in a boiling water-bath for fully 4 hours, then cooled and diluted to the mark with water. A convenient quantity of this solution is diluted to 100 times its volume with water and 10 ml. of the resulting solution treated with 5 ml. of the alkaline picrate reagent described on p. 205. As nearly as possible at the same time suitable quantities (from 1 ml. upwards) of the dilute standard solution (0·005 mg. creatinine per ml., see p. 205) are diluted to 10 ml. with water and mixed with 5 ml. of alkaline picrate solution. After standing for 10 minutes, in order to allow the colours to develop, the sample is matched against the series of standards by direct visual observation or, alternatively, is compared with a standard of closely similar intensity by means of a colorimeter.

Discussion. A genuine meat extract of good quality may be expected to contain from about 5 to 12 per cent. of total creatinine (i.e. creatine plus creatinine). The determination is of service to the analyst since it may assist towards the detection

of yeast extract, the latter not containing either creatine or creatinine.

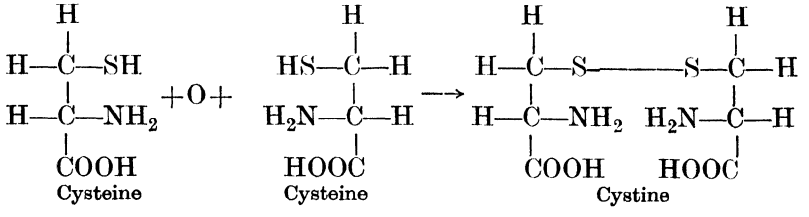
Benedict and Behre[22] have suggested 3:5-dinitrobenzoic acid as an alternative to picric acid as a reagent for creatinine with which it gives a purplish-rose colour in alkaline solution. The coloured product is photosensitive and after reaching its maximum intensity the tint fades, while in the absence of light the rate of development of the colour and its subsequent fading are influenced by the concentration of alkali present. Creatinine can be detected in a concentration of 0·01 mg. per 100 ml. and colorimetric readings can be taken in concentrations of about 0·2 mg. per 100 ml. It possesses an advantage over picric acid in that it is itself almost colourless but this has to be offset against the rather rapid fading which characterises the reaction colour. The reagent has been adopted by Komm and Pinder[23] for the determination of creatinine in meat extracts and broth cubes.

REFERENCES

1. M. Jaffe, *Z. physiol. Chem.*, 1886, **10**, 391.
2. O. Folin, *Z. physiol. Chem.*, 1904, **41**, 223.
3. A. Chaston Chapman, *Analyst*, 1909, **34**, 475.
4. I. Greenwald and J. Gross, *J. Biol. Chem.*, 1924, **59**, 601.
5. I. Greenwald, *J. Amer. Chem. Soc.*, 1925, **47**, 1148.
6. I. Greenwald, *J. Biol. Chem.*, 1928, **77**, 539.
7. I. Greenwald, *J. Biol. Chem.*, 1929, **80**, 103.
8. O. Folin, *J. Biol. Chem.*, 1914, **17**, 463.
9. O. Folin and J. L. Morris, *J. Biol. Chem.*, 1914, **17**, 469.
10. W. C. Rose, O. M. Helmer and A. Chanutin, *J. Biol. Chem.*, 1927, **75**, 543.
11. O. Folin, *J. Biol. Chem.*, 1914, **17**, 469.
12. O. Folin and E. A. Doisy, *J. Biol. Chem.*, 1917, **28**, 349.
13. S. R. Benedict, *J. Biol. Chem.*, 1929, **82**, 1.
14. L. Heitzmann, " Urinary Analysis and Diagnosis," 6th Ed., London: Baillière, Tindall and Cox, 1934.
15. E. J. King, G. A. D. Haslewood and G. E. Delory, *Lancet*, 1937, **232**, 886.
16. M. Somogyi, *Proc. Soc. Expt. Biol. Med.*, 1929, **26**, 353.
17. M. Somogyi, *J. Biol. Chem.*, 1930, **86**, 655.
18. M. Somogyi, *J. Biol. Chem.*, 1930, **87**, 339.
19. G. E. Delory and J. Jacklin, *Biochem. J.*, 1942, **36**, 281.
20. E. J. King and G. A. D. Haslewood, *J. Soc. Chem. Ind.*, 1937, **56**, 97.
21. J. A. Behre and S. R. Benedict, *J. Biol. Chem.*, 1922, **52**, 11.
22. S. R. Benedict and J. A. Behre, *J. Biol. Chem.*, 1936, **114**, 515.
23. E. Komm and H. Pinder. *Z. Unters. Lebensm.*, 1939, **78**, 113.

CYSTINE AND CYSTEINE

Cysteine, which is β-thiol-α-aminopropionic acid, is readily oxidised to cystine, two molecules of the former condensing together with the elimination of water in accordance with the equation :—



These sulphur containing amino acids are widely distributed in protein matter and because of their biological significance considerable attention has been paid to their determination. Early methods were based upon measurement of the blue colour resulting from the reduction by cysteine of phosphotungstic acid in the presence of sodium sulphite[1–11] while the quantitative application of Arnold's qualitative test for cystine using sodium nitroprusside[12] has also been attempted[13, 14]. Other proposals include the formation of a yellow cobalt complex with cysteine in the presence of hydrogen peroxide[15], the use of *o*-benzoquinone[16] and the method introduced by Sullivan depending upon the formation of a red colour when sodium 1:2-naphthoquinone-4-sulphonate is added to cystine followed by sodium sulphite, alkali and sodium hydrosulphite[17–19]. The latter method has been quite widely used but is subject to interference by aldehydes, sugars and many other substances[20–26]. The procedure[27] based upon Fleming's observation[28] that cystine in the presence of ferric ions reacts with *p*-aminodimethylaniline (*asym.* dimethyl-*p*-phenylenediamine) to form a coloured compound analogous to methylene blue (possibly 3-carboxy-7-dimethylaminobenzothiazine) is more specific and has been comprehensively studied by B. Vassel[29].

Method (*Cystine*)[29]. A volume of the protein hydrolysate to be tested not exceeding 1 ml. and expected to contain between 0·05 and 0·2 mg. cystine is measured into a test tube (15×1·8 cm.). If necessary, the volume should be made up to 1 ml. with acid of the same composition and normality as that used for the hydrolysis then 3 ml. of a freshly prepared 0·035 per cent. solution of *p*-aminodimethylaniline monohydrochloride in 6N sulphuric acid and 0·165 g. of zinc dust are added in succession and, after stand-

ing 2 to 4 minutes, 2 ml. of a 20 per cent. w/v solution of ferric ammonium sulphate, $Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$, in N sulphuric acid is added. With occasional mixing to counteract the tendency of the zinc dust to float, the reducing action of the latter is allowed to proceed for 45 minutes. At the end of this time an additional 3 ml. of ferric ammonium sulphate solution is added, the test tube lightly stoppered and immersed at once in a boiling water-bath and held there for 45 minutes. In order to ensure that none of the zinc dust is left undissolved at the end of the heating period, the walls of the test tube are carefully wetted twice with the hot mixture after 5 and 10 minutes' heating. After having been heated for 45 minutes the tube is placed in a cold water-bath, during which time the greyish-blue colour of the solution changes to purple. The liquid is then transferred to a 25-ml. graduated flask, made up to volume with water and the intensity of the stable purple colour measured, preferably at its maximum absorption band (5750 to 5800 Å) by means of a spectrophotometer. A calibration curve is prepared using known quantities of cystine dissolved in acid of the same strength and composition as that employed to prepare the protein hydrolysate under examination.

(*Cysteine*). Zinc dust (0·165 g.) is added to a mixture of 3 ml. of a freshly prepared 0·035 per cent. solution of *p*-aminodimethylaniline monohydrochloride in 6N sulphuric acid and 2 ml. of a 20 per cent. w/v solution of ferric ammonium sulphate in N sulphuric acid contained in a test tube. After standing at room temperature for 10 minutes, the tube is immersed in a boiling water-bath for 35 minutes and precautions taken to ensure that all the zinc dissolves. The tube and its contents are quickly cooled by immersion in cold water, 1 ml. of the solution to be examined added, followed by 3 ml. of the ferric ammonium sulphate solution. The mixture is then heated in boiling water for 45 minutes and the procedure outlined for the determination of cystine followed from this point on. Under these conditions two molecules of cysteine produce the same intensity of colour as one molecule of cystine. While it is easier to prepare standard curves for cysteine from cystine, the former can be used successfully provided that the hydrochloric acid in the cysteine hydrochloride is neutralised and the solvent used is an oxygen-free acid of a strength and composition equal to that of the unknown solution.

Discussion. The zinc dust, apart from exerting its reducing

power, appears to act also as a condensing agent. If cystine is first reduced in acid solution by metallic zinc and the other reagents are then added, very little colour with a maximum absorption at 5800 Å is produced. However, if ferric ions are present during the reduction, whether *p*-aminodimethylaniline has been added or not, a maximum colour develops on completion of the test provided that an excess of ferric ions is maintained during the heating period. The addition of a mixture of ferrous and ferric ions after the reduction of cystine with zinc in the absence of the other reagents will not produce the same intensity of colour as when the zinc acts in the presence of the other reagents. Since cystine gives no colour under the conditions outlined for the determination of cysteine the concentration of both substances in protein hydrolysates can be measured by applying the two procedures given above.

Ergothioneine and methionine do not give any colour under the conditions of this test but *iso*cystine, cystineamine, *l*-cystinyldiglycine, cystinylcystine and S-carboxymethylcystine react positively. All the more common organic constituents of blood and urine, as well as *l*-tyrosine, *l*-histidine, *l*-tryptophan and *dl*-serine give negative tests. Similarly, no colour is yielded by homocystine or reduced glutathione but the presence of either of these substances in concentrations comparable with that of cystine or cysteine leads to low results in the determination of the latter two substances. Ascorbic acid does not interfere but if it is added after the blue colour due to cystine or cysteine has formed the colourless leuco compound of the dyestuff is produced. For some unexplained reason the values obtained by Vassel[29] for the content of cystine in insulin were some 20 per cent. lower than those reported by other investigators[30] using alternative methods.

REFERENCES

1. O. Folin and J. M. Looney, *J. Biol. Chem.*, 1922, **51**, 421.
2. J. M. Looney, *J. Biol. Chem.*, 1922, **54**, 171.
3. G. Hunter and B. A. Eagles, *J. Biol. Chem.*, 1927, **72**, 177.
4. P. Criston and C. Benoit, *Bull. Soc. sci. med.*, 1928, **8**, 246.
5. O. Folin and A. D. Marenzi, *J. Biol. Chem.*, 1929, **83**, 103.
6. C. Rimington, *Biochem. J.*, 1930, **24**, 1114.
7. S. L. Tompsett, *Biochem. J.*, 1931, **25**, 2014.
8. J. W. H. Lugg, *Biochem. J.*, 1932, **26**, 2144.
9. K. Shinohara, *J. Biol. Chem.*, 1935, **109**, 665.
10. K. Shinohara, *J. Biol. Chem.*, 1935, **110**, 263.
11. K. Shinohara, *J. Biol. Chem.*, 1936, **112**, 671.
12. V. Arnold, *Z. physiol. Chem.*, 1911, **70**, 300.
13. E. Abderhalden and E. Wertheimer, *Arch. Ges. Physiol.* (*Pflügers*), 1923, **198**, 122.

14. A. Blankenstein, *Biochem. Z.*, 1930, **218**, 321.
15. L. Michaelis and E. S. G. Barron, *J. Biol. Chem.*, 1929, **83**, 191.
16. E. Hazeloop, *Ann. P. van der Wielen*, 1934, 159.
17. M. X. Sullivan, *U. S. Public Health Repts.*, 1926, **41**, 1030.
18. M. X. Sullivan, *U.S. Public Health Repts.*, 1929, **44**, 1421 and 1599: also Suppl. No. 78.
19. M. X. Sullivan and W. C. Hess, *U.S. Public Health Repts.*, 1931, **46**, 390.
20. J. W. H. Lugg, *Biochem. J.*, 1932, **26**, 2144.
21. F. T. G. Prunty, *Biochem. J.*, 1933, **27**, 387.
22. J. W. H. Lugg, *Biochem. J.*, 1933, **27**, 668.
23. J. H. Bushill, L. H. Lampitt and L. C. Baker, *Biochem. J.*, 1934, **28**, 1293.
24. S. Rossouw and T. J. Wilken-Jorden, *Onderstepoort J. Vet. Sci.*, 1934, **2**, 361.
25. W. C. Hess and M. X. Sullivan, *J. Biol. Chem.*, 1935, **108**, 195.
26. J. C. Andrews and K. C. Andrews, *Amer. J. Med. Sci.*, 1936, **191**, 594.
27. H. Toyoda, *Bull. Chem. Soc. Japan*, 1934, **9**, 263.
28. R. Fleming, *Biochem. J.*, 1930, **24**, 965.
29. B. Vassel, *J. Biol. Chem.*, 1941, **140**, 323.
30. M. X. Sullivan, W. C. Hess and E. R. Smith, *J. Biol. Chem.*, 1939, **130**, 741.

ETHYL ALCOHOL (IN BLOOD AND URINE)

Efforts to establish cases of alleged intoxication by determining the concentration of alcohol in the blood or in the urine have long been made in all parts of the world. The greater majority of the proposed procedures depend upon the method originally devised by Nicloux which consists essentially in treating distillates from deproteinised samples with measured quantities of potassium dichromate, adding sulphuric acid, heating in a hot water-bath then titrating the excess dichromate which remains[1]. There can be no doubt that volumetric methods are much to be preferred but some investigators have proposed colorimetric modifications based upon observation of the extent to which the orange-yellow colour of the dichromate is changed to green[2–4]. One such procedure is given below.

Method (*for Blood*)[4]. Exactly 2 ml. of oxalated whole blood is mixed with 14 ml. of water and 2 ml. of a 10 per cent. aqueous solution of sodium tungstate. The liquid is acidified by the addition of 2 ml. of 18N sulphuric acid, filtered, 10 ml. of the filtrate transferred to a 100-ml. round-bottomed flask and 5 to 6 ml. distilled off through a glass tube leading into a 25-ml. ground-glass stoppered measuring cylinder containing 5 ml. of

18N sulphuric acid and 2 ml. of a 0·3194 per cent. aqueous solution of potassium dichromate. The volume of this mixture should be close to 15 ml. in order that it may be approximately 6N with respect to sulphuric acid. During distillation the receiver should be cooled in an ice-bath and the open end of the condenser tube should reach nearly to the bottom of the cylinder. After distillation, the ground-glass stopper is sealed in place with a drop of concentrated sulphuric acid and the contents heated in a water-bath at a temperature of 75° to 80° C. for 20 minutes, then cooled to room temperature and diluted to exactly 20 ml. The extent of the colour change from yellow to green is matched against a series of standards prepared by adding 1 ml. of a solution of alcohol of known strength to a mixture of 2 ml. of potassium dichromate solution (0·3194 per cent.), 5 ml. of 18N sulphuric acid and 7 ml. of water, diluting to 15 ml., heating at 75° to 80° C. for 20 minutes, cooling, and diluting to 20 ml. Standard solutions of alcohol of strengths varying from 15 to 150 mg. per 100 ml. provide a suitable range of colours.

(*for Urine*)[4]. The sample is treated in the same way as a blood filtrate, 10 ml. being taken for the distillation when the alcohol content is expected to be low while an appropriate quantity diluted to 10 ml. with water is employed when relatively high values are anticipated.

Discussion. Considerable care is necessary in interpreting results obtained by this method since, of course, any volatile reducing substance, such as acetone, will give a positive result. The investigators responsible for the above procedure meet the difficulty of matching the change of colour from yellow to green by the use of an Evelyn photoelectric colorimeter[5] with composite filter 440. Kozelka and Hine[6] have evolved a technique in which interference due to aldehydes, ketones and phenols is eliminated by passing the vapours from the steam-distilled filtrates through a mixture of equal volumes of saturated aqueous solutions of mercuric chloride and sodium hydroxide maintained at a temperature of 100° C. In this method the excess of dichromate is measured by adding potassium iodide and titrating with sodium thiosulphate solution, but the modification might well be applicable to colorimetric work.

A small proportion of alcohol is frequently found in the blood and urine of subjects who have not ingested alcohol: thus, in thirty normal cases Gibson and Blotner[4] found 0 to 1 mg. per 100 ml. in the blood and 0 to 0·9 mg. per 100 ml. in the urine

while, in a further group of twenty-two people who had not taken any alcohol, the blood contained from 1 to 6·5 mg. per 100 mg. and the urine from 0 to 2·4 mg. per 100 ml. Another team of investigators found 1·7 to 4·0 mg. per 100 ml. of alcohol in normal blood[7]. Southgate and Carter, who have collected a considerable amount of data on the concentration of alcohol in blood and urine following its ingestion under various conditions, state that the proportion in urine is about 1·35 times that in the blood, the ratio being remarkably constant[8]. The results of further similar studies have been reported by J. Evans and A. O. Jones[9]. It is generally conceded that a state of inebriety exists when the concentration of alcohol in the blood exceeds 180 mg. per 100 ml. although, of course, subjects differ quite widely in their tolerance, while the reaction of the same person will vary according to the time and circumstances of ingestion.

REFERENCES

1. M. Nicloux, *Compt. rend. Soc. Biol.*, 1896, **48**, 841.
2. H. A. Heise and B. Halporn, *Penn. Med. J.*, 1932, **36**, 190.
3. H. A. Heise, *Amer. J. Clin. Path.*, 1934, **4**, 182.
4. J. G. Gibson, 2nd, and H. Blotner, *J. Biol. Chem.*, 1938, **126**, 551.
5. K. A. Evelyn, *J. Biol. Chem.*, 1936, **115**, 63.
6. F. L. Kozelka and C. H. Hine, *Ind. Eng. Chem., Anal. Ed.*, 1941, **13**, 905.
7. A. O. Gettler, J. B. Niederl and A. A. Benedetti-Pichler, *J. Amer. Chem. Soc.*, 1932, **54**, 1476.
8. H. W. Southgate and G. Carter, *Brit. Med. J.*, 1926, i, 463.
9. J. Evans and A. O. Jones, *Analyst*, 1929, **54**, 134.

FRUCTOSE (IN BLOOD AND URINE)

Various methods have been described for determining small amounts of fructose (lævulose) in the presence of other carbohydrates. The Seliwanoff colour test using resorcinol[1] has been applied to quantitative work by Folin and Berglund[2] and by Roe[3] while the reaction between fructose and diphenylamine in the presence of concentrated hydrochloric acid at 100° C.[4], which results in the production of a blue colour, has been variously modified for quantitative requirements[5–11]. Scott has suggested that the diphenylamine be substituted by bile salt[12]. More recently, Jordan and Pryde[13] discovered that hexoses, substituted hexoses and their derivatives produce an intense purple colour in

the presence of skatole (3-methyl-indole) and hydrochloric acid at 80° to 100° C. and that fructose and its derivatives give the reaction much more readily than aldohexoses, so much so that, on heating at 40° C. for 15 minutes, fructose, its derivatives (including hexosediphosphate from yeast fermentation) and sorbose alone yield a colour. This method has been improved by Reinecke[14] but, since the procedure involves the use of a freshly prepared solution of hydrogen chloride gas in ice-cold ethyl alcohol of 10N strength, it is inconvenient, and the much simpler technique using the resorcinol reaction, as recommended by Roe[3], will generally be preferred.

Method[3] (*for Blood*). To 1 part of the blood sample is added 7 parts of water and after allowing the mixture to stand a few minutes until hæmolysis is complete the proteins are precipitated by adding 1 part of a 10 per cent. aqueous solution of zinc sulphate, $ZnSO_4.7H_2O$, and 1 part of 0·5N sodium hydroxide. The mixture is filtered and 2 ml. of the filtrate (equivalent to 0·2 ml. of the sample) is transferred to a test tube. Into each of three similar test tubes, 2 ml. of standard fructose solution is introduced, the standards used containing 0·1, 0·05 and 0·025 mg. fructose per ml. To each of the tubes is added 2 ml. of a 0·1 per cent. solution of resorcinol in alcohol (90 per cent.) and 6 ml. of strong hydrochloric acid (30 per cent. HCl). The tubes are vigorously shaken, immersed in a water-bath maintained at 80° C. for 8 minutes, quickly cooled in running water and the stable cherry-red colour of the test mixture compared with the standard which most closely matches it, using a suitable colorimeter.

In order to prepare the standards a stock solution is made by dissolving 1 g. of pure fructose in 100 ml. of saturated aqueous solution of benzoic acid and the three working standards are made by diluting portions with the same solvent.

(*for Urine*). To 2 ml. of the sample is added 18 ml. of a 1 per cent. aqueous solution of acetic acid and 0·2 g. of acid-washed, activated charcoal. The mixture is vigorously shaken for 5 minutes then filtered and the colour test applied to 2 ml. of the filtrate in the manner described above for blood. In this case the standards should be prepared from the stock solution by dilution with 1 per cent. acetic acid instead of with saturated solution of benzoic acid.

The acid washed, activated charcoal should be specially prepared by placing 50 g. of a good grade of absorbent charcoal on a filter, pouring over it 250 ml. of a 10 per cent. aqueous

solution of acetic acid, washing through with 500 ml. of water, transferring the charcoal to a suitable dish and heating, at first gently, then to redness for 15 minutes.

If protein is present in the sample of urine to be examined it may be removed by the method given for the preliminary treatment of blood. To each 10 ml. of deproteinised urine filtrate 0·1 ml. of glacial acetic acid and 0·1 g. of acid-washed activated charcoal is added and the determination then completed as for a normal sample.

Discussion. The depth of colour obtained is dependent upon the concentration of alcohol and hydrochloric acid in the test mixture, hence it is important that the amount of these substances should be equal in both sample and standards. The intensity of the colour produced may be considered as directly proportional to the amount of fructose present provided the deviation between sample and standard is within fairly narrow limits. This method for the determination of fructose is not disturbed by the presence of glucose up to 300 mg. per 100 ml., or by galactose up to 500 mg. per 100 ml. Furfuraldehyde gives a green colour under the conditions of the test, hence the method is inapplicable in the presence of appreciable concentrations of pentoses.

REFERENCES

1. T. Seliwanoff, *Ber.*, 1887, **20,** 181.
2. O. Folin and H. Berglund, *J. Biol. Chem.*, 1922, **51,** 209.
3. J. H. Roe, *J. Biol. Chem.*, 1934, **107,** 15.
4. A. Ihl and A. Pechmann, *Chem-Ztg.*, 1885, **9,** 451.
5. F. Kronenberger and P. Radt, *Biochem. Z.*, 1927, **190,** 161.
6. S. van Creveld, *Klin. Wochenschr.*, 1927, **6,** 697.
7. P. Radt, *Biochem. Z.*, 1928, **198,** 195.
8. R. C. Corley, *J. Biol. Chem.*, 1929, **81,** 81.
9. W. W. Oppel, *Biochem. Z.*, 1930, **229,** 85.
10. H. Steinitz and I. von Riesen, *Biochem. Z.*, 1932, **252,** 201.
11. J. Patterson, *Biochem. J.*, 1935, **29,** 1398.
12. L. D. Scott, *Biochem. J.*, 1935, **29,** 1012.
13. R. C. Jordan and J. Pryde, *Biochem. J.*, 1938, **32,** 279.
14. R. M. Reinecke, *J. Biol. Chem.*, 1942, **142,** 487.

GLUCOSE (IN BLOOD)

Glucose (dextrose) is the principal sugar present in blood, the normal concentration approximating to 0·1 per cent., and its accurate determination in patients suffering from diabetes mellitus is of profound importance; in addition, it is necessary to carry out

numerous determinations on the blood of test animals in the biological assay of insulin. Several methods have been described such as the volumetric copper-reduction procedure due to MacLean[1] and the volumetric ferricyanide method of Hagedorn and Jensen[2] but the colorimetric methods, based upon the original proposal of Folin and Wu[3], are simpler and more widely employed. Here, the original Folin and Wu method is described and also a modified procedure due to Benedict[4]. In principle, the colorimetric method consists in heating a protein-free filtrate from the blood with an alkaline cupric tartrate solution then treating with a solution of phosphomolybdic acid which is reduced in proportion to the amount of cuprous salt, and, therefore, in proportion to the quantity of sugar. The compound formed by reduction of phosphomolybdic acid is blue, and the intensity of this colour is compared with that produced by a standard solution of pure glucose similarly treated.

The volumetric ferricyanide method of Hagedorn and Jensen[2] has been ingeniously transformed into a colorimetric method by R. F. Milton[5] who recommends converting the ferrocyanide formed in the reaction into a colloidal suspension of the brown uranium salt and measuring the colour produced by means of a Hilger-Spekker absorptiometer using the green filter No. 5. The present author has found this to be an excellent procedure and the gradation of colour produced is so good that accurate determinations can readily be made by direct visual comparison against natural standards.

Method of Folin and Wu[3]. The following are required :—

1. An Alkaline Copper Solution made by dissolving 40 g. of anhydrous sodium carbonate in about 400 ml. of water, transferring to a litre graduated flask, adding 7·5 g. of tartaric acid then adding precisely 4·5 g. of crystalline copper sulphate previously dissolved in about 100 ml. of water, mixing and diluting to the mark. If a sediment forms on standing the clear supernatant solution should be decanted.

2. A Phosphomolybdic Acid Solution. This is made by dissolving 35 g. of molybdic acid and 5 g. of sodium tungstate in 400 ml. of a 5 per cent. aqueous solution of sodium hydroxide contained in a large beaker, boiling the resulting solution for 20 to 40 minutes in order to remove as completely as possible the ammonia present in the molybdic acid, allowing to cool, then transferring to a 500-ml. graduated flask and washing in with

sufficient water to bring the volume of the mixture to about 350 ml. After adding 125 ml. of phosphoric acid (s.g. 1·75) the solution is diluted to the mark with water.

3. Stock Standard Glucose Solution. A saturated solution of benzoic acid in water is prepared by adding 2·5 g. of the acid to about a litre of boiling water, allowing to cool and filtering; this filtrate is employed as solvent in order to make a solution of the sugar containing precisely 0·1 per cent. of anhydrous glucose. This solution will keep almost indefinitely.

4. Diluted Standard Glucose Solution. This is prepared freshly each time by diluting 5 ml. of the stock glucose solution to 200 ml. with the saturated benzoic acid solution: it contains 0·0025 per cent. of sugar.

To a small test tube (about 20×75 mm.) containing 3·5 ml. of water is added 0·1 ml. of the blood to be examined followed by 0·2 ml. of a 10 per cent. aqueous solution of sodium tungstate, $Na_2WO_4.2H_2O$, then 0·2 ml. of 2/3N sulphuric acid. After shaking, the contents of the tube are allowed to stand for 10 minutes, or until the protein precipitate coagulates, then filtered through a 7-cm. paper (Whatman No. 41) and 2 ml. of the filtrate transferred to a Folin's blood sugar tube (Fig. 11) followed by 2 ml. of the alkaline copper solution. In another Folin's tube is placed 2 ml. of the diluted standard glucose solution and 2 ml. of the copper solution and into a third Folin's tube, 1 ml. of the standard glucose solution, 1 ml. of water and 2 ml. of the copper solution. After mixing the contents, all the tubes are immersed in a boiling water-bath for exactly 6 minutes, cooled quickly under the tap, care being taken not to agitate the tubes lest aërial oxidation of the cuprous oxide should occur, and 2 ml. of the phosphomolybdic acid solution added to each. Finally, the solution in each tube is diluted with water to the 12·5 ml. mark, the contents mixed and, after allowing to stand a few minutes in order that bubbles of carbon dioxide may escape, the blue colour produced by the sample under examination is matched against that of whichever standard colour is the nearer. The stronger standard contains the equivalent of 100 mg. of glucose per 100 ml. of blood, the weaker containing half this quantity. The colour measurement is most conveniently conducted in a colorimeter of the Duboscq type and it is customary to consider the concentration of glucose in the sample as being inversely proportional to the height of the plunger, the colour being matched against a standard

conveniently set at, say, 20 or 40 mm. The colour intensity resulting from the examination of the sample should not differ from that of the standard used for matching by more than 20 per cent. When the blood sugar content of the sample is high a smaller quantity of the blood filtrate should be taken for the test and the difference in volume made up by adding the appropriate amount of water. A blank experiment, which should be performed with each new set of reagents employed, using 0·1 ml. of

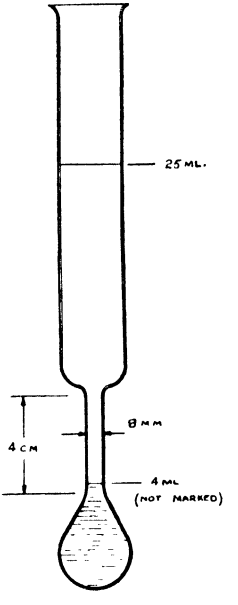


FIG. 11.—FOLIN'S SUGAR TUBE

The special reaction tube used for the determination of sugar in blood. (After O. Folin and H. Wu, *J. Biol. Chem.*, 1920, **41**, 367.)

water instead of 0·1 ml. of blood, should not show more than a just detectable pale blue tint.

Method of Folin and Wu Using Permanent External Standards. Two discs, each containing nine glass colour standards, are available for use with the Lovibond Comparator. They cover a range from 60 to 400 mg. glucose per 100 ml. of blood at increments of 20 mg. The technique adopted for use in conjunction with these standards is identical with that described above. While the degree of precision attainable by the use of permanent standards cannot be expected to equal that given by a colorimeter they are, nevertheless, sufficiently accurate for routine clinical control work and

obviate the need for applying the test to standard glucose solutions.

Discussion. The above procedure gives slightly higher values in insulin hypoglycæmia than the volumetric method of either MacLean[1] or of Hagedorn and Jensen[2]: these latter give results for the blood-sugar concentration which approximate closely to "true glucose" values. The true concentration of sugar cannot be calculated by deducting the non-fermentable reducing substance (approximating to 20 or 30 mg. per 100 ml. as glucose) from the "enhanced" values because there is uncertainty as to what proportion of this non-fermentable reducing matter is included by the test. In human blood the chief substance, other than glucose, responsible for the reduction is glutathione, which is confined to the corpuscles. Herbert and Bourne[6, 7] have suggested diluting the blood with an isotonic solution of sodium sulphate so that the corpuscles are subsequently precipitated intact with the plasma proteins. In their procedure 0·2 ml. of the sample of fresh blood is introduced into a centrifuge tube containing 3·6 ml. of sodium sulphate solution (3·0 per cent. w/v $Na_2SO_4.10H_2O$), 0·1 ml. of 10 per cent. aqueous solution of sodium tungstate is added followed by 0·1 ml. of 2/3N sulphuric acid. The bright red mixture, in which the corpuscles must not be hæmolysed, is centrifuged, the supernatant fluid filtered through a 5·5 cm. paper (Whatman No. 41) and the colour test applied to 2 ml. of the filtrate exactly as described above, except that, as twice as much blood is initially taken, the diluted standard glucose solution should contain 0·01 per cent. of sugar instead of 0·005 per cent.

Benedict[4] has overcome the tendency for the colorimetric method to yield "enhanced" values by introducing a modification into the formula for the alkaline copper solution whereby the reducing power of substances other than sugar is suppressed by the inclusion of alanine and sodium bisulphite.

Method of Benedict[4]. The technique is the same as that given in the description of the Folin and Wu method excepting that the formulæ for the copper reagent and the phosphomolybdic acid are modified :—

1. Stock Alkaline Copper Reagent. This is made by dissolving 15 g. of anhydrous sodium carbonate, 3 g. of alanine (α-aminopropionic acid) and 2 g. of sodium potassium tartrate in about 250 ml. of water and adding 3 g. of copper sulphate, $CuSO_4.5H_2O$,

previously dissolved in about 100 ml. of water; the mixed solution is then diluted to 500 ml. This solution will remain in good condition for about 6 weeks.

2. The Working Copper Bisulphite Reagent is prepared just prior to use by adding 1 vol. of a 1 per cent. aqueous solution of sodium bisulphite (sodium metabisulphite)* to 20 vols. of the stock alkaline copper reagent.

3. Phosphomolybdic Acid Solution. To 150 g. of molybdic acid and 75 g. of anhydrous sodium carbonate contained in a large Erlenmeyer flask is added 500 ml. of water in small portions while shaking. The mixture is heated to boiling, filtered, the insoluble residue on the paper washed with water until the total volume of the combined filtrate and washings amounts to 600 ml. To this solution is added 300 ml. of phosphoric acid (s.g. 1·75) and the reagent diluted to 1 litre with water.

The procedure for the determination of blood sugar is identical with that already described for the Folin and Wu methods excepting that the copper-bisulphite reagent is used in place of the alkaline copper solution prescribed for the original technique and that the phosphomolybdic acid solution is made in accordance with Benedict's instructions as given above. If it is preferred to work with 0·2 ml. of blood, then the final colour solution may be diluted to 25 ml. instead of 12·5 ml., but in this case the standard should contain 0·01 per cent. of glucose.

Discussion. There is little doubt that Benedict's modification constitutes an improvement upon the original procedure and in this form the method is widely employed. In determining sugar in blood by any method it is important to guard against the possibility of glycolysis. It has been shown by Tolstoi[8] that when normal blood has been drawn the glucose disappears quite rapidly although it remains more constant in diabetic blood. His observations indicate that the fall in sugar concentration during 3 hours is quite appreciable, therefore estimations should be performed at once if accurate results are to be obtained. This is particularly important when a sugar tolerance curve is being prepared, since, if the specimens are all left until the end of the test period before the sugar determinations are undertaken, the shape of the curve will be naturally altered by glycolysis in the earlier specimens. If it is not possible to undertake sugar determinations immediately it is a good plan to add a trace of formaldehyde to the specimen (approximately in the ratio of 1 drop of the 38 per

* See footnote on p. 22.

cent. solution to 10 ml. of blood) which prevents glycolysis and ensures constancy of sugar concentration during 48 hours. The formaldehyde combines with protein matter in the blood and therefore does not affect the alkaline copper solution.

Method of Milton[5]. The following special reagents are required :—

1. A 0·6 per cent. Aqueous Solution of Potassium Ferricyanide which should be stored in a dark glass bottle, or, preferably, be freshly made. It is a good plan to rinse the crystals of ferricyanide once with cold water and reject the washing before using them to prepare the solution: this will ensure freedom of the reagent from ferrocyanide while the slight uncertainty as to the strength of the reagent is immaterial.
2. A Cyanide-Carbonate Solution made by dissolving 1·5 g. of sodium cyanide (iron free) in 150 ml. of water, adding 8 g. of sodium carbonate, Na_2CO_3, previously dissolved in about 100 ml. of water and diluting the mixture to 1 litre.
3. A Uranium Solution prepared by dissolving 1 g. of uranium acetate, $UO_2(C_2H_3O_2)_2.2H_2O$, in water, adding 10 ml. of a 2 per cent. solution of gum Ghatti and 20 ml. of glacial acetic acid and diluting the mixture to 100 ml. with water. The gum Ghatti solution may be made by suspending 10 g., contained in a muslin bag, in the top of a 500-ml. cylinder of water for about 16 hours.

Exactly 4·9 ml. of a 0·45 per cent. aqueous solution of zinc sulphate, $ZnSO_4.7H_2O$, is transferred to a test tube, 0·1 ml. of fresh blood taken directly from a pricked finger (or oxalated venous blood) is added, the blood pipette rinsed once or twice with the mixture, precisely 1 ml. of 0·1N sodium hydroxide added and the tube, after being given a swirling motion in order to mix the contents, immersed in a boiling water-bath for 3 minutes. At the same time, a control tube containing 5 ml. of the zinc sulphate solution and 1 ml. of 0·1N sodium hydroxide is similarly treated. After cooling, the mixture is filtered through a pledget of cotton-wool inserted in the apex of a small funnel, 3 ml. of the filtrate (equivalent to 0·05 ml. of blood) transferred to a dry tube of standard dimensions, 2 ml. of the ferricyanide solution and 2 ml. of cyanide-carbonate solution added, and the tube, after being swirled in order to mix the contents, immersed in a boiling water-bath for exactly 10 minutes. The whole is then cooled under

running water and 5 ml. of the uranium reagent added. The control is similarly treated and the intensity of the brown colour of the test liquid measured by means of a suitable instrument and the difference between the reading so obtained and that due to the control referred to a calibration curve based on determinations conducted on known quantities of glucose. Alternatively, the colour obtained in the test may be matched visually against natural standards containing up to 0·12 mg. of anhydrous glucose (equivalent to 240 mg. per 100 ml. of sample). If the concentration of sugar in the blood sample exceeds this amount the test should be repeated using 1·5 ml. of the blood filtrate mixed with 1·5 ml. of water.

Discussion. In this method the non-glucose reducing substances are removed by Somogyi's zinc hydroxide method for the precipitation of proteins[9]. Special care is necessary to avoid contamination by particles of dust or traces of grease ; reaction tubes should be cleaned with chromic acid and the uniformity of reaction mixtures ensured by swirling the tubes and not by closing with the thumb and shaking. The presence of cellulose particles from filter papers may cause slightly high results and all reagents should, therefore, be filtered twice through the same paper. The uranium ferrocyanide colour develops to its full intensity in 30 seconds and thereafter remains constant for some hours. Gelatin can be used as a protective colloid in place of gum Ghatti but it is less effective and a higher concentration is needed. If it is to be employed, a 2·5 per cent. solution should be prepared and preserved with a crystal of thymol and a mixture of 2 vols. of this solution with 1 vol. of 5 per cent. aqueous solution of uranium acetate and 2 vols. of glacial acetic acid used in place of reagent No. 3 described above. If this modified solution is adopted it is necessary to prepare it freshly each day and, where the colours are measured by means of a photometer, the calibration curve must also be checked. Finally, it is most important to ensure that the sodium cyanide employed is free from traces of iron. In cases where highly purified specimens of this salt are not available 1·2 g. of potassium cyanide (AnalaR grade or equivalent reagent quality) should be used for making the cyanide-carbonate solution.

REFERENCES

1. H. MacLean, *Biochem. J.*, 1919, **13**, 135.
2. H. C. Hagedorn and B. N. Jensen, *Biochem. Z.*, 1923, **135**, 46.
3. O. Folin and H. Wu, *J. Biol. Chem.*, 1920, **41**, 367.

4. S. R. Benedict, *J. Biol. Chem.*, 1931, **92**, 141.
5. R. F. Milton, *Analyst*, 1942, **67**, 183.
6. F. K. Herbert and M. C. Bourne, *Biochem. J.*, 1930, **24**, 299 and 1787.
7. F. K. Herbert and M. C. Bourne, *Brit. Med. J.*, 1931, i, 94.
8. E. Tolstoi, *J. Biol. Chem.*, 1924, **60**, 69.
9. M. Somogyi, *J. Biol. Chem.*, 1930, **86**, 655.

HÆMOGLOBIN AND CARBOXYHÆMOGLOBIN (IN BLOOD)

The most accurate method for determining active hæmoglobin in blood consists in saturating the sample with air and measuring the oxygen content gasometrically; alternatively, the hæmoglobin may be converted into carboxyhæmoglobin and the carbon monoxide content determined by gas analysis. The oxygen capacity method was introduced by J. S. Haldane and J. L. Smith[1] and it is the standard by which all the other procedures are controlled. For routine clinical purposes determinations by colorimetric methods are preferred since, although they lack the precision of the gasometric procedures, they can be rapidly performed on very small samples of blood. Gowers[2] was the first to develop a colorimetric method, which merely consisted in diluting the sample and comparing the colour with artificial standards of gelatin coloured with picrocarmine. However, owing to its instability, oxyhæmoglobin was found to be a less satisfactory form of hæmoglobin for colorimetric determination than some of the other derivatives, hence methods based upon preliminary conversion to carboxyhæmoglobin[3–5] acid hæmatin[6–8], alkaline hæmatin[9], cyanhæmoglobin[10, 9] and methæmoglobin[9] have all been utilised. Determination of the iron content of blood has also been recommended as providing a measure of the total hæmoglobin[11–14]. Four procedures are given here: first, the technique recommended by Palmer[5] for applying the method depending upon conversion to carboxyhæmoglobin; secondly, Wu's[9] alkaline modification of the acid hæmatin method of B. Cohen and A. H. Smith[7]; thirdly, a simple procedure devised by G. A. Harrison[15] for directly viewing the colour of undiluted blood using a special cell in conjunction with the Lovibond Comparator; finally, Wong's method[14] involving a determination of the iron content of the blood. The first method is readily applicable to the approximate determination of carb-

oxyhæmoglobin in samples derived from cases of carbon monoxide poisoning and the appropriate technique is presented below as well as the colorimetric method of Sayers and Yants[16] using tannic acid and pyrogallol.

Method for Hæmoglobin by Conversion to Carboxyhæmoglobin[5]. The following reagent and standard solutions of hæmoglobin are required :—

1. Ammonia Solution made by diluting 1 ml. of concentrated ammonia (s.g. 0·880) to 250 ml. with water.

2. Stock Standard Hæmoglobin Solution. A quantity of fresh human blood (or ox blood) is stirred with a rod until defibrinated then filtered through gauze and its oxygen capacity determined gasometrically by the method of Van Slyke and Hiller[17, 18]. The blood is then diluted with the above ammonia solution to an oxygen capacity of 4 vols. per cent. and the resulting solution transferred to a dark glass bottle and saturated with carbon monoxide by bubbling through it 2 or 3 vols. of the gas. Alternatively, coal gas may be passed through for 10 minutes after adding a drop of capryl alcohol (*sec.*-octyl alcohol) to prevent foaming. In either case, when the blood is fully saturated the tube through which the gas was passed into the solution is slowly withdrawn and the bottle is immediately closed with a glass stopper or cork (rubber is inadmissible) and sealed with hard paraffin. If stored in an ice-box the solution will keep for months provided the bottle is refilled with carbon monoxide or coal gas every time the stopper is removed : if air is allowed to replace the gas in the bottle methæmoglobin begins to form thus rendering the solution inaccurate as a colorimetric standard.

3. Diluted Standard Solution of Hæmoglobin. This is made by diluting 5 ml. of the stock standard to 101 ml. with the above ammonia solution. It is advisable to prepare this freshly just prior to conducting a determination. Since the oxygen capacity of normal human adult blood approximates to 20 vols. per cent. it follows that this working standard is about equal to a 1 in 100 dilution of a normal sample.

In order to conduct the determination, 0·05 ml. of the sample is drawn into a blood pipette and transferred into 5 ml. of the ammonia solution contained in a test tube. The pipette is rinsed by alternately drawing the ammonia solution into it and blowing out the liquid two or three times. After adding a minute drop

of capryl alcohol, coal gas is bubbled through the mixture for about 30 seconds. The pink colour of the resulting liquid is then compared with that of the diluted standard solution of hæmoglobin using a colorimeter of the Duboscq type. If the blood contains less than 60 or 70 per cent. of the normal hæmoglobin content then 0·1 ml. of sample should be taken instead of 0·05 ml.

If 0·05 ml. of sample has been used it will be diluted 1 in 100 and therefore the oxygen capacity can be calculated by multiplying the ratio of its colour intensity with respect to that of the standard by 20. Similarly, if 0·1 ml. of the sample is used the ratio should be multiplied by 10.

Discussion. It is customary to express the content of hæmoglobin in the blood in terms of the Haldane scale. On this system an oxygen capacity of 18·5 ml. per 100 ml. is represented as " 100 per cent. hæmoglobin " because, as a result of the work of Haldane[4], it was thought that this value characterised normal adult blood. These early determinations were conducted by introducing blood into a chamber connected with a manometer, or a micro-gas burette, and containing air at atmospheric pressure, then adding alkaline ferricyanide to liberate the oxygen which was measured by the increase in the pressure, or the volume, of the supernatant air. The alkaline reagent favours a slow consumption of oxygen by some reducing agent in the blood-reagent mixture and, furthermore, the maximum increase in pressure, or volume, is only reached after some time and is not maintained. These difficulties have been overcome by Van Slyke and his collaborators who have introduced a method which provides for the liberation of the oxygen by neutral ferricyanide solution in an evacuated chamber and its absorption by a solution of sodium hydrosulphite after the preliminary removal of carbon dioxide by sodium hydroxide[19]. Determinations by this method, which have been conducted by investigators throughout the world, have established that the normal oxygen capacity of blood for a human male adult averages 20·7 vols. per cent., the corresponding value for the female being 19·0. The normal values vary considerably according to age and sex as shown in Table XIV which is based on the work of C. S. Williamson[20] who determined the hæmoglobin spectrophotometrically in 919 subjects of all ages among both sexes. Hence, in assessing the normality, or otherwise of a sample the influence of age and sex should be taken into account.

TABLE XIV. THE NORMAL HÆMOGLOBIN CONTENT OF HUMAN BLOOD ACCORDING TO AGE AND SEX

Age	Males			Females		
	Oxygen Capacity vol. per cent.	Hæmoglobin g. per 100 ml.	Haldane Scale "per cent."	Oxygen Capacity vol. per cent.	Hæmoglobin g. per 100 ml.	Haldane Scale "per cent."
1 day	32·0±4	23·9	173	32·0±4	23·9	173
2 to 3 days	29·0±4	21·6	157	29·0±4	21·6	157
4 to 8 days	26·0±4	19·4	141	26·0±4	19·4	141
9 to 13 days	23·0±4	17·2	125	23·0±4	17·2	125
2 to 8 weeks	20·5±4	15·3	111	20·5±4	15·3	111
3 to 5 months	17·5±3	13·1	95	17·5±3	13·1	95
6 to 11 months	16·0±3	11·9	86	16·0±3	11·9	86
1 to 2 years	15·5±2	11·6	84	15·5±2	11·6	84
3 to 5 years	16·4±2	12·2	88	16·4±2	12·2	88
6 to 10 years	17·3±2	12·9	94	17·3±2	12·9	94
11 to 15 years	18·0±2	13·4	97	18·0±2	13·4	97
16 to 60 years	20·7±2	15·5	112	19·0±2	14·2	103
60 to 70 years	19·9±2	14·8	107	19·0±2	14·2	103
70 + years	19·0±2	14·2	103	18·7±2	13·9	101

Method for Hæmoglobin by Conversion to Carboxyhæmoglobin Using a Permanent External Standard. A coloured glass standard equivalent to 100 per cent. hæmoglobin (Haldane scale) and mounted in a Lovibond Comparator disc is available. (The other eight glasses are used for the determination of carboxyhæmoglobin as described on p. 235.) In order to conduct a determination 2 ml. of the dilute solution of ammonia already described above is introduced into a special Comparator tube graduated in tenths of a millilitre and 0·04 ml. of the sample delivered into the ammonia in the tube, the pipette being rinsed by alternately filling it with the ammonia solution and then emptying it. A minute drop of capryl alcohol is added and the mixture saturated with coal gas and diluted in steps until its colour matches that of the standard glass : coal gas is passed in after each addition of ammonia in order to maintain a full saturation. If the final volume of the liquid is 10 ml. then the sample contains 100 per cent. of hæmoglobin (Haldane scale) or, if the colour of the mixture matches that of the standard after dilution to 8 ml. then the blood under examination would be returned as containing 80 per cent. of hæmoglobin. With normal bloods, and certainly for specimens rich in hæmoglobin, it is preferable to take only 0·02 ml. and to double the observed reading.

Method for Hæmoglobin : Alkaline Hæmatin Technique[9]. The following standard solutions are required :—

1. Stock Standard Hæmoglobin Solution. A quantity of fresh human blood (or ox blood) is stirred with a rod until defibrinated then filtered through gauze and its oxygen capacity determined gasometrically by the method of Van Slyke and Hiller[17, 18]. The blood is then diluted with 0·1N hydrochloric acid to an oxygen capacity of 4 vols. per cent. and the resulting solution transferred to a dark glass bottle ; if kept cool and free from mould growth it will remain in good condition for several months.
2. Diluted Standard Solution of Hæmoglobin. This should be freshly prepared by diluting 5 ml. of the stock standard to 101 ml. with 0·1N hydrochloric acid thus giving a standard equivalent to a hæmoglobin content of 0·2 vol. per cent.

To carry out the determination 0·1 ml. of the sample is drawn into a blood pipette and transferred into 10 ml. of 0·1N hydrochloric acid. The pipette is rinsed by drawing the acid into it and expelling the liquid back into the solution from which it was drawn. The mixture is allowed to stand for 40 minutes for the colour to develop or, alternatively, it may be maintained at 40° C. for 10 to 15 minutes. The reaction mixture is then rendered alkaline by adding 1 ml. of a 10 per cent. aqueous solution of sodium hydroxide and the resulting colour matched against standards of suitable strength. Since, under the above conditions, the colour produced will vary from a pale straw (20 per cent. on the Haldane scale) to a brownish-red (130 per cent. on the Haldane scale) it is important that the standard used for comparison should approximate to the colour of the test solution. If 1 ml. of a 10 per cent. sodium hydroxide solution is added to 10 ml. of the diluted standard described above the colour will correspond to a hæmoglobin content equivalent to 20 vols. per cent. (108 per cent. on the Haldane scale). To prepare much weaker colours, quantities of standard solution less than 10 ml. should be diluted to 10 ml. with 0·1N hydrochloric acid and then rendered alkaline with 1 ml. of the sodium hydroxide solution.

Method for Hæmoglobin : Alkaline Hæmatin Technique Using Permanent External Standards. A set of eighteen standard glasses is available for use with the Lovibond Comparator. The standards, which cover a range from 20 per cent. to 130 per cent. (Haldane scale), are housed in two discs each carrying nine glasses.

The technique described above should be employed in conjunction with these standards.

Discussion. Owing to its extreme simplicity the alkaline-hæmatin method is widely favoured. The original acid-hæmatin technique was found by Wu[9] to be much affected by non-hæmoglobin substances in both plasma and cell stroma and it was observed that purified hæmoglobin dissolved in plasma gave a considerably higher reading than when dissolved in saline solution. On the other hand, red cells which had merely been washed gave less colour when suspended in plasma and submitted to the acid-hæmatin test than a corresponding suspension in saline. These anomalies disappear when the acidified hæmoglobin and blood solutions are rendered alkaline prior to measuring the colours, an effect which may be attributed to the fact that in acid media the hæmoglobin derivatives are in colloidal suspension rather than in true solution, whence the shade and intensity of colour may be influenced by factors affecting the state of dispersion.

It has been suggested by J. W. Clegg and E. J. King[21] that the difficulty appertaining to the preparation of a standard based upon the gasometric determination of oxygen capacity may be obviated by using an alkaline solution of hæmin of known iron content. As already indicated, the figure of 13·8 g. of hæmoglobin per 100 ml. proposed by Haldane[4] as representative of normal blood is almost certainly too low and Clegg and King favour the adoption of Haden's scale[22] based upon the assumption that healthy blood contains 15·6 g. of hæmoglobin per 100 ml. whence a stable standard solution having a colour equal to 100 per cent. hæmoglobin (Haden scale) can be prepared by dissolving 79·4 mg. of pure hæmin (containing 8·57 per cent. Fe) in 1 litre of 0·1N sodium hydroxide.

Hæmin may be prepared[23] by allowing 1 litre of citrated blood to settle, removing the clear plasma by means of a siphon, adding to the residue sufficient of a 0·9 per cent. aqueous solution of sodium chloride to produce about 800 ml. and transferring the mixture to a separator. A mixture of 2 litres of glacial acetic acid, 100 ml. of water and 0·25 g. of sodium chloride is heated to 90° C., the blood preparation is added slowly with continuous stirring, the mixture maintained at 90° C. for 15 minutes and allowed to stand overnight. Most of the supernatant fluid is removed by siphoning, the residue suspended in dilute acetic acid and, after allowing to stand for several hours, the supernatant fluid is again removed, water added and the crystalline hæmin

filtered off on a Buchner funnel and washed, first with dilute hydrochloric acid, then with water, and dried in a warm cupboard. The hæmin thus produced may be purified by dissolving 5 g. in 25 ml. of pyridine, adding 40 ml. of chloroform, shaking for 15 minutes, filtering the solution by suction and washing the insoluble residue with 15 ml. of chloroform ; the filtrate is poured in a steady stream into a mixture of 350 ml. of glacial acetic acid and 4 ml. of concentrated hydrochloric acid previously heated to boiling, the flask rinsed with a further 15 ml. of concentrated hydrochloric acid, the mixture set aside for 12 hours, the precipitated hæmin collected on a Buchner funnel and washed successively with 50 ml. of 50 per cent. acetic acid, 100 ml. of water, 25 ml. of alcohol and 25 ml. of ether, the pure hæmin being finally dried by suction. The iron content of the material should be checked by one of the usual gravimetric methods or by the volumetric procedure using titanous chloride as described by G. E. Delory[23].

It has been shown by Clegg and King[21] that, in applying the alkaline hæmatin test, it is not necessary to include a preliminary treatment with acid and they recommend diluting 0·05 ml. of blood to 5 ml. with 0·1N sodium hydroxide, heating in a boiling water-bath for 5 minutes, allowing to cool and matching the brownish-red colour against hæmin standards prepared in the manner indicated above. A Lovibond Comparator disc for use with this method is now available covering a range from 30 to 120 per cent. hæmoglobin on the Haden scale.

When adult human blood is added to 0·1N sodium hydroxide the brown colour of alkaline hæmatin quickly develops without heating but fœtal blood, some of which is present in the blood of infants up to the age of 7 months[24], remains red in the presence of soda while at about the age of 3 years another form of hæmoglobin which resists alkali denaturation develops and persists in small concentration throughout life. Both these resistant forms are converted to alkaline hæmatin by heating with 0·1N sodium hydroxide for 5 minutes whence this step is included in Clegg and King's procedure. The adoption of the hæmin standard in place of that based upon the oxygen capacity is to be favoured in view of the work of Ammundsen[25] who has shown that, under conditions of modern town life, inactive hæmoglobin not reacting with ferricyanide (carboxyhæmoglobin, methæmoglobin, sulphæmoglobin) may be present in the blood of normal individuals to the extent of from 2 to 12 per cent.

Method for Hæmoblobin by Directly Measuring the Colour of Undiluted Blood Using Permanent External Standards. This is simply an application of the Lovibond Comparator for assessing the colour of the blood. The sample, obtained by pricking the skin, is run into a special blood cell consisting of a base-plate and a cover-plate constructed of white optical glass (Fig. 12, *a*). Fused on the cover-plate are three small beads of glass, which have been ground until they project 0·0045 in. (±0·00015 in.) and

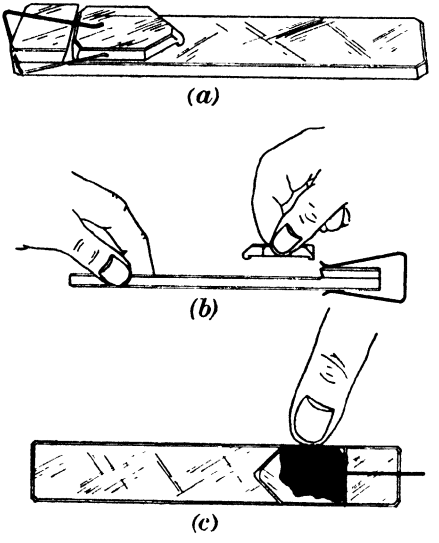


FIG. 12.—CELL FOR DIRECTLY MEASURING THE COLOUR OF UNDILUTED BLOOD

The sample is allowed to flow between the base-plate and the cover-plate from a small puncture as shown at *c*. The cover-plate is seen being lowered on to the base-plate *b* and the arrangement for holding the two plates together is shown at *a*. The cell is designed for use in conjunction with the Lovibond Comparator. (After G. A. Harrison, *Lancet*, 1938, **235**, 621.)

thus when the two plates are in apposition and separated by these three points of glass a cell is formed 0·0045 in. in thickness.

In order to conduct a determination the base-plate and cover-plate are cleaned, first with water, then with alcohol and finally dried with a clean non-fluffy cloth. The two plates are placed in apposition and clipped together (Fig. 12, *b* and *c*). The cell thus produced is filled from the side with capilliary blood as shown in Fig. 12, *c*, then introduced into the right-hand slot of the Comparator and the colour of the film of blood viewed by transmitted light from a North window and matched against the permanent glass standards. Two discs, each containing nine

glasses, are available which together cover a range from 20 per cent. to 130 per cent. (Haldane scale).

Discussion. By the above procedure errors due to dilution of the sample are avoided and the simplicity of the method allows of determinations being conducted at the bedside. Oxalated venous blood may be used but is liable to appear bluer than the standards, which renders estimation of the colour difficult and, if it has been allowed to stand, it may, in spite of thorough mixing, give a film which is speckled owing to rouleaux formation and thus lead to errors. The use of the Harrison cell in conjunction with the appropriate discs converts the Lovibond Comparator into a hæmoglobinometer somewhat on the lines of the well-known instrument designed by Dare[26]. In practice it is capable of giving results which are generally sufficiently accurate for routine clinical work.

Method for Hæmoglobin by Determination of the Iron Content[14]. The solutions listed below are required:—

1. A Saturated Aqueous Solution of Potassium Persulphate made by shaking 7 g. of the solid salt with 100 ml. of water and allowing the undissolved portion to settle. The excess solid material serves to replace persulphate lost from the solution as a result of the slow decomposition of the dissolved salt which takes place during storage.
2. An Approximately 3N Solution of Potassium Thiocyanate prepared by dissolving 146 g. of the solid salt of AnalaR grade in water, diluting to 500 ml. and adding 20 ml. of acetone.
3. Standard Iron Solution containing 0·1 mg. Fe per ml. Ferric ammonium sulphate (0·861 g.) is dissolved in water, 20 ml. of a 10 per cent. w/v aqueous solution of sulphuric acid added and the mixture diluted to 1 litre.

The sample of blood (0·5 ml.) is transferred to a 50-ml. graduated flask, 2 ml. of concentrated sulphuric acid added and, after agitating for 2 minutes, 2 ml. of the saturated solution of potassium persulphate is added, the mixture well shaken, adjusted to about 25 ml. with water and 2 ml. of a 10 per cent. aqueous solution of sodium tungstate added. The mixture is cooled to room temperature under the tap, diluted to 50 ml. with water, well shaken and filtered through a dry filter paper into a dry receiving vessel. Exactly 20 ml. of the filtrate is transferred to a large test tube graduated at 20 ml. and 25 ml.; into another similar test tube 1 ml. of the standard iron solution is added

followed by 0·8 ml. of concentrated sulphuric acid and sufficient water to dilute the liquid to 20 ml. After cooling the standard to room temperature by holding the tube under the tap, 1 ml. of saturated potassium persulphate solution followed by 4 ml. of approximately 3N potassium thiocyanate is added to both tubes, the contents of each well mixed and the relative intensities of the red colours determined by means of a Duboscq pattern colorimeter.

The 20 ml. of filtrate taken correspond with 0·2 ml. of the original blood and since 0·1 mg. Fe is used to produce the standard colour it follows that :—

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Unknown}} \times 50 = \text{mg. Fe per 100 ml. of blood.}$$

To obtain the result in terms of vols. per cent. of oxygen capacity the figure representing mg. Fe per 100 ml. is multipled by $\frac{22{\cdot}4}{55{\cdot}9} = 0{\cdot}4$. This follows from the fact that 55·9 mg. of hæmoglobin contains 1 mg. of iron and also binds 1 mg. molecule of oxygen (22·4 ml. at N.T.P.). Hence, the calculation of hæmoglobin in terms of oxygen capacity becomes :—

$$\frac{20 \times \text{Reading of the Standard}}{\text{Reading of the Unknown}} = \text{vols. per cent.}$$

Discussion. This procedure possesses the obvious advantage of dispensing with the necessity for preparing standard hæmoglobin solutions ; indeed, it is sometimes recommended as an alternative to the gasometric procedure for the purpose of preparing the standards for use in applying the other methods described above. After examining in detail the principal proposals for the determination of hæmoglobin in blood Marenzi and Lida[27] concluded that the methods based on the formation of hæmatin hydrochloride were not altogether satisfactory but that Wong's procedure[14], based on the determination of the iron content, gives results concordant with the gasometric methods.

Method for Carboxyhæmoglobin Based Upon the Preliminary Determination of Hæmoglobin by the Carboxyhæmoglobin Method Using Permanent External Standards. A disc containing nine coloured glasses for use with the Lovibond Comparator is available. The standards cover the range from 0 to 100 per cent. carboxyhæmoglobin, the 100 per cent. glass being the one employed for the determination of hæmoglobin (see p. 229). In

order to make an approximate estimation of carboxyhæmoglobin the total hæmoglobin is determined by the method already described on p. 229, and then a second sample of blood, of the same volume, is diluted to the same extent as in the previous determination and the colour matched against the permanent external standards, no carbon monoxide or coal gas being added.

The method may conveniently be described by supposing that a sample of blood was found to contain 80 per cent. total hæmoglobin (Haldane scale). The carboxyhæmoglobin is then determined by transferring 0·04 ml. of the sample into 2 ml. of dilute ammonia solution (1 ml. of concentrated ammonia solution in water to 250 ml.) contained in a graduated comparator tube, making the volume of the mixture up to 8 ml. (corresponding to 80 per cent. total hæmoglobin already found) with more dilute ammonia and matching the colour against those of the glasses in the disc. If there is no carboxyhæmoglobin it will match the 0 per cent. carboxyhæmoglobin glass. In order to illustrate the calculation let it be supposed that 30 per cent. carboxyhæmoglobin was indicated in the sample previously found to contain 80 per cent. total hæmoglobin. Since 100 per cent. on the Haldane scale is equivalent to 13·8 g. of hæmoglobin per 100 ml. of blood we have $80 \times 0{\cdot}138 = 11{\cdot}04$ g. total hæmoglobin per 100 ml. and carboxyhæmoglobin $11{\cdot}04 \times 0{\cdot}3 = 3{\cdot}312$ g. per 100 ml. whence the oxyhæmoglobin content = (11·040 − 3·312) g. per 100 ml.

Discussion. The above method only yields approximate results and it is not capable of detecting less than 10 per cent. of carboxyhæmoglobin. Difficulty may be experienced in applying this technique to stale blood which has been obtained more than 24 hours post mortem since such samples are sticky and liable to contain clots and, in addition, chemical changes in the oxyhæmoglobin may have occurred. The standard glass representing 0 per cent. carboxyhæmoglobin, or 100 per cent. hæmoglobin (Haldane scale) is designed to match blood of oxygen capacity 18·5 vols. per cent. diluted 1 in 250 and placed in a Comparator tube. The 100 per cent. carboxyhæmoglobin standard corresponds with a similar preparation which has been saturated with coal gas. The 10 per cent. carboxyhæmoglobin standard glass matches the colour of a pair of Comparator tubes, one of which contains 1 ml. of 1 in 250 blood and 9 ml. of dilute ammonia solution and saturated with coal gas and the other 9 ml. of 1 in 250 blood and 1 ml. of dilute ammonia but no coal gas and so on for the other values. Obviously, carboxyhæmoglobin could be determined by

the application of this principle using natural standards in place of the permanent coloured glasses.

Method for Carboxyhæmoglobin Using Tannic Acid[16]. A series of standards is prepared as follows :—

About 5 ml. of normal blood is drawn and clotting prevented by adding 5 mg. per ml. of potassium citrate and half of this specimen is diluted to 10 ml. with water. The hæmoglobin of the other half is entirely converted to carboxyhæmoglobin by rotating the blood about the walls of a 200-ml. bottle filled with air containing 3 to 5 per cent. of carbon monoxide and is then diluted to 10 ml. with water. For saturating the blood a mixture of air and coal gas may be used. The blood is thus treated before it is diluted with water in order to minimise the volume of carbon monoxide gas physically dissolved in the final solution. From these two solutions ten standards are made, each of 1 ml. volume, by mixing 0·1, 0·2 ml., etc., of the carboxyhæmoglobin solution with 0·9, 0·8 ml., etc., of the oxyhæmoglobin solution. The mixtures are placed in test tubes of approximately 15 mm. internal diameter. Equal volumes of 2 per cent. aqueous solution of tannic acid and 2 per cent. aqueous solution of pyrogallol are mixed and 1 ml. of the mixture added to each of the ten standard solutions. Each tube is inverted twice to mix the contents and is then sealed by pouring in about 2 ml. of melted hard paraffin in order to prevent the action of aërial oxygen. The paraffin should only be heated to a temperature just above its melting point and the tubes partially immersed in cold water while the paraffin is poured into them. After the paraffin has hardened the seal is rendered permanent by placing a disc of paper over the paraffin in each tube and filling the remainder of the tube with sealing wax. Standards prepared in this manner develop their full colour in 30 minutes and, if stored in a cool place, will retain their correct value for 2 weeks.

In order to conduct a determination, a sample of 0·1 ml. of blood is drawn by ear or finger prick and is diluted to 2 ml. with water. To the diluted blood approximately 40 mg. of a dry mixture of equal parts of tannic acid and pyrogallol is immediately added and the contents of the tube mixed by inverting several times, but no more than is necessary, for escape of significant portions of carbon monoxide must be avoided. After allowing to stand for 30 minutes the colour of the test liquid is

compared with that of the standards. The observations should be made by reflected daylight and not in direct sunlight.

Discussion. Once the standards have been prepared the above constitutes a simple approximate test suitable for use in the field in cases of suspected carbon monoxide poisoning. It depends upon the fact that whereas the addition of tannic acid to normal blood merely results in a grey suspension, the same reagent forms a carmine red solution in the presence of carboxyhæmoglobin.

REFERENCES

1. J. S. Haldane and J. L. Smith, *J. Physiol.*, 1899–1900, **25**, 331.
2. W. R. Gowers, *Lancet*, 1878, **115**, 882.
3. F. Hoppe-Seyler, *Z. physiol. Chem.*, 1892, **16**, 505.
4. J. S. Haldane, *J. Physiol.*, 1900–01, **26**, 497.
5. W. W. Palmer, *J. Biol. Chem.*, 1918, **33**, 119.
6. H. Haessler and H. Newcomer, *Ann. Internal Med.*, 1916, **17**, 806.
7. B. Cohen and A. H. Smith, *J. Biol. Chem.*, 1919, **39**, 489.
8. E. E. Osgood and H. D. Haskins, *J. Biol. Chem.*, 1923, **57**, 107.
9. H. Wu, *J. Biochem. (Japan)*, 1922, **2**, 173.
10. W. C. Stadie, *J. Biol. Chem.*, 1920, **41**, 237.
11. O. Wolter, *Biochem. Z.*, 1910, **24**, 108.
12. L. Berman, *J. Biol. Chem.*, 1918, **35**, 231.
13. A. L. Brown, *J. Amer. Chem. Soc.*, 1922, **44**, 423.
14. S. Y. Wong, *J. Biol. Chem.*, 1928, **77**, 409.
15. G. A. Harrison, *Lancet*, 1938, **235**, 621.
16. R. R. Sayers and W. P. Yants, *U.S. Bureau of Mines*, 1925, Tech. Paper No. 373.
17. D. D. Van Slyke and A. Hiller, *J. Biol. Chem.*, 1929, **84**, 211.
18. J. P. Peters and D. D. Van Slyke, " Quantitative Clinical Chemistry," London : Baillière, Tindall and Cox, 1932, vol. 2, p. 348.
19. D. D. Van Slyke and W. C. Stadie, *J. Biol. Chem.*, 1921, **49**, 1.
20. C. S. Williamson, *Ann. Internal Med.*, 1916, **18**, 505.
21. J. W. Clegg and E. J. King, *Brit. Med. J.*, 1942, ii, 329.
22. R. L. Haden, *J. Amer. Med. Assoc.*, 1922, **79**, 1496.
23. G. E. Delory, *Analyst*, 1943, **68**, 5.
24. R. Brinkmann and J. H. P. Jonxis, *J. Physiol.*, 1935, **85**, 117.
25. E. Ammundsen, *Ugesk. Laeger*, 1937, **99**, 908.
26. A. Dare, *Philadelphia Med. J.*, 1900, **6**, 557.
27. A. Marenzi and E. Lida, *Compt. rend. Soc. Biol.*, 1938, **129** 1267.

HISTIDINE (IN URINE)

A colour reaction was suggested by Knoop[1] which involves bromination of histidine in acid solution, followed by neutralisation of the acid with excess of ammonia and heating of the alkaline solution, whereupon a blue or violet colour develops. Hunter[2] attempted to stabilise the colour by extracting excess of

bromine with chloroform and Kapeller-Adler[3–5] suggested modifications of the test in attempts to render it quantitative. W. D. Langley[6], in a careful study of the problem, has proposed many improvements and emphasised the necessity for introducing a correction for the error due to suppression of the colour produced by urea and by other substances present in urine. The test has some value in that it provides presumptive evidence of pregnancy.

Method[6]. The following standard solutions of histidine are required :—

1. A Strong Standard, 1 mg. histidine per ml., made by dissolving 0·124 g. of histidine monohydrochloride in water, adding a drop of chloroform and diluting to 100 ml.
2. A Weak Standard, 1 mg. histidine per 6 ml., made by diluting 50 ml. of the strong standard with exactly 200 ml. of water and adding 50 ml. of a 10 per cent. w/v solution of sulphuric acid.

To 25 ml. of the sample of urine to be tested is added 5 ml. of a 10 per cent. w/v solution of sulphuric acid and about 75 mg. of powdered potassium permanganate, the latter being added in small portions with stirring until the purple colour persists for about 15 seconds. About 0·5 g. of acid-washed charcoal is then introduced, the mixture stirred, filtered and 6 ml. of the nearly colourless filtrate pipetted into each of a pair of test tubes graduated at 10 ml. Into a third test tube 6 ml. of the weak standard is introduced and to one of the tubes containing the filtrate from the sample is added 0·4 ml. of the strong standard. The tubes are now protected from exposure to light and into the solution is aspirated a 1 per cent. v/v solution of bromine in carbon tetrachloride (about 1·5 ml. for each sample) at such a rate that a brown colour of bromine is always present. After 20 minutes, 1 ml. of a 0·5 per cent. aqueous solution of phenol is added to each tube and the aspiration is stopped. The colourless, bromine-free solution, if kept cold, is stable for at least 3 hours. To each tube is added 1 ml. of a saturated aqueous solution of sodium acetate, the contents shaken and the tubes immersed in a boiling water-bath for 1 minute, light being excluded. The solutions are cooled to room temperature in an ice-bath, diluted with water to 10 ml. and the colours compared by means of a colorimeter, the calculations being made in the usual manner to give the histidine content per 5 ml. of urine. A correction is then applied, based on the recovery of the added histidine ; e.g. 6 ml. of filtrate

analysed gave 1·09 mg. histidine, 6 ml. of filtrate+0·4 mg. gave 1·37 mg. histidine, whence 1·37−1·09=0·28 mg.=70 per cent. of the 0·4 mg. added, thus, correcting in the same ratio, 1·09÷0·70 =1·56 mg. histidine in 5 ml. of urine.

When an amount of histidine larger than could be matched with 1 mg. is present, the determination should be repeated with diluted urine. More dilute standards are often required but with pregnancy urines the colours obtained are usually too intense to be compared with the standard and the following volumes of sample are diluted to 25 ml. for analysis: urines of s.g. 1·005 to 1·010, 20 ml.; 1·010 to 1·020, 15 ml.; 1·020 to 1·028, 10 ml.; 1·028 and higher, 5 ml. Within the usual limits of colorimetric methods the intensity of colour produced by the test is proportional to the histidine present.

Discussion. The reaction between histidine and bromine is affected by so many different environmental factors that it is advisable to ascertain from time to time that maximal colours are being obtained by comparing them with artificial standards composed of indicators dissolved in specified media in accordance with the data presented in Table XV. The diluted colour standards should not be more than 2 days old as the methyl red fades. In a test such as this a high degree of precision cannot be expected but the method has the advantage of being reasonably selective. The only substances, other than histidine, which have been found to give the reaction are histamine, which produces a yellow, and methylhistidine (α-amino-β-methylimidazolepropionic acid), which yields a pale red of about a fifth the intensity of that given by histidine.

TABLE XV. ARTIFICIAL STANDARDS APPROXIMATELY MATCHING THE COLOURS PRODUCED IN THE DETERMINATION OF HISTIDINE (After W. D. Langley, *J. Biol. Chem.*, 1941, **137**, 255)

The stock indicator solution consists of bromo-cresol green, 0·04 per cent., 5 ml.; methyl red, 0·02 per cent., 7 ml.; phenol red, 0·02 per cent., 1 ml.: all mixed and diluted to 50 ml. with a phthalate buffer (*p*H 4·6) made by adding 9·72 ml. of 0·2M sodium hydroxide to 50 ml. of 0·2M potassium hydrogen phthalate and diluting with carbon dioxide-free water to 200 ml.

Stock Indicator Solution ml.	Phthalate Buffer, *p*H 4·6 ml.	0·1N acid (HCl) or base (NaOH) ml.	Water (Carbon Dioxide-Free) ml.	Histidine equivalent mg.
6	8	0·7 base	1·3	1·0
3	5	0·1 acid	3·0	0·8
3	5	0·2 acid	6·3	0·6
3	5	0·2 acid	14·3	0·4

In an attempt to determine whether or not the histidine excretion may be used as an index of pregnancy, Langley[6] determined the specific gravity, creatinine and histidine content of 43 samples from male donors, 61 from non-pregnant females and 107 pregnancy urines. The ratios of creatinine to histidine, each in mg. per ml. of urine were determined, this being a convenient means of characterising the urines with regard to their histidine content. In this way, the samples, regardless of their water content, should fall into either of two groups: if the histidine concentration is relatively high, as in pregnancy, the urine would be characterised as having a low creatinine to histidine ratio as compared with that of the male or the non-pregnant female. It was found that during pregnancy the ratios range from 0·9 to 6·4 with an average value of 2·71 and in non-pregnancy from 2·5 to 17·7 with a mean approximating to 8·9. However, Langley concludes that the estimation of histidine in relation to creatinine of the urine cannot be recommended at present as a reliable test for pregnancy, as about 16 per cent. of the samples studied could not be definitely classified. Nevertheless, the method may well prove to be a useful aid for establishing pregnancy when used in association with other evidence.

REFERENCES

1. F. Knoop, *Beitr. chem. Physiol. Path.*, 1908, **11**, 356.
2. G. Hunter, *Biochem. J.*, 1922, **16**, 638.
3. R. Kapeller-Adler, *Biochem. Z.*, 1933, **264**, 131.
4. R. Kapeller-Adler, *Klin. Wochenschr.*, 1934, **13**, 21.
5. R. Kapeller-Adler, *Klin. Wochenschr.*, 1936, **15**, 1728.
6. W. D. Langley, *J. Biol. Chem.*, 1941, **137**, 255.

ICTERUS INDEX (OF BLOOD PLASMA OR SERUM)

The icterus index expresses the intensity of the colour of blood plasma or serum relative to that of a 0·01 per cent. aqueous solution of potassium dichromate. The test, which was developed by Meulengracht[1, 2] and Bernheim[3], serves as a rough clinical guide for observing the progression or retrogression of jaundice.

Method. An artificial standard is prepared by dissolving 0·1 g. of potassium dichromate in water containing 0·1 ml. of concentrated sulphuric acid and diluting the solution to 1 litre with water. The colour of this solution is arbitrarily taken as unity

(Index=1). Serum or plasma is obtained in the usual way and its colour compared with the standard by introducing each into separate tubes of identical bore. If the sample is more yellow than the standard, 1 ml. is taken and diluted with physiological saline solution (0·9 per cent. sodium chloride), adding 0·5 or 1 ml. at a time until the colour matches that of the standard. Thus, if 6 ml. of saline has to be added, then the plasma or serum will have been diluted 1 to 7 and the icterus index will be 7. If a colorimeter is used for making the colour comparison it is necessary that an approximate match be first obtained by the dilution method.

Method Using Permanent External Standards. The Lovibond Comparator series includes a disc covering the range from an index of 1 to an index of 17. In conducting a determination the sample should be diluted until its colour matches the glass with an index of 1. The other glasses should only be employed for the purpose of obtaining a rough estimate of the dilution that will be required ; often it will be found that the undiluted serum or plasma will have a different tint from that of the corresponding glass with the higher index.

Discussion. The icterus index of normal blood plasma ranges from 4 to 6. Although the test is a simple one there are certain sources of error, such as staining of the serum or plasma due to pigments other than bile, cloudiness of the serum, or fading of the standard. The latter trouble may be obviated by storing the dichromate solution in the dark and avoiding the use of old stocks. It is most important to prevent hæmolysis: to ensure this it is essential that apparatus used to collect the sample be quite dry, while excessive venous constriction should not be applied, neither should blood be squirted forcibly through the needle nor centrifuged at high speed lest hæmolysis result from mechanical injury to the corpuscles. While it is generally easier to employ serum for the test, if plasma is to be used care must be exercised to avoid undue excess of anti-coagulant. An alternative method consists in the precipitation of hæmoglobin, together with proteins, by adding to 1 vol. of plasma or serum, 1 or 2 vols. of acetone and, after filtering or centrifuging, making the appropriate dilutions of the clear filtrate or supernatant fluid with 67 per cent. aqueous solution of acetone instead of with saline. This method may be of service in cases where the serum is hazy due to lipæmia. Difficulties due to the latter cause, and to the occurrence of coloured pigments other than hæmoglobin or bile (carotinæmia),

may be minimised by taking the samples in the morning before the patient has broken the night's fast. If the icterus index exceeds 15, clinical jaundice is usually demonstrable but values as high as this have been reported in patients with carotinæmia who had no excess of bilirubin in their serum and it is therefore important that when high results are obtained by this test, they should be carefully correlated with other data.

REFERENCES

1. E. Meulengracht, *Deut. Arch. klin. Med.*, 1920, **132**, 285.
2. E. Meulengracht, *Ber. Ges. Physiol. expt. Pharmakol.*, 1921, **7** 65.
3. A. R. Bernheim, *J. Amer. Med. Assoc.*, 1924, **82**, 291.

LACTIC ACID (IN BLOOD)

The existing colorimetric methods for the determination of lactic acid depend upon the preliminary formation of acetaldehyde by the action of warm sulphuric acid and a colour reaction for the detection of the aldehyde. Several reagents have been suggested for the latter purpose, including thiophen[1, 2], codeine[3, 4], guaiacol[5], hydroquinone[6] and phenyl-phenol[7–10], but by far the greater proportion of the investigations have been directed to the use of veratrole (*o*-dimethoxy-benzene)[11–21] which was originally suggested by Mendel and Goldscheider[11]. This method, as modified by R. F. Milton[19], is described below.

Method[19]. The sample is collected in a tube containing ammonium fluoride sufficient to allow of there being 1 mg. per ml. of the salt present, and then 1 ml. of the blood is transferred to a centrifuge tube and 3 ml. of water added. To this mixture, 1 ml. of a 10 per cent. aqueous solution of zinc sulphate, $ZnSO_4.7H_2O$, is added followed by the addition, drop by drop with shaking, of 1 ml. of 0·5N sodium hydroxide. After allowing to stand for a few minutes, the mixture is centrifuged for about 15 minutes, 3 ml. of the clear supernatant liquid transferred to another centrifuge tube provided with a 5-ml. graduation mark, 1 ml. of a 15 per cent. aqueous solution of copper sulphate, $CuSO_4.5H_2O$, added and, after adding 1 g. of finely powdered calcium hydroxide[22], the contents of the tube mixed by careful inversion and diluted to 5 ml. with water. After further shaking, the tube is allowed to stand for 30 minutes and then centrifuged. The

supernatant fluid is poured into a filter stick packed with a layer of acid washed asbestos and forced through the filter by gentle blowing. The first few drops of filtrate are discarded, the bulk collected in a convenient vessel, 1 ml. of the filtrate (equivalent to 0·1 ml. of blood) transferred to a clean dry test tube and 3 ml. of concentrated nitrogen-free sulphuric acid introduced down the side of the vessel, the contents carefully mixed, the tube placed in a boiling water-bath for 5 minutes and then cooled to 20° C. After the addition of 0·15 ml. of a 0·125 per cent. solution of veratrole in absolute alcohol the contents are again mixed and the tube is immersed for 20 minutes in a beaker of water maintained at 20° C. The intensity of the magenta colour produced is then compared in a colorimeter with that of suitable standards developed at the same time as the test.

In order to prepare a standard solution, 0·171 g. of calcium lactate, $CaC_6H_{10}O_6.5H_2O$, is dissolved in water and the solution diluted to 100 ml.; this solution will contain the equivalent of 1 mg. per ml. lactic acid. From this standard a series of dilutions are made containing from 0·005 to 0·03 mg. per ml. and 1 ml. of each of these is treated in the manner described above, starting with the cautious addition of 3 ml. of sulphuric acid.

Discussion. The reaction of veratrole with the acetaldehyde complex appears to be continuous but the colour development tends to slow down for a period between 15 and 25 minutes from the time of its commencement. A high degree of accuracy cannot be expected from any of the known procedures for the colorimetric determination of lactic acid, possibly owing to variations in the initial reaction with sulphuric acid. Clearly, the acetaldehyde formed behaves in an atypic manner, since, although it normally distils at 21° C., the acid mixture is heated to 100° C. without any apparent loss of aldehyde. The amount of veratrole specified in the above procedure is insufficient for quantities of lactic acid above 0·03 mg. and if more is present initial dilutions should be made before proceeding with the colour reaction. Elgart and Harris[21] state that by this method they could only obtain recoveries of lactic acid equivalent to about 79 per cent. of that originally added but, on the other hand, Milton tested the procedure against the volumetric method of von Fürth and Charnass[23] as modified by Friedemann and Kendall[24] and obtained results which were never more than 7 per cent. lower; the same investigators also criticise the adoption of Somogyi's zinc hydroxide method[25] for the removal of the blood proteins and

prefer the use of trichloracetic acid but their procedure is extremely complicated and even if greater accuracy is attainable it is questionable if, in this instance, the end justifies the means.

Human blood normally contains between 5 and 35 mg. of lactic acid per 100 ml. but during severe exercise the concentration may rise to 200 mg. per 100 ml.

REFERENCES

1. W. Fletcher and F. Gowland Hopkins, *J. Physiol.*, 1907, **35**, 247.
2. C. N. H. Long, *J. Physiol.*, 1924, **58**, 455.
3. L. Chelle, *Bull. Soc. pharm. Bordeaux*, 1914, **53**, 1.
4. G. Servantie, *Compt. rend. Soc. Biol.*, 1925, **92**, 700.
5. G. A. Harrop, Jr., *Proc. Soc. Expt. Biol. Med.*, 1919, **17**, 162.
6. Z. Dische and D. Laszlo, *Biochem. Z.*, 1927, **187**,, 344.
7. E. Eegriwe, *Z. anal. Chem.*, 1933, **95**, 323.
8. B. F. Miller and J. A. Muntz, *J. Biol. Chem.*, 1938, **126**, 413.
9. R. H. Koenemann, *J. Biol. Chem.*, 1940, **135**, 105.
10. S. B. Barker and W. H. Summerson, *J. Biol. Chem.*, 1941, **138**, 535.
11. B. Mendel and I. Goldscheider, *Biochem. Z.*, 1925, **164**, 163.
12. M. Vas and A. Lang, *Biochem. Z.*, 1927, **172**, 428.
13. A. Hansen, O. Riesser and T. Nagaya, *Biochem. Z.*, 1928, **196**, 301.
14. H. Jungman, *Biochem. Z.*, 1928, **201**, 258.
15. B. Mendel, *Biochem. Z.*, 1928, **202**, 390.
16. H. J. Fuchs, *Biochem. Z.*, 1930, **217**, 405.
17. B. J. Krÿgsman, *Arch. neérland. Physiol.*, 1933, **18**, 503.
18. R. Nordbö, *Biochem. Z.*, 1934, **271**, 213.
19. R. F. Milton, *Analyst*, 1936, **61**, 91.
20. F. Rappaport and I. Reifer, *Mikrochem. Acta*, 1937, **2**, 62.
21. S. Elgart and J. S. Harris, *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 758.
22. D. D. Van Slyke, *J. Biol. Chem.*, 1917, **32**, 455.
23. O. von Fürth and D. Charnass, *Biochem. Z.*, 1910, **26**, 199.
24. T. E. Friedemann and A. I. Kendall, *J. Biol. Chem.*, 1929, **82**, 23.
25. M. Somogyi, *J. Biol. Chem.*, 1930, **86**, 655.

METALS (IN ORGANIC MATERIAL, BODY FLUIDS AND TISSUES)

See Section I—Metals :—

Arsenic, p. 22. Copper, p. 49. Iron, p. 53. Lead, p. 64. Manganese, pp. 76 and 78. Mercury, p. 81. Potassium, p. 98. Sodium, p. 105. Tin, p. 110. Zinc, p. 122.

NON-PROTEIN NITROGEN (IN BLOOD)

In order to conduct this determination the proteins of laked blood, or plasma, are precipitated with trichloracetic acid, the precipitate is filtered off and part of the filtrate digested with sulphuric acid until all the nitrogen is converted into ammonium

sulphate. The proportion of ammonium salt is then determined colorimetrically with Nessler's reagent, excess of the latter being employed in order to neutralise the sulphuric acid. The following technique has been recommended by E. J. King, *et al.*[1, 2].

Method[1, 2]. A sample (0·2 ml.) of the blood, or plasma, is pipetted into 3·2 ml. of a 3·0 per cent. w/v aqueous solution of sodium sulphate, $Na_2SO_4.10H_2O$, and 0·3 ml. of a 10 per cent. aqueous solution of sodium tungstate and 0·3 ml. of 2/3N sulphuric acid are added. The tube containing the mixture is stoppered, well shaken, and after 5 minutes the proteins are separated by filtration through a dry paper ; then 1 ml. of the filtrate (equivalent to 0·05 ml. of the sample) is transferred to a convenient tube and evaporated with 0·5 ml. of 30 per cent. v/v sulphuric acid until the liquid turns dark and white fumes are evolved. The cooled liquid is then treated with 1 drop of 30 per cent. hydrogen peroxide to destroy any coloured products and boiled for about 5 minutes. To the cooled solution is added 5 ml. of water and 3 ml. of Nessler's reagent made according to the formula given in the description of the method for determining urea in blood (see p. 279). The brownish-yellow colour produced is matched against standards made by diluting appropriate quantities (from 1 to 5 ml.) of an ammonium chloride solution containing 0·01 mg. N per ml. to 5 ml. with water and adding 3 ml. of Nessler's reagent. The standard ammonium chloride solution is prepared by dissolving 0·153 g. of the pure salt in water, making up to 100 ml. and diluting 25 ml. of this solution previously mixed with 10 ml. of N sulphuric acid to 1 litre.

Discussion. The nitrogen of the proteins of blood constitutes about 98 to 99 per cent. of the total, the remaining 1 or 2 per cent. being termed non-protein nitrogen, or N.P.N., because it is the nitrogen in the filtrate after precipitating the proteins. The non-protein nitrogen includes the nitrogen of urea, uric acid, creatinine, creatine, ammonia, amino-acids and that contained in unspecified nitrogenous bodies which is usually termed " rest nitrogen," but it does not include lipoid nitrogen since lipoids are precipitated along with the proteins. Urea nitrogen accounts for roughly half (33 to 65 per cent.) of the non-protein nitrogen of normal blood while ammonia nitrogen occurs in such low concentration (0·1 to 0·2 mg. per 100 ml.) that it may generally be disregarded. Hammett[3] studied the nitrogen partition of the whole-blood of 60 normal individuals 3 hours after a meal and his observations indicated that urea nitrogen constitutes about

48 per cent. of the total non-protein nitrogen, the corresponding percentage proportions of nitrogen for the other nitrogenous constituents being creatinine 1, creatine 4, uric acid 2, amino-acid 14 and " rest nitrogen " 31. Researches by numerous investigators have all gone to show that in diseases accompanied by an increase in non-protein nitrogen most of the additional nitrogen occurs as urea, the absolute amount of the " rest nitrogen " being hardly altered, whence a determination of the concentration of urea gives about the same clinical guidance as the figure for non-protein nitrogen. The normal range of the latter is from about 25 to 40 mg. per 100 ml. of blood. The determination is often conducted on a larger scale using 2 ml. of sample, the ammonia being distilled from a Pregl-Kjeldahl apparatus into centinormal acid, the excess of the latter being determined by titration with alkali of corresponding strength.

REFERENCES

1. E. J. King, G. A. D. Haslewood and G. E. Delory, *Lancet*, 1937, **232**, 886.
2. E. J. King, G. A. D. Haslewood, G. E. Delory and D. Beal, *Lancet*, 1942, **242**, 207.
3. F. S. Hammett, *J. Biol. Chem.*, 1920, **41**, 611.

PHENOLS

See Section V : Miscellaneous Substances, p. 408.

PHENYLPYRUVIC ACID (IN URINE)

The presence of this substance in the urine is associated with metabolic disease and, so far, has only been found in cases of mental deficiency. The following simple technique for its approximate determination is due to Penrose and Quastel[1].

Method[1]. The sample is filtered and 5 ml. treated with 5 ml. of a freshly filtered saturated solution (approximately 0·28 per cent.) of 2:4-dinitrophenylhydrazine in N hydrochloric acid and the mixture shaken. If phenylpyruvic acid is present a yellow opalescence or precipitate forms almost immediately. After standing at room temperature for 30 minutes, the mixture is again shaken and 2 ml. transferred to a tube containing 4 ml. of

N sodium hydroxide and after allowing to stand for a few minutes the alkaline liquid is added to 24 ml. of water. The intensity of any persistent red colour which is produced (normal urine only yields a transient red, quickly fading to light yellow under these conditions) is matched against standards similarly prepared from normal urine containing known amounts of added phenylpyruvic acid, from 1 to 5 mg. giving a suitable intensity of colour.

Method Using Permanent External Standards. A disc containing coloured glass standards ranging from 20 to 100 mg. of phenylpyruvic acid per 100 ml. and based upon the above procedure is issued for use with the Lovibond Comparator. In this instance the reaction mixture is introduced into a 0·5-cm. cell and not into a test tube while a glass standard approximately corresponding to the colour obtained with normal urine is included in the disc. In cases where the concentration of phenylpyruvic acid exceeds 100 mg. per 100 ml. the determination should be repeated after suitably diluting the sample with water.

Discussion. Before carrying out the above procedure it is important to ensure the absence of acetoacetic acid by applying Rothera's modification[2] of Legal's sodium nitroprusside test[3] as this substance reacts similarly to phenylpyruvic acid. So far as is known the presence of glucose or protein does not interfere but the development of alkalinity in the sample quickly decomposes the ketonic acid. If the examination cannot be carried out at once the urine should be preserved by the addition of a few crystals of thymol and stored in an ice chest.

The presence of phenylpyruvic acid in the urine of mentally deficient patients was first observed by Fölling[4] who noticed that such samples give a transient green colour with ferric chloride. Penrose and Quastel found that it is not essential to collect 24-hour samples since the excretion continues with fair regularity and in a particular case of a female imbecile, 126 mg. of phenylpyruvic acid per 100 ml. was found, equivalent to a daily total of about 1 g. In another typical example, the urine of a male idiot was found to contain between 40 and 50 mg. per 100 ml. The condition, which it is proposed to term phenylketonuria, appears to be connected with defective metabolism and it was found that the excretion of phenylpyruvic acid by a mentally deficient person could be increased by adding phenylalanine to the diet whereas the urine of normal people undergoing the same treatment does not give any reaction for phenylpyruvic acid. The condition is familial: more than one child in a family may be affected but the

parents and remaining children are usually normal. Genetically, the abnormality appears to be inherited as a single Mendelian recessive characteristic.

REFERENCES

1. L. Penrose and J. H. Quastel, *Biochem. J.*, 1937, **31**, 266.
2. A. C. H. Rothera, *J. Physiol.*, 1908, **37**, 491.
3. E. Legal, *Breslauer Arztliche Z.*, 1883, **5**, 38.
4. A. Fölling, *Z. physiol. Chem.*, 1934, **227**, 169.

PHOSPHATASE (IN BLOOD PLASMA OR SERUM AND IN MILK)

Phosphatase is the name given to an enzyme which catalyses the scission, or formation, of linkages involving completely oxidised ortho-, meta-, or pyro-phosphoric acids. Among substrates for their activity are esters such as glycerophosphates, hexosephosphates, nucleotides and phenylphosphates. Phosphatases are widely distributed in the animal and vegetable kingdoms and their presence in mammalian organs, tissues and secretions has been established. R. Robison[1] has demonstrated the presence of phosphatase in bone and ossifying cartilage and has shown that the enzyme is intimately related with the process of bone formation. The blood plasma of normal individuals contains small amounts of phosphatase but in conditions of generalised bone disease the enzyme appears to leak out of the bone into the blood plasma[2, 3]. High concentrations are also found in the plasma of persons suffering from obstructive jaundice and, in a much lesser degree, in the toxic and infective varieties of the same disease[4–7]. Hence, the determination of the concentration of phosphatase in plasma has some clinical value, particularly in helping towards the differentiation of the various kinds of jaundice, although it is important that the results be carefully correlated with other diagnostic features. The original methods for the determination of phosphatase depended upon addition of the material to be tested to a standardised substrate consisting of a mono-ester of phosphoric acid, such as sodium β-glycerophosphate, either with or without addition of a buffer solution, followed by incubation at a chosen temperature for a definite period of time and subsequent determination of the liberated inorganic phosphate[8]. Later, it was shown by King

and Armstrong[9] that disodium phenylphosphate is quickly hydrolysed by the phosphatase of blood plasma and that the amount of phenol so liberated may be taken as a measure of the enzyme present. Not only is phenol more conveniently determined than phosphate but the amount liberated exceeds the latter by three times. A method for the examination of plasma or serum based upon this principle and using Folin and Ciocalteu's[10] phenol reagent is given here, the technique being a slight modification of that advocated by King, Haslewood and Delory[11].

In 1933 Graham and Kay[12] established the fact that phosphatase is always present in cow's milk, their work confirming earlier observations made by Demuth[13] and by Wilson and Hart[14]. In further investigations on milks from a great number of cows of different breeds, ranging over the whole lactation period, Folley and Kay[15] proved conclusively that phosphatase is invariably present in raw milk. While the enzyme appears to be stable for days when milk is kept under storage conditions it is destroyed with increasing rapidity as the temperature rises above body heat and extended observation has shown that when milk is pasteurised, that is to say, retained at a temperature of not less than 145° F. and not more than 150° F. for 30 minutes and immediately cooled to a temperature of not more than 55° F., the phosphatase is almost completely destroyed. It has also been shown by Kay and Graham that the thermostability of the enzyme is just slightly greater than that of the pathogenic organisms likely to occur in milk[16]. Consequently, by determining the phosphatase content of milk it is possible to distinguish between pasteurised and raw milk and, further, to detect defective pasteurisation, or, alternatively, the presence of added raw milk. Two procedures for the determination of phosphatase in milk are described here, the first being the widely used method of Kay and Graham[17] employing Folin and Ciocalteu's[10] reagent and the second, a modification due to Aschaffenburg and Neave[18] using the reagent suggested by Gibbs[19], 2:6-dibromoquinone chloroimide, for the colorimetric determination of the liberated phenol.

Method for Blood Plasma or Serum. The following reagents are required :—

1. Buffer Substrate made by dissolving 1·09 g. of disodium phenylphosphate and 10·3 g. of sodium barbitone (sodium diethyl barbiturate) in water and diluting to 1 litre. This

solution is 0·005M with respect to the phenylphosphate and the strength of the sodium barbitone is 0·05M.

2. Folin and Ciocalteu's Reagent. For details of the preparation, see p. 414. For use in the test for phosphatase this reagent is diluted with twice its volume of water.

3. An Aqueous Solution of Sodium Carbonate, 20 per cent. w/v Na_2CO_3. This should be stored in a warm place in order to prevent the salt from crystallising out.

4. Stock Standard Phenol Solution made by dissolving 1 g. of crystalline phenol in 0·1N hydrochloric acid and making up to 1 litre with the same solvent.

5. Diluted Standard Phenol Solution containing 0·1 mg. per ml. phenol is made by suitably diluting the stock solution.

6. Standard-Phenol-Solution-and-Reagent containing 0·01 mg. per ml. phenol is made by mixing 5 ml. of the diluted standard and 15 ml. of the diluted Folin and Ciocalteu's reagent and making up to 50 ml. with water. This solution should be made freshly each day.

In a conical centrifuge tube are placed 4 ml. of buffer substrate, the tube is immersed in a water-bath maintained at 38° C. and after 5 minutes, without removing the tube from the bath, exactly 0·2 ml. of plasma or serum (which must be cell free) is added and mixed. The tube is stoppered and allowed to remain in the bath for 30 minutes. At the end of this time 1·8 ml. of the diluted Folin and Ciocalteu's reagent is added and the mixture centrifuged or filtered through a 5·5-cm. paper. A control test is also prepared by introducing into another centrifuge tube 4 ml. of buffer substrate, 0·2 ml. of plasma and at once adding 1·8 ml. of the diluted Folin and Ciocalteu's reagent and centrifuging or filtering the mixture without previous heating. Exactly 4 ml. of the supernatant fluid, or filtrate from the test is transferred to a suitable tube and a similar amount from the control is introduced into a second tube and to each is added 1 ml. of the sodium carbonate solution and the tubes are then immersed in the water-bath at 38° C. for 10 minutes in order to develop the colour. The intensity of the blue colour produced in the test and in the control is compared by means of a suitable colorimeter with a standard colour made up at the same time by taking 4 ml. of the standard-phenol-solution-and-reagent (containing 0·01 mg. per ml. phenol) and 1 ml. of the 20 per cent. sodium carbonate solution. The phosphatase activity of a plasma is expressed as units per

100 ml. and is numerically equal to the number of mg. of phenol which would be set free from the phenylphosphate by 100 ml. of plasma under the standard conditions. If the determination is conducted as described above, the reading of the standard against the control deducted from the reading of the standard against the test will give the result in units of phosphatase per 100 ml.

Method for Blood Plasma or Serum Using Permanent External Standards. Glass standards to fit the above technique are available in a Lovibond Comparator disc which covers a range from 8 to 60 units of phosphatase per 100 ml. of plasma. In cases where the enzyme content of plasma or serum is above 60 units it is customary to dilute the sample with normal saline (0·9 per cent. w/v aqueous solution of sodium chloride) so that the number of units per 100 ml. of the diluted specimen will not be in excess of 60. This practice should be adopted irrespective of the method of colour measurement employed.

Discussion. The results obtained by the above procedure agree closely with those given by the method of King and Armstrong[9], of which it is a modification, and they also show satisfactory accord with the figures given by the original technique of Jenner and Kay[8]. The blood of normal individuals is found to contain 5 to 10 units of phosphatase per 100 ml. of plasma.

Method of Kay and Graham for Milk[17]. (*Long Test.*) The undermentioned reagents are required :—

1. Buffer Substrate * made by dissolving 1·09 g. of disodium phenylphosphate and 11·54 g. of sodium barbitone (sodium diethyl barbiturate) in water saturated with chloroform and diluting to 1 litre.
2. Folin and Ciocalteu's Reagent. This is made by the method described on p. 414. For use in the test for phosphatase the reagent is diluted with twice its volume of water.
3. An Aqueous Solution of Sodium Carbonate the strength of which should be equivalent to 14 per cent. w/v Na_2CO_3. The concentration of this solution should be fairly accurately adjusted.

To 10 ml. of the buffer substrate solution contained in a 25-ml. stoppered test tube is added 0·5 ml. of the sample of milk under

* The British Drug Houses, Ltd., issue buffer substrate tablets for the preparation of this solution. If these are used, 1 tablet is dissolved in 50 ml. of boiling water and, after allowing the solution to boil for exactly 1 minute, it is rapidly cooled and is then ready for use.

examination followed by 3 drops of chloroform. After mixing the contents, the tube is placed in an incubator maintained at 37°–38° C. and left for 24±2 hours. At the end of this time the liquid in the tube is cooled, 4·5 ml. of the diluted Folin and Ciocalteu's reagent added and, after mixing, the whole is allowed to stand for 3 minutes then filtered using a 9-cm. Whatman No. 40 paper. To 10 ml. of the filtrate is added 2 ml. of sodium carbonate solution (reagent No. 3 above), the tube immersed in a boiling water-bath for 5 minutes and then again filtered. The filtrate is transferred to a 13-mm. cell and the colour measured by means of a Lovibond tintometer. In expressing the intensity of the colour, account should only be taken of the blue units although, in practice, small values on the slides carrying the red and yellow glasses are needed to effect a good match. Alternatively, the colour may be assessed by the use of an All Purposes Lovibond Comparator or in a B.D.H. Lovibond Nessleriser in conjunction with the special discs appertaining to the phosphatase test for milk.

A control test, omitting the incubation, should always be conducted either by storing the filtrate obtained after adding the diluted Folin and Ciocalteu's reagent in a refrigerator until the next day or by similarly storing a portion of the milk under examination and carrying out the test, apart from the incubation, on the second day. The colour of the final filtrate derived from the control test should not exceed 1·5 Lovibond blue units. Blank tests should also be made on each fresh batch of reagents without adding any milk but including the incubation treatment and such trials should not yield colour readings in excess of 0·5 Lovibond blue unit.*

(*Short Test*). This is carried out as above excepting that instead of incubating for 24 hours at 37° C. the tube containing the buffer substrate solution with the milk is immersed in a water-bath maintained at 45° to 49° C. for 10 minutes. The remainder of the procedure, both for the determination and the control test is identical with that described for the *long test*.

* In an Addendum (1943) to the Memorandum 139/Foods issued by the Ministry of Health under the Milk (Special Designations) Order, 1936 (S. R. & O., 1936, No. 356), a technique for carrying out the phosphatase test on heat-treated milk is given. This differs from that described above in two particulars only:

(i) the test solution is prepared by diluting the phenol reagent with twice its volume of a 5 per cent. w/v aqueous solution of sodium hexametaphosphate instead of with twice its volume of water; and

(ii) in the production of the final colour, the heating in a boiling water-bath is continued for 2 minutes only.

This has since been incorporated in the Milk (Special Designations) Regulations, 1946 (S. R. & O., 1946, No. 10).

Discussion. In order to comply with the milk (Special Designations) Order, 1936 (Statutory Rules and Orders, 1936, No. 356), milk that is labelled " pasteurised " must have been held at a temperature of not less than 145° F. and not more than 150° F. for 30 minutes and immediately cooled to a temperature of not more than 55° F. Considering first the interpretation of the *short test*, if the control shows more than a trace of blue colour and the reagents contain no free phenol, it is probable that a phenol-producing organism is present in the milk. This does *not* occur in

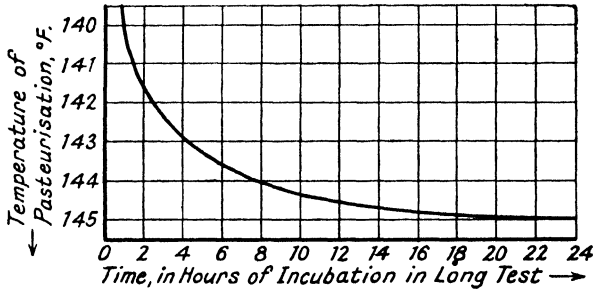


FIG. 13.—RELATION BETWEEN TEMPERATURE OF PASTEURISATION AND TIME OF INCUBATION

Curve showing the time during which milk pasteurised at different temperatures may be incubated with the phosphoric ester under the conditions of the *long test* without producing more than the standard colour of 2·3 Lovibond blue units. (H. D. Kay and W. R. Graham, Jr., *J. Dairy Res.*, 1935, **6**, 191.)

pasteurised milk which has been kept at a satisfactorily low temperature following pasteurisation. With fresh, properly pasteurised milk, that is, milk which has been cooled to 55° F. immediately after pasteurisation and maintained between that temperature and 65° F. for not more than 18 hours, the control tube should show only a trace of blue colour. If with such a control the incubated tube shows a definite blue colour, *i.e.* over 2·3 Lovibond blue units, it can be said with certainty that the milk has not been adequately pasteurised, and if the colour is over 6·0 blue units, the milk has probably not been heated at all. With some experience, it will be possible to omit control tubes for the majority of samples submitted to this test.

While the production of a colour exceeding 2·3 blue units may be taken as evidence of imperfect pasteurisation or admixture of raw milk, the converse is not invariably true. For example, if the milk has been heated at 140° F. instead of at (or above) the statutory minimum temperature of 145° F. the colour produced in the *short test* will not exceed 2·3 blue units. In cases where the

colour approaches very near to 2·3 blue units, or where more accurate information is required regarding the efficiency of pasteurisation, and generally, when time permits, the *long test* should be applied.

Considering the results of the *long test*, milks which, with controls below 1·5 blue units, give colours below 2·3 units may be classified as properly pasteurised. Those giving colours between 2·3 and 6·0 blue units should be considered as improperly pasteurised, while those giving colours of greater intensity than 6·0 blue units show evidence of serious errors in pasteurisation performance. Raw milk usually gives more than 30 units of colour. It is not possible by means of the phosphatase test alone to decide the cause of a high figure. All that can be said is that the milk has not been correctly pasteurised. The *long test* is capable of detecting with certainty the following faults :—

(*a*) Holding at 145° F. for 20 minutes instead of 30 minutes

(*b*) Holding at 143·5° F. (or less) instead of at 145° F.

(*c*) Admixture of 0·25 per cent. of raw milk with correctly pasteurised milk.

The minimum statutory temperature of pasteurisation in the United States of America varies from State to State and may be as low as 140° F. In Canada the range allowed is 140° to 145° F., and exposure of the milk to a temperature of 140° F. for a period of 30 minutes is generally accepted as being sufficient to destroy any pathogenic organisms present, but is insufficient to completely destroy the phosphatase. By decreasing the time of incubation, however, the *long test* can be adapted to suit American and Canadian standards. Thus, no milk which has been pasteurised at 140° F. for 30 minutes will give a blue colour in the *long test* greater than 2·3 Lovibond blue units provided the incubation time does not exceed 1 hour but many samples will give colours greater than 2·3 units when incubated for 75 minutes. A decrease in the incubation time renders the test less sensitive to the detection of the presence of traces of raw milk. Fig. 13 is a graph indicating the duration of the incubation in the *long test* corresponding to the temperature at which the milk is alleged to have been pasteurised. In all cases, if the blue colour which is developed exceeds 2·3 blue units the milk has not been heated as claimed or has been improperly handled.

An alternative way of pasteurising milk, i.e. by heating at higher temperatures, for example, at 160° to 167° F. for short

" holding " periods of from 10 to 20 seconds, is permissible in the United States. Several investigators have studied the response of the phosphatase test to this method of pasteurisation and it has appeared that only rather gross errors in time and temperature will be detectable if processing at 167° F. for 20 seconds is adopted as standard treatment. However, the test promises to give full satisfaction when pasteurisation is conducted at temperatures between 160° and 162° F. for 15 to 20 seconds and, fortunately, this milder treatment is now more generally favoured * as it is realised that the commercial properties of milk (cream line, etc.) are less seriously affected while it has been shown that there is still an adequate margin to allow of the complete destruction of pathogenic flora.

Method of Aschaffenburg and Neave for Milk[18]. The following reagents are required for this procedure :—

1. Buffer Substrate which must be freshly prepared by dissolving 0·11 g. of disodium phenylphosphate in about 80 ml. of water, adding 3 ml. of a 2·65 per cent. aqueous solution of sodium carbonate, Na_2CO_3, and diluting to 100 ml. with more water. Chloroform should not be added to this reagent.
2. Gibbs' Phenol Reagent made by dissolving $23 \pm 0{\cdot}5$ mg. of 2 : 6-dibromoquinone chloroimide in 5 ml. of absolute ethyl alcohol. This solution will keep for a week at room temperature or for about a month if stored below 7° C. away from light.

In order to conduct the test 5 ml. portions of the buffer substrate are introduced into each of two test tubes (150 mm. long by $13{\cdot}5 \pm 0{\cdot}5$ mm. internal diameter) and into one tube 0·5 ml. of the milk to be examined is introduced. The tubes are closed with rubber stoppers (which should be kept in water and boiled out before use) and, after being inverted about ten times, are introduced into an incubator at 37° to 38° C. for 1 hour. The tubes are then removed and, before the contents have been allowed to cool below 30° C., 5 drops (50 ± 2 drops=1 ml.) of Gibbs' phenol reagent is added to the test mixture and 1 drop of the same to the blank. The contents of the tubes are mixed, first by giving them a circular motion, and then by replacing the stoppers and turning

* In accordance with Provisional Regulations, dated 9th July, 1941, made by the Minister of Health under the Food and Drugs Act, 1938 (1 & 2 Geo. 6, c. 56), which may be cited as the Milk (Special Designations) Regulations, 1941, it is now permissible to pasteurise milk in this country by holding it at a temperature of not less than 162° F. for at least 15 seconds and immediately cooling to a temperature of not more than 55° F.

up and down ten times. The stoppers are again removed and 2 ml. of normal butyl alcohol (previously neutralised if necessary) added and the stoppers replaced. The contents of the tubes are cooled to 15° to 20° C. and after about 7 minutes the indophenol colour is gradually transferred to the alcoholic layer by slowly inverting the tubes eight times (four complete turns), the immiscible liquids being allowed to separate between each inversion.

In addition to the blank tests which should be carried out with each batch of milk samples under examination it is necessary to perform a control test in all cases in which it is suggested that the colour development in the test proper might be due to causes other than milk phosphatase. To do this, 0·5 ml. of the milk is added to 5 ml. of a solution made by diluting 3 ml. of a 2·65 per cent. aqueous solution of sodium carbonate, Na_2CO_3, to 100 ml. with water, incubating at 37° to 38° C. for 1 hour and then proceeding as already described using 5 drops of Gibbs' reagent. Normal milk should give a colourless or yellowish butyl alcohol layer. A green or blue colour indicates the presence of phenol.

The intensity of the indophenol colour may be assessed by means of the Lovibond tintometer using a $\frac{1}{2}$-inch cell or by comparing it with artificial standards made in accordance with the data given in Table XVI. In the former case the butyl alcohol

TABLE XVI. ARTIFICIAL COLOUR STANDARDS FOR USE IN ASCHAFFENBURG AND NEAVE'S PHOSPHATASE TEST ON MILK (After H. Scharer.)

Standard	*Blue* Copper Sulphate ($5H_2O$), 30 per cent. in 1 per cent. hydrochloric acid vols.	*Yellow* Ferric Chloride ($6H_2O$), 4·51 per cent. in 1 per cent. hydrochloric acid vols.	*Red* Cobalt Chloride ($6H_2O$), 5·96 per cent. in 1 per cent. hydrochloric acid vols.	Water vols.
1	4·6	3·0	0·9	1·5
2	5·5	2·7	0·8	1·0

layer is syphoned off and transferred to the cell but, as 2 ml. is insufficient to give the depth of liquid required to cover the opening of the tintometer, it is necessary to perform the phosphatase test in duplicate and to combine the butyl alcohol layers. The indophenol colour is matched by the use of blue, yellow and neutral glasses. The results should be interpreted in accordance with the scheme presented in Table XVII. In the blank test on

TABLE XVII. INTERPRETATION OF THE RESULTS OBTAINED IN ASCHAFFENBURG AND NEAVE'S PHOSPHATASE TEST ON MILK

Colour of Butyl Alcohol Layer	Lovibond Blue Units	Blue Compared with Artificial Colour Standards	Classification of Sample
Yellow or greenish-yellow	2·0	Less than 1	Properly pasteurised
Yellowish-green . .	2·0 to 2·5	Between 1 and 2	Doubtful
Green or bluish-green .	2·5	More than 2	Under-pasteurised

the reagents a green or blue colour should not develop either before or after extraction with butyl alcohol. If, in the course of the test, a blue or grey colour is produced in the reaction mixture before extraction with butyl alcohol gross errors in pasteurising technique or a serious contamination with raw milk is indicated.

Discussion. The above procedure is a modification of the method originated by H. Scharer[20–22]. The need for paying meticulous attention to details of technique must be accounted a disadvantage but, nevertheless, Scharer's test, or modifications of it, find much favour in the United States. A quick test to distinguish raw from heated milk can be obtained by adding 5 drops of dibromoquinone chloroimide solution to a mixture of 5 ml. of buffer substrate and 0·5 ml. of milk at room temperature. In the presence of raw milk a bluish colour develops within less than 3 minutes.

Note on the Application of the Phosphatase Test to Dairy Products Other than Milk. Investigations have been undertaken with a view to utilising the phosphatase test for the control of pasteurisation as applied to cream. However, several factors demand careful consideration, among which may be mentioned the possible protective action of the fat, the higher viscosity of cream and the fact that the concentration of the enzyme in raw cream is much higher than in milk. Experiments so far conducted have yielded somewhat conflicting results and there can be little doubt that some modifications in technique are necessary in order to use the phosphatase test as a reliable and sensitive method of controlling the efficiency of cream pasteurisation. It seems unlikely that this can be achieved by simply altering the colour standard, since cream may vary considerably in composition and because a close relationship undoubtedly exists between butter fat content and phosphatase activity. If it can be proved that these properties are fairly closely proportional to each other, then it might be satisfactory to recommend diluting cream with water to a butter-

fat content of 4 to 6 per cent. and then treating it similarly to milk[23].

It has been shown[16, 17, 23] that the Kay and Graham short test will clearly distinguish butter made from pasteurised cream and that originating from raw cream. The technique evolved for milk is applied to butter serum prepared either by simply melting and centrifuging the sample or by adding water to butter contained in a large test tube in the proportion of 1 to 2, melting and cooling the stoppered, inverted tube in a refrigerator until the fat has solidified.

Preliminary work has shown that the phosphatase test can be applied to cheese by intimately mixing, say, 20 g. of the sample with 20 ml. of buffer and an equal amount of water and submitting 1 ml. of this mixture to the full test as if it were milk. No reduction in enzyme activity of cheese made from raw milk occurs after 18 months' storage whereas cheese made from pasteurised milk gives no phosphatase reaction.

The whole subject of the application of the phosphatase test to milk and other dairy products is comprehensively discussed by H. D. Kay, R. Aschaffenburg and F. K. Neave in Technical Communication No. 1 (1939) of the Imperial Bureau of Dairy Science, Shinfield, near Reading, England.

REFERENCES

1. R. Robison, *Biochem. J.*, 1923, **17**, 286.
2. H. D. Kay, *Brit. J. Expt. Path.*, 1929, **10**, 253.
3. C. C. Franseen, C. C. Simmons and R. McLean, *Surg. Gyn. Obstet.*, 1939, **68**, 1038.
4. W. M. Roberts, *Brit. Med. J.*, 1933, i, 734.
5. A. R. Armstrong, E. J. King and R. I. Harris, *Canad. Med. Assoc. J.*, 1934, **31**, 14.
6. A. R. Armstrong and E. J. King, *Canad. Med. Assoc. J.*, 1935, **32**, 379.
7. F. K. Herbert, *Brit. J. Expt. Path.*, 1935, **16**, 365.
8. H. D. Jenner and H. D. Kay, *Brit. J. Expt. Path.*, 1932, **13**, 22.
9. E. J. King and A. R. Armstrong, *Canad. Med. Assoc. J.*, 1934, **31**, 376.
10. O. Folin and V. Ciocalteu, *J. Biol. Chem.*, 1927, **73**, 627.
11. E. J. King, G. A. D. Haslewood and G. E. Delory, *Lancet*, 1937, **232**, 886.
12. W. R. Graham, Jr., and H. D. Kay, *J. Dairy Res.*, 1933, **5**, 54.
13. F. Demuth, *Biochem. Z.*, 1925, **159**, 415.
14. L. T. Wilson and E. B. Hart, *J. Dairy Sci.*, 1932, **15**, 116.
15. S. J. Folley and H. D. Kay, *Enzymologia*, 1936, **1**, 48.
16. H. D. Kay and W. R. Graham, Jr., *J. Dairy Res.*, 1933, **5**, 63.
17. H. D. Kay and W. R. Graham, Jr., *J. Dairy Res.*, 1935, **6**, 191.
18. R. Aschaffenburg and F. K. Neave, *J. Dairy Res.*, 1939, **10**, 485.
19. H. D. Gibbs, *J. Biol. Chem.*, 1927, **72**, 649.
20. H. Scharer, *J. Dairy Sci.*, 1938, **21**, 21.
21. H. Scharer, *J. Milk Tech.*, 1938, **1**, 35.
22. H. Scharer, *J. Milk Tech.*, 1939, **2**, 16.
23. H. D. Kay and F. K. Neave, *Dairy Ind.*, 1937, **2**, 5.

PHOSPHATES

See Section II : Acid Radicles, p. 158.

PROTEINS (IN BLOOD SERUM, PLASMA, URINE, CEREBROSPINAL FLUID, ETC.)

Two methods are available for the colorimetric determination of proteins ; the one involves preliminary wet oxidation of the precipitated proteins followed by a determination of the resulting ammonium salt using Nessler's reagent and the other is a quantitative modification of the biuret test. Procedures for the application of both these methods to blood plasma, or serum, are given and of the biuret method to urine and cerebrospinal fluid. Plasma protein is divided into two main fractions namely, globulin (including fibrinogen) and albumin and when making determinations it is usual to include an estimate of the proportions of each kind present, advantage being taken of the fact that the first mentioned fraction is precipitated by saturated aqueous solutions of salts such as ammonium or sodium sulphate. The technique below for applying the wet oxidation principle follows that recommended by King, Haslewood and Delory[1].

J. L. Kantor and W. J. Gies[2] were the first to propose the application of the biuret test for the colorimetric determination of proteins and later Autenrieth and Mink[3, 4] applied the method to the examination of the body fluids. The idea has not been widely adopted owing to the difficulty of preparing suitable standard protein solutions of reasonable permanence. However, some progress has been made in this direction and two procedures are described : the first, employing the Lovibond Comparator with external glass standards ; the second, a modified technique using as standard a fairly stable solution of serum made from rabbit blood which is evaluated by the Kjeldahl method.

Wet Oxidation Method[1]. *Total Proteins.* A measured quantity (0·2 ml.) of plasma derived from oxalated blood is transferred to a 10-ml. graduated flask and diluted to 10 ml. with isotonic sodium chloride solution (0·9 g. NaCl per 100 ml.). Of this solution, 0·2 ml. (equivalent to 0·004 ml. of the sample) is introduced into 4 ml. of water contained in a Pyrex glass centrifuge tube,

0·1 ml. of a 10 per cent. aqueous solution of zinc sulphate, $ZnSO_4.7H_2O$, added followed by 0·1 ml. of 0·5N sodium hydroxide and the mixture centrifuged. After the supernatant liquid has been carefully decanted, the inverted tube is drained on a filter paper then 0·5 ml. of a 30 per cent. v/v aqueous solution of sulphuric acid is added, together with a small piece of porous pot. The mixture is gently boiled until blackening occurs and white fumes appear, allowed to cool, 1 drop of hydrogen peroxide (30 per cent.) added and again boiled for 4 minutes. To the cooled, colourless solution is added 5 ml. of water and 3 ml. of Nessler's reagent made according to the formula given in the description of the method for determining urea in blood (see p. 279). The brownish-yellow colour produced is matched against standards made by diluting appropriate quantities (from 1 to 5 ml.) of ammonium chloride solution containing 0·01 mg. N per ml. to 5 ml. with water and adding 3 ml. of Nessler's reagent. The standard ammonium chloride solution is prepared in the same manner as given in the method for determining non-protein nitrogen in blood (see p. 246). The result is multiplied by 6·25 in order to convert nitrogen into proteins.

Fibrin. To 5 ml. of the solution of plasma in isotonic sodium chloride, as used above for determining the total proteins and contained in a narrow tube, is added 0·1 ml. of calcium chloride solution and the mixture maintained at a temperature of 37° C. until clotting occurs. The fibrin is collected on a thin glass rod, pressed to remove liquid, washed with water and dropped into a test tube preparatory to applying the wet oxidation procedure. This, and also the colorimetric comparison, is conducted in the manner already described above for determining total proteins. The amount of solution prescribed above is equivalent to 0·1 ml. of the original sample.

Albumin. A measured quantity (0·2 ml.) of plasma derived from oxalated blood is transferred to a 5-ml. graduated flask and diluted to the mark with a 22 per cent. aqueous solution of sodium sulphate, Na_2SO_4. The mixture is maintained at a temperature of 37° C. for 3 hours, then filtered through a fine filter paper and 0·2 ml. of the filtrate (equivalent to 0·008 ml. of the original sample) is added to 4 ml. of water contained in a Pyrex glass centrifuge tube. The protein is precipitated with zinc sulphate and sodium hydroxide, separated, wet oxidised and the colour comparison made in the same way as described for total proteins.

Globulin. The proportion of this protein is derived by deduct-

ing the sum of the fibrin and albumin from the value found for the total proteins.

Discussion. The quantity of total proteins varies in normal individuals from about 6 to 8 g. per 100 ml. The concentrations of the different proteins in plasma are approximately: albumin, 3·4 to 6·0 g. per 100 ml.; globulin (excluding fibrinogen) 1·5 to 3·0 g. per 100 ml.; fibrinogen, 0·2 to 0·4 g. per 100 ml. Where there is a decrease of plasma proteins, as a result, for example, of proteinuria or malnutrition, the albumin is chiefly affected and there is often a reduction of the albumin-globulin ratio (normally 1·3 to 4·0). A reduction of this kind is characteristic of nephrosis while an increase in the globulin, especially fibrinogen, may accompany inflammatory conditions.

Biuret Method Using Permanent External Standards (*for Blood Serum or Plasma*). For the determination of total proteins in serum (albumin and globulin) or in plasma (albumin, globulin and fibrinogen) 0·2 ml. of the sample is transferred to a graduated 15-ml. centrifuge tube, 4·8 ml. of water added followed by 5 ml. of a 10 per cent. aqueous solution of trichloracetic acid. After allowing to stand for a few minutes the mixture is centrifuged and the clear supernatant fluid decanted from the precipitate. The tube is drained by inverting it upon filter paper then 1 ml. of water and 1 ml. of a 30 per cent. aqueous solution of sodium hydroxide added, the mixture thoroughly agitated until the protein has dissolved, 1 ml. of a 5 per cent. aqueous solution of copper sulphate, $CuSO_4.5H_2O$, added and sufficient water to bring the volume of the liquid to exactly 10 ml. The mixture is well shaken for at least a minute then centrifuged and the clear supernatant liquid transferred to a Comparator tube and the latter placed in the right-hand compartment of the instrument. The violet-coloured liquid is matched against the permanent glass standards. Two discs are available which are marked from 20 mg. to 360 mg. of protein per 100 ml. calculated on the basis that 5 ml. of the sample is taken for the test; hence, for 0·2 ml. of serum or plasma the disc readings are multiplied by 25. The glass standards have been prepared by applying the biuret test to samples of diluted human serum, the protein content of which was determined by the Kjeldahl method, due allowance being made for the non-protein nitrogen.

In order to determine albumin in blood serum or plasma 0·5 ml. of the sample is mixed with 9·5 ml. of a 27·79 per cent. aqueous solution of ammonium sulphate and the precipitated globulin

removed by filtration through two thicknesses of filter paper (Whatman No. 44). If necessary, the filtration should be repeated until a clear solution is obtained, then 5 ml. of the filtrate is mixed with 5 ml. of trichloracetic acid solution and the determination continued exactly as for total proteins, the final disc reading being multiplied by 20.

(*for Urine*). In applying the method for total proteins it is necessary in the case of highly alkaline specimens to adjust the reaction by the addition of glacial acetic acid until the sample is neutral or slightly acid. The determination is carried out by mixing 5 ml. of the sample with 5 ml. of the trichloracetic acid solution, centrifuging and continuing as described above. In this case the readings on the discs indicate the actual proportion of proteins present in the sample.

Preparatory to making a differential determination of the urinary proteins it is important to adjust the reaction of the sample to *p*H 7·4 (using phenol red as indicator) by adding to a relatively large bulk either glacial acetic acid or strong solution of sodium hydroxide. The globulin, together with any mucus which may be present, is then precipitated by mixing 10 ml. of the neutralised urine with 10 ml. of a saturated aqueous solution of ammonium sulphate (52·8 per cent.). After filtering off the precipitate, 10 ml. of the filtrate is treated with 10 ml. of trichloracetic acid solution, the mixture centrifuged and the colour test applied as described above. The disc reading gives directly the number of mg. of albumin per 100 ml.

(*for Cerebrospinal Fluid*). The external standards may be employed for the approximate determination of proteins in cerebrospinal fluid. For this test 2 ml. of the sample is mixed with 2 ml. of trichloracetic acid solution and, after centrifuging, the precipitated proteins are dissolved in a mixture of 1 ml. of water and 1 ml. of 30 per cent. sodium hydroxide solution then 0·5 ml. of 5 per cent. copper sulphate solution added followed by exactly 4 ml. of water. After mixing thoroughly for about a minute the precipitated cupric hydroxide is centrifuged to the bottom and the colour of the supernatant liquid matched in the comparator. The concentration of proteins is given directly by the disc readings.

In pathological cerebrospinal fluids an approximate estimate of the proportion of albumin and globulin may be made by mixing 2 ml. of the sample with 2 ml. of saturated ammonium sulphate solution, centrifuging, mixing 2 ml. of the supernatant liquid with

2 ml. of trichloracetic acid solution, again centrifuging and decanting the clear liquid. The precipitated albumin is then dissolved in a mixture of 1 ml. of water and 0·5 ml. of sodium hydroxide solution and, after treating with 0·5 ml. of copper sulphate solution, the mixture diluted to 4 ml. and the test completed as before. In this case the readings on the disc should be multiplied by 2.

Discussion. Care should be exercised to ensure that the reagents do not become contaminated with ammonia absorbed from the laboratory atmosphere since this impurity will cause the final colour to be too blue. In the determination for total proteins a control test on the reagents should yield a colourless blank. Moreover, in the differential determinations it is essential that, after precipitation with trichloracetic acid, the supernatant liquid should be decanted as completely as possible, thus guarding against the possibility of leaving sufficient ammonium sulphate behind to yield, on the subsequent addition of sodium hydroxide, enough ammonia to maintain some of the cupric hydroxide in solution and thereby influence the final colour. Experiment shows that, provided the decantation is carefully conducted, only a slight positive error is introduced.

The normal range of total proteins in cerebrospinal fluid is 10 to 35 mg. per 100 ml. and the lower disc provides standards of 20 and 40 mg., hence fluids yielding a colour less intense than the 40-mg. glass are of normal protein content while those yielding the same or a deeper colour are pathological. It is unfortunate that the technique does not provide smaller steps over the normal range, but this could only be done by using more than 2 ml. of the sample and larger quantities of cerebrospinal fluid are seldom available owing to the usual need to apply several other tests on each specimen. However, the procedure may be modified to suit special conditions.

Biuret Method Using Natural Standards[7, 8]. An alternative technique using a strong solution of copper sulphate was originated by Hiller[5, 6] and later modified by Robinson and Hogden[7, 8]. It is claimed to yield coloured test solutions entirely free from any trace of opalescence and to give more reliable results. Thus, by using a strong copper sulphate solution, the precipitated cupric hydroxide is aggregated into larger particles, consequently loss of protein by adsorption is minimised.

(*for Blood Serum or Plasma*). For the determination of total serum proteins, 0·2 ml. of the sample is transferred to a 15-ml.

graduated centrifuge tube, then diluted to 5 ml. with physiological saline (0·9 per cent., aqueous solution of sodium chloride) and 5 ml. of a 10 per cent. aqueous solution of trichloracetic acid added. The contents of the tube are mixed with a fine glass rod, centrifuged until the supernatant liquid is clear and the latter poured off, the tube being well drained by inverting it on filter paper. The protein precipitate is mixed with a drop of 3 per cent. aqueous solution of sodium hydroxide by means of a fine stirring rod until a smooth paste is formed, a little more alkali solution added, stirring being continued until all gel-like particles have dissolved, and the volume then made up to 9 ml. with the alkali. To this solution 0·25 ml. of a 20 per cent. aqueous solution of copper sulphate $CuSO_4.5H_2O$ is added, the final volume adjusted to 10 ml. with sodium hydroxide solution, the tube closed with a rubber stopper, vigorously shaken, allowed to stand for 10 minutes and then centrifuged for 30 minutes. The violet colour of the supernatant liquid is compared with that of a standard prepared from 5 ml. of a serum solution in which the protein content has been determined by the Kjeldahl method. The comparisons are conveniently made by means of a colorimeter of the Duboscq pattern.

For the preparation of the standards Robinson and Hogden[8] recommend rabbit serum because they found that stock solutions could be made which lasted for several months without becoming turbid, whereas solutions prepared from human or dog serum often developed turbidity after storage for a week. A solution containing approximately 0·25 per cent. of total proteins is made by diluting 10 ml. of blood serum from a normal rabbit to 250 ml. with a 0·9 per cent. aqueous solution of sodium chloride. The total nitrogen concentration of this solution is determined[9] by transferring 2·5 ml. to a 100-ml. Pyrex Kjeldahl flask containing two glass beads, adding 3 ml. of sulphuric acid-selenium oxychloride digestion mixture (1 ml. of selenium oxychloride in a mixture of 250 ml. of concentrated sulphuric acid and 250 ml. of a saturated aqueous solution of potassium sulphate) and heating over a flame until a colourless fuming residue results. After allowing to cool, the flask is attached to a Pregl's micro-Kjeldahl distillation apparatus, excess of sodium hydroxide solution introduced and the ammonia distilled into 0·01N sulphuric acid in the usual way. The non-protein nitrogen is determined on the original serum by transferring 2·5 ml. to a 25-ml. volumetric flask containing 20 ml. of a 10 per cent. aqueous solution of trichlor-

acetic acid, mixing, diluting to the mark with more acid solution, filtering and determining the nitrogen on 10 ml. of the filtrate as above. The protein content of the standard solution is then obtained by subtracting the proportion of non-protein nitrogen from that of the total nitrogen and converting the difference to protein by applying the usual factor 6·25. The standardised stock solution is then distributed in 50-ml. containers, preserved with a crystal of thymol and stored in a refrigerator when not in use. This diluted rabbit serum standard is usually faintly opalescent ; if any increase in turbidity or any flocculation occurs, the standards should be discarded.

In order to determine serum albumin, 1 vol. of the sample is diluted with 30 vols. of a 22 per cent. aqueous solution of sodium sulphate, Na_2SO_4, the mixture allowed to stand for 4 hours, then centrifuged and 7 ml. of the clear supernatant liquid transferred to a 15-ml. centrifuge tube[10]. The protein is precipitated by the addition of 7 ml. of a 10 per cent. aqueous solution of trichloracetic acid and the determination continued as already described for the determination of the total serum proteins. The final colour is compared with that developed by treating 4 ml. of the standard serum solution in the manner described for the determination of the total serum proteins.

(*for Urine*). The total proteins may be determined by the technique described above for the total serum proteins except that 5 ml. of the sample is taken and mixed directly with 5 ml. of trichloracetic acid solution.

Discussion. The question as to whether the various proteins in the body fluids respond similarly in the biuret test has been the subject of some speculation. Autenrieth[4] noted that serum albumin and serum globulin have the same biuret colour value per unit weight. On the other hand, Fine's results[11] suggested that there might be a small difference ; when a globulin standard was used to determine albumin, the values were inclined to be low, and when an albumin standard was used to estimate globulin they tended to be high. Lieben and Jesserer[12], in studying the reaction, concluded that under the proper conditions the colour intensity and colour tone of the biuret mixtures of a variety of proteins and protein derivatives are equal at equal weight concentrations of the solutions and independent of the molecular size and amino acid content. Yet again, Sizer[13], when determining the transmission spectra of biuret colour solutions with a Hardy photoelectric spectrophotometer, found that the percentage trans-

mission at a given wavelength and also the shape of the curve vary with the nature and concentration of the respective proteins. Finally, Robinson and Hogden[7] report that the optical density at 560 mμ of biuret colour solutions is essentially the same per unit weight of protein for total serum protein, serum albumin and also protein recovered from pathological urine and ascitic fluid.

REFERENCES

1. E. J. King, G. A. D. Haslewood and G. E. Delory, *Lancet*, 1937, **232**, 886.
2. J. L. Kantor and W. J. Gies, *J. Biol. Chem.*, 1909, **9**, xvii.
3. W. Autenrieth and F. Mink, *Münch. med. Woch.*, 1915, **62**, 1417.
4. W. Autenrieth, *Münch. med. Woch.*, 1917, **64**, 241.
5. A. Hiller, *Proc. Soc. Exp. Biol. Med.*, 1927, **24**, 358.
6. A. Hiller, J. F. McIntosh and D. D. van Slyke, *J. Clin. Investig.*, 1927, **4**, 235.
7. H. W. Robinson and C. G. Hogden, *J. Biol. Chem.*, 1940, **135**, 707.
8. H. W. Robinson and C. G. Hogden, *J. Biol. Chem.*, 1940, **135**, 727.
9. H. W. Robinson, J. W. Price and C. G. Hogden, *J. Biol. Chem.*, 1937, **120**, 481.
10. P. E. Howe, *J. Biol. Chem.*, 1921, **49**, 109.
11. J. Fine, *Biochem. J.*, 1935, **29**, 799.
12. F. Lieben and H. Jesserer, *Biochem. Z.*, 1936, **285**, 36.
13. I. W. Sizer, *Proc. Soc. Exp. Biol. Med.*, 1937, **37**, 107.

PYRUVIC ACID (IN BLOOD)

A colorimetric method for the determination of pyruvic acid based upon the isolation of the 2 : 4-dinitrophenylhydrazine derivative and the reaction of the latter with strong alkali to form a red compound was suggested by E. M. Case[1]. Subsequent investigators have found that the concentration of this constituent rapidly falls in freshly drawn blood and, with a view to overcoming this difficulty, Bueding and Wortis[2] advocated running the sample into a vessel containing sodium monoiodoacetate. When ketonæmia exists, the subject's blood may contain sufficient acetoacetic acid to disturb the results and it is claimed by Klein[3] that this source of error can be obviated by allowing the ethyl acetate extract of the pyruvate-hydrazone to stand for 18 to 24 hours. The complete procedure recommended by Klein[3] is given here.

Method[3]. A solution of 50 per cent. monoiodoacetic acid in water is adjusted with an aqueous solution of sodium hydroxide to

*p*H 7·8 and a measured volume corresponding to 25 mg. of monoiodoacetic acid (in general, 0·1 ml. of a 25 per cent. solution of the sodium salt) is transferred to a bottle containing 20 mg. of dried potassium oxalate. About 5 ml. of blood, obtained by venipuncture, is caught directly in this bottle. After mixing, 1 vol. of the blood is added to 7 vols. of water, 1 vol. of a 10 per cent. aqueous solution of sodium tungstate added followed by 1 vol. of 2/3N sulphuric acid. The mixture is agitated, filtered through dry paper, 10 ml. of the protein free filtrate transferred to a small separator and about 1 ml. of a 0·5 per cent. solution of 2 : 4-dinitrophenylhydrazine in 2N hydrochloric acid added. The latter solution is made by heating the acid under a refluxing condenser, adding the 2 : 4-dinitrophenylhydrazine and when solution is complete allowing to cool and finally filtering. After the mixture in the separator has stood for 15 minutes it is extracted with three successive 5-ml. portions of ethyl acetate, the combined extracts washed once with 2 ml. of water and then kept in a separator over 2 ml. of water for 18 to 24 hours; after removal of the water the pyruvate hydrazone is transferred from the ethyl acetate to 1 per cent. aqueous solution of sodium carbonate (Na_2CO_3) by successive extractions with 2-, 2- and 1-ml. portions. To prevent any loss of hydrazone from the stem of the separator between the first and second shakings the ethyl acetate is washed with 0·5 ml. of sodium carbonate solution. All four fractions are transferred to a separator and, in order to remove any free dinitrophenylhydrazine or interfering hydrazones the combined alkaline solution is shaken with 1 ml. of ethyl acetate. The sodium carbonate extract is transferred to a 10-ml. glass-stoppered cylinder and the ethyl acetate remaining in the separator is washed with two 0·5-ml. portions of sodium carbonate solution which are also added to the contents of the cylinder. The volume of the alkaline extract is made up to 8 ml. with more sodium carbonate solution and finally 2 ml. of 4N sodium hydroxide added. After mixing, the intensity of the red colour is compared with that of standards similarly prepared.

Discussion. The pyrophosphoric ester of vitamin B_1 (cocarboxylase) is the co-enzyme necessary for the normal katabolism of pyruvic acid in body tissues and it has been shown that when there is a deficiency of the former substance there is a corresponding accumulation of the latter[4]. Hence the determination of blood pyruvate may have clinical significance. In an examination of sixty normal subjects Bueding and Wortis[2] found that the con-

centration of pyruvic acid varied from 0·77 to 1·16 mg. per 100 ml. The method given above should be accepted with some reserve since there may be other α-keto acids present in blood which would react to produce 2 : 4-dinitrophenylhydrazones; thus oxaloacetic and α-ketoglutaric acids are known to give colorations under the conditions of the test. Again, high concentrations of acetoacetic acid, say above 50 mg. per 100 ml. of blood, may be expected to interfere notwithstanding the precautions included in the above procedure.

REFERENCES

1. E. M. Case, *Biochem. J.*, 1932, **26**, 753.
2. E. Bueding and H. Wortis, *J. Biol. Chem.*, 1940, **133**, 585.
3. D. Klein, *J. Biol. Chem.*, 1941, **137**, 311.
4. B. S. Platt and G. D. Lu, *Quart. J. Med.*, 1936, **5**, 355.

SILICATES

See Section II : Acid Radicles, p. 172.

SULPHANILAMIDE AND OTHER SULPHONAMIDES
(IN BLOOD AND URINE)

The success attendant upon the therapeutic application of sulphanilamide (*p*-aminobenzenesulphonamide) created the need for an accurate method for its determination in blood and urine. Fuller[1] described a procedure for determining sulphanilamide in the latter fluid by diazotising and coupling with β-naphthol in alkaline solution while for blood he recommended removal of the proteins with trichloracetic acid followed by diazotising and coupling in alkaline solution with thymol. An improvement on this was effected by Marshall and his collaborators[2–4] who proposed diazotisation and coupling in acid solution with dimethyl-α-naphthylamine. However, this method is subject to the disadvantage that successive supplies of dimethyl-α-naphthylamine yield colours of different intensity, some giving highly tinted blank reactions while other samples react too slowly with diazotised sulphanilamide. This difficulty led to further search for a more satisfactory compound with which to effect the coupling reaction and the method was greatly improved when

Bratton, Marshall and their assistants[5] replaced dimethyl-α-naphthylamine by N-(1-naphthyl)-ethylenediamine dihydrochloride. The technique employing this reagent is described below. In the body, sulphanilamide is partly conjugated with acetic acid and excreted as the acetyl derivative, hence it is sometimes desirable to make determinations before and after hydrolysis in order to assess the proportion of free and acetylated base present in urine or blood. According to Marshall, Cutting and Emerson[6] acetylation of sulphanilamide does not occur in dogs.

An alternative method in which the yellow colour produced by the addition of *p*-dimethylaminobenzaldehyde to sulphanilamide in an acidic medium is measured[7] has, owing to its simplicity, attained some popularity with clinicians[8]. Apart from the fact that numerous organic bases yield a similar tint under the same conditions, it is difficult to measure the intensity of the bright yellow colour produced by sulphanilamide, and since its reliability has also been questioned on other grounds[9, 10] it is not considered that the method warrants description here.

Method[5] (*for Blood*). To 30 ml. of a 0·05 per cent. aqueous solution of saponin is added 2 ml. of oxalated blood and after 2 minutes 8 ml. of a 15 per cent. aqueous solution of trichloracetic acid is added and the mixture filtered through a dry paper. To 10 ml. of the filtrate (equivalent to 0·5 ml. of the original sample) is added 1 ml. of a freshly prepared 0·1 per cent. aqueous solution of sodium nitrite, the mixture stirred, allowed to stand for 3 minutes and 1 ml. of a 2 per cent. aqueous solution of ammonium sulphamate, $NH_2SO_3NH_4$, added. After being stirred and allowed to stand for 10 minutes, 1 ml. of a 0·1 per cent. aqueous solution of N-(1-naphthyl)-ethylenediamine dihydrochloride is added and any red colour produced matched against standards similarly prepared. The result so obtained is a measure of the free sulphanilamide in the sample; in order to determine the total sulphanilamide 10 ml. of the filtrate from the trichloracetic acid precipitation is treated with 0·5 ml. of 4N hydrochloric acid, the mixture heated in a boiling water-bath for 1 hour then allowed to cool, the volume adjusted to 10 ml. and the colour test again applied in the same way as before.

Standard solutions of sulphanilamide are made by adding quantities varying from 0·25 ml. to 2·0 ml. of a 0·02 per cent. aqueous solution to a series of 18 ml. portions of the 15 per cent. solution of trichloracetic acid and diluting each to 100 ml. If the colour test is applied individually to 10 ml. of each of these

standards a series will be obtained varying from 1 to 8 mg. per 100 ml. of the sample under test.

(*for Urine*). Sufficient water is added to a measured volume of the sample in order to reduce the sulphanilamide content to between 1 and 4 mg. per 100 ml. and the degree of dilution noted. A dilution of 1 in 5 or 1 in 10 may be tried in a rough preliminary test. If the sample contains proteins 2 ml. of the diluted urine is added to 30 ml. of water and 8 ml. of the 15 per cent. trichloracetic acid solution, the mixture filtered and the colour test applied to 10 ml. of the filtrate as already described. A further 10 ml. of the filtrate may be hydrolysed by heating with hydrochloric acid and the colour test applied in order to determine the total sulphanilamide. If the original sample is free from protein, 5 ml. of the diluted urine is transferred to a 100-ml. graduated flask, 5 ml. of 4N hydrochloric acid added and the mixture diluted to the mark with water. For free sulphanilamide, the colour test is applied directly to 10 ml. while for total sulphanilamide 10 ml. is heated in a boiling water-bath without further addition of acid and the colour test then conducted as before. In calculating the results the degree to which the sample has been diluted must, of course, be taken into account.

Discussion. The above is a slight modification of the method described by the originators since a 2 per cent. solution of ammonium sulphamate is employed in place of one only a quarter this strength. The naphthyl-ethylenediamine dihydrochloride solution should be stored in an amber bottle and is then reasonably stable. The red colour produced in the test attains its maximum intensity at once and is permanent for an hour or more.

Precisely the same procedure is applicable to the colorimetric determination of most of the other drugs belonging to the sulphanilamide group. It has been suggested that the intensity of colour produced in the test by any one of these compounds is inversely proportional to the molecular weight; this does not quite accord with the facts since, apart from variations in shade, while the relation between the quantity of sulphanilamide and the intensity of the colour is linear, that for the other common drugs of this series is not. Actual observations of the colours produced by a number of these compounds when submitted to the above test are presented in Table XVIII.

Method Using Permanent External Standards. A disc for use with the Lovibond Comparator is available covering the range from 0·5 to 8·0 mg. of sulphanilamide per 100 ml. of the sample

TABLE XVIII. RELATION BETWEEN THE QUANTITY OF SULPHANILAMIDE, OR OTHER SULPHONAMIDE,* AND THE COLOUR PRODUCED

Weight of Drug (in 10 ml.) mg.	Sulphanilamide — Colour observed in 1-cm. cell — Lovibond Units			Sulphapyridine M. and B. 693 — Colour observed in 1-cm. cell — Lovibond Units			Sulphathiazole M. and B. 760 — Colour observed in 1-cm. cell — Lovibond Units			Sulphaguanidine — Colour observed in 1-cm. cell — Lovibond Units			Sulphadiazine — Colour observed in 1-cm. cell — Lovibond Units		
	Red	Blue †	Brightness †	Red	Blue †	Brightness †	Red	Blue †	Brightness †	Red	Blue †	Brightness †	Red	Blue †	Brightness †
0·005	1·1	0·2	0·1	0·6	0·2	—	0·7	0·1	0·1	0·9	0·1	0·1	0·8	0·1	0·1
0·010	2·7	0·5	0·6	1·5	0·3	0·1	1·5	0·5	0·2	1·8	0·2	0·3	1·5	0·3	0·2
0·015	4·2	1·0	0·9	2·4	0·4	0·2	2·5	0·8	0·4	3·1	0·5	0·6	2·5	0·5	0·5
0·020	6·1	1·3	1·1	3·6	0·6	0·4	3·8	1·0	0·8	4·3	0·8	0·8	3·7	0·8	0·7
0·025	7·7	1·7	1·4	4·8	0·9	0·6	5·3	1·4	1·1	5·7	1·0	1·0	4·8	0·9	0·8
0·030	9·7	1·9	1·8	6·1	1·5	1·0	6·5	1·8	1·2	7·1	1·4	1·1	6·1	1·3	1·1
0·035	11·1	2·1	2·1	7·6	1·9	1·4	7·7	2·0	1·4	9·0	1·8	1·2	7·8	1·7	1·3
0·040	12·4	2·1	2·4	9·2	2·0	1·6	9·0	2·2	1·7	10·7	2·0	1·5	9·3	2·0	1·6

* The constitution of these substances is indicated by the following chemical names: 2-(*p*-aminobenzenesulphonamido)-pyridine [sulphapyridine]: 2-(*p*-aminobenzenesulphonamido)-thiazole [sulphathiazole]: *p*-aminobenzenesulphonguanidine [sulphaguanidine]: 2-(*p*-aminobenzenesulphonamido)-pyrimidine [sulphadiazine].

† These values are subject to slight variation: they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

under test. The standards are calibrated to give the correct reading when the determination is started with 2 ml. of the original sample and applying the colour test to a fourth part of the filtrate resulting from the separation of the proteins. Owing to the brightness of the final coloured solution, it is necessary to use a yellow " brightness screen " in front of the right-hand aperture of the comparator. The technique adopted is precisely as given above excepting that a control solution must be prepared, substituting 2 ml. of distilled water for the sample, in order to allow for any slight tint produced by the reagents.

By using sulphosalicylic acid instead of trichloracetic acid as protein precipitant Sheftel[11] has found it possible to apply a modification of the above method of Marshall *et al.*[5] in which all reagents are added in the form of tablets, enabling a determination to be conducted at the bedside. The intensity of the colour produced is measured by matching against a lucite wedge mounted between two scales calibrated in mg. per 100 ml. of blood of sulphanilamide and sulphathiazole respectively, the concentration of sulphapyridine being derived by multiplying the reading on the sulphathiazole scale by 0·8.

REFERENCES

1. A. T. Fuller, *Lancet*, 1937, **232**, 194.
2. E. K. Marshall, Jr., K. Emerson, Jr., and W. C. Cutting, *J. Amer. Med. Assoc.*, 1937, **108**, 953.
3. E. K. Marshall, Jr., *Proc. Soc. Expt. Biol. Med.*, 1937, **36**, 422.
4. E. K. Marshall, Jr., and D. Babbitt, *J. Biol. Chem.*, 1937, **122**, 263.
5. A. C. Bratton and E. K. Marshall, Jr., D. Babbitt and A. R. Hendrickson, *J. Biol. Chem.*, 1939, **128**, 537.
6. E. K. Marshall, Jr., W. C. Cutting and K. Emerson, Jr., *Science*, 1937, **85**, 202.
7. A. E. A. Werner, *Lancet*, 1939, **236**, 18.
8. A. T. Fuller, *Lancet*, 1942, **242**, 760.
9. G. V. James, *Analyst*, 1940, **65**, 206.
10. C. J. Morris, *Biochem. J.*, 1941, **35**, 952.
11. A. G. Sheftel, *J. Amer. Med. Assoc.*, 1941, **117**, 439.

SULPHATES (IN BLOOD)

Advantage has been taken of the insolubility of benzidine sulphate in dilute alcohol or acetone to evolve methods for the colorimetric determination of sulphates involving solution of the precipitate in dilute hydrochloric acid followed by treatment with hydrogen peroxide and ferric chloride[1–3] or, alternatively, diazoti-

sation of the benzidine sulphate and coupling with phenol[4]. The method has been developed for the determination of sulphates in blood and, so far as the writer is aware, has not been described for use in other connections; however, its adaptation to many other purposes would merely involve the introduction of obvious modifications. In the following procedure, due to Cuthbertson and Tompsett[5], the diazotised benzidine is coupled with thymol.

Method[5]. To 2 ml. of blood, serum, or plasma, is added 6 ml. of water and 2 ml. of a 20 per cent. aqueous solution of trichloracetic acid, the mixture shaken and then centrifuged. Into another centrifuge tube 2·5 ml. of the supernatant liquid is measured, followed by 5 ml. of a freshly prepared 0·5 per cent. solution of benzidine in acetone. After mixing and standing for 30 minutes the contents of the tube are centrifuged, the supernatant liquid is poured off, the precipitate of benzidine sulphate is washed twice with acetone and the tube then inverted over filter paper until the interior has dried. The precipitate in the tube is dissolved by warming with 1 ml. of N hydrochloric acid. After cooling, 0·5 ml. of a freshly prepared 0·1 per cent. aqueous solution of sodium nitrite is added and also, after the elapse of 1 minute, 2·5 ml. of a 15 per cent. aqueous solution of sodium hydroxide followed by 2·5 ml. of a 1 per cent. solution of thymol in 10 per cent. aqueous sodium hydroxide. The red colour produced is allowed to develop during 15 minutes in order to attain its full intensity and then compared with standards; these are made by mixing 2 ml. of standard benzidine hydrochloride solution with 1 ml. of the sodium nitrite solution and, after standing 1 minute, adding 5 ml. of 15 per cent. sodium hydroxide, shaking and mixing with 5 ml. of the alkaline thymol solution. The standard benzidine hydrochloride solution is prepared by diluting a 0·4014 per cent. solution of the hydrochloride in N hydrochloric acid (equivalent to 0·5 mg. S per ml.) so that a series of standards is obtained in which 1 ml. contains benzidine equivalent to 0·0025 up to 0·08 mg. S.

Discussion. The presence of phosphates to the extent of about 20 mg. P does not interfere and the test is not disturbed by quite large quantities of sodium chloride or potassium oxalate. After attaining its maximum intensity the red colour produced in the test is stable for about an hour. The concentration of sulphate in the plasma of normal human adults varies from 0·1 to 0·5 mg. S per 100 ml. while the cellular constituents of blood contain only a minute amount of sulphate. According to Cuthbertson and Tompsett[5], cases of nephritis and cardio-renal disease exhibit, on

the average, an increased retention of sulphates coincident with a corresponding diminution in the rate of elimination of urea and non-protein nitrogen.

REFERENCES

1. R. S. Hubbard, *J. Biol. Chem.*, 1927, **74**, v.
2. E. G. Wakefield, *J. Biol. Chem.*, 1929, **81**, 713.
3. R. S. Hubbard, *J. Biol. Chem.*, 1930, **88**, 663.
4. B. S. Kahn and S. L. Leiboff, *J. Biol. Chem.*, 1928, **80**, 623.
5. D. P. Cuthbertson and S. L. Tompsett, *Biochem. J.*, 1931, **25**, 1237.

SURAMIN (IN BLOOD)

Suramin, also known as Antrypol, Bayer 205 or Germanin, is the symmetrical urea of the sodium salt of *m*-benzoyl-*m*-amino-*p*-methyl - benzoyl - 1 - aminonaphthalene - 4 : 6 : 8 - trisulphonic acid. This substance is extensively employed by intravenous injection in the treatment of trypanosomiasis and also as a protective against the disease, consequently some value attaches to data relating to the concentration of the drug attained in the blood during treatment. The method given below was worked out by Dangerfield, Gaunt and Wormall[1].

Method[1]. From 1 to 3 ml. of plasma (which may conveniently contain between 0·018 and 0·36 mg. of the drug) separated by centrifuging from oxalated blood, or the same quantity of serum derived from naturally clotted blood, is transferred to a 10-ml. graduated tube, 3 ml. of concentrated hydrochloric acid added and, when necessary, sufficient water to bring the volume of the liquid to 6 ml. The tube is immersed in a boiling water-bath for 6 hours, the cooled brown or brownish-black mixture diluted with water to 10 ml., 3 g. of previously ignited acid-washed kaolin (Society of Leather Trades Chemists' specification) added, the mixture shaken and then filtered. To 2 ml. of the filtrate is added 1 drop of a 0·5 per cent. aqueous solution of sodium nitrite and, after 1 minute, 2 drops of a saturated aqueous solution of urea. After shaking and allowing to stand a further 2 minutes, 3 ml. of a 30 per cent. w/v aqueous solution of sodium acetate, $NaC_2H_3O_2.3H_2O$, is added and this is immediately followed by 1 ml. of a 0·2 per

cent. solution of monomethyl-α-naphthylamine hydrochloride in 50 per cent. v/v acetic acid. After standing for 20 minutes the intensity of the stable red colour is compared with standards made by adding known amounts of the drug to similar quantities of normal plasma or serum.

Method Using Permanent External Standards. Discs are available for use with the Lovibond Comparator covering a range from 0·18 to 3·60 mg. per 100 ml. of solution to be diazotised. The coloured glasses have been standardised by application of the above procedure.

Discussion. One of the main products of the hydrolysis of suramin is *m*-aminobenzoic acid and it is this substance which couples with monomethyl-α-naphthylamine hydrochloride to produce the red colour of the test. It is most important to use good quality distilled water since it has been observed[2] that impurities from this source have led to the formation of turbidities in the final matching solution. A procedure for the determination of these drugs in urine has been developed by Boursnell, Dangerfield and Wormall[2] but the method is complicated since it is necessary to effect separation by dialysis from diazotisable substances normally present. Fortunately, such determinations are not generally needed since the more important value, so far as treatment is concerned, is the concentration in the blood plasma. The protective level against trypanosomiasis is not known with certainty but may be 1 to 1·5 mg. per 100 ml. which can be generally maintained for a total period of 4 or 5 months by administering a series of three injections each of 2 g. every 4 weeks. It appears that suramin is retained in the body tissues for long periods[3] and its value as a prophylactic agent against sleeping sickness is attributable to this phenomenon.

REFERENCES

1. W. G. Dangerfield, W. E. Gaunt and A. Wormall, *Biochem. J.*, 1938, **32**, 59.
2. J. C. Boursnell, W. G. Dangerfield and A. Wormall, *Biochem. J.*, 1939, **33**, 81.
3. J. C. Boursnell and A. Wormall, *Biochem. J.*, 1939, **33**, 1191.

TRYPTOPHAN

Under appropriate conditions the indole portion of the tryptophan molecule will react with various aldehydes to form coloured compounds and procedures for the quantitative determination of this protein unit utilising formaldehyde with nitrous acid[1–4], formaldehyde with sulphuric acid[5–7], glyoxylic acid[8–10], *p*-dimethylaminobenzaldehyde[11–14], benzaldehyde with nitrous acid[3], and vanillin[15, 16] have been proposed. The method[17, 18] depending upon reduction of Folin's phosphotungstic-phosphomolybdic acid reagent is not specific and is unsuited for application to protein hydrolysates. The quantitative colorimetric procedure described below is based upon the thymol reaction of Jolles[19, 20] for indoxyl sulphuric acid (indican of the urine) as modified by Sharlit[21] and has been developed by Albanese and Frankston[22].

Method[22]. The sample of protein to be examined is hydrolysed by treatment for 22 hours under a reflux condenser with 5 ml. of a boiling 20 per cent. w/v aqueous solution of sodium hydroxide. The hydrolysate is neutralised to *p*H 7 with glacial acetic acid, any silica derived from the glass vessel is separated by filtration, and the volume of the liquid adjusted so that 2 ml. may be expected to contain between 1 and 2 mg. of tryptophan. A 2-ml. sample of the filtrate is transferred to a tube graduated at the 5-ml. mark, 0·3 ml. of a 3 per cent. aqueous solution of sodium nitrite added followed by 0·1 ml. of a 10 per cent. aqueous solution of acetic acid and the mixture shaken intermittently for 10 minutes. Then, in succession, is added 0·3 ml. of a 1 per cent. aqueous solution of potassium persulphate, 0·5 ml. of a 1 per cent. solution of thymol in alcohol (95 per cent.) and 5 ml. of a mixture of 3 parts of a 40 per cent. w/v aqueous solution of trichloracetic acid and 2 parts of concentrated hydrochloric acid. After the addition of each of the foregoing ingredients the mixture is well agitated. The tube is immersed in a boiling water-bath for 5 minutes and then cooled in an ice-bath for the same period of time. All but about 0·3 ml. of the nearly colourless upper layer of liquid is removed by means of a capillary pipette from the deep red layer of ethyl trichloracetate below, and the latter then diluted to 5 ml. with glacial acetic acid. Any brownish-red colour produced is matched against a series of standards prepared from known quantities of tryptophan varying from 0·5 to 3·5 mg.

Discussion. This method depends upon preliminary deamini-

sation of tryptophan by the treatment with sodium nitrite and dilute acetic acid to produce α-hydroxy-β-indolyl-propionic acid which gives Jolles' reaction for indoxyl sulphuric acid, whence Sharlit's technique for the colorimetric determination of the latter compound can be applied. The colours produced are permanent during several days, the control being a pale emerald green which is changed to an olive green in the presence of 0·5 mg. of tryptophan and thence to brownish-red with more of the protein unit; above 3·5 mg. the colour gradation is not well marked. This test is not specific, being given by various indole compounds, but in the case of proteins and protein hydrolysates, in which tryptophan is the only indole compound present, the reaction is useful. None of the known amino acids or humin-free sulphuric acid hydrolysates of casein give any red colour, the solution in all cases being pale emerald green similar to the control test on the reagents.

REFERENCES

1. E. Voisenet, *Bull. Soc. chim.*, 1905, [3], **33**, 1198.
2. O. Fürth and F. Liebin, *Biochem. Z.*, 1920, **109**, 124.
3. E. Lüscher, *Biochem. J.*, 1922, **16**, 556.
4. J. M. Looney, *J. Biol. Chem.*, 1926, **69**, 519.
5. J. Tillmans and A. Alt, *Biochem. Z.*, 1925, **164**, 135.
6. J. Tillmans and A. Alt, *Biochem. Z.*, 1926, **178**, 243.
7. T. Ruemele, *Z. anal. Chem.*, 1931, **84**, 82.
8. H. Fasal, *Biochem. Z.*, 1912, **44**, 392.
9. C. A. Cary, *J. Biol. Chem.*, 1928, **78**, 377.
10. S. Winkler, *Z. physiol. Chem.*, 1934, **228**, 50.
11. E. Kurchin, *Biochem. Z.*, 1914, **65**, 451.
12. C. E. May and E. R. Rose, *J. Biol. Chem.*, 1922, **54**, 213.
13. C. A. Cary and E. B. Meigs, *J. Agric. Res.*, 1924, **29**, 603.
14. W. J. Boyd, *Biochem. J.*, 1929, **23**, 78.
15. I. Kraus, *J. Biol. Chem.*, 1925, **63**, 157.
16. I. K. Ragins, *J. Biol. Chem.*, 1928, **80**, 543.
17. O. Folin and J. M. Looney, *J. Biol. Chem.*, 1922, **51**, 421.
18. O. Folin and V. Ciocalteu, *J. Biol. Chem.*, 1927, **73**, 627.
19. A. Jolles, *Z. physiol. Chem.*, 1913, **84**, 310.
20. A. Jolles, *Z. physiol. Chem.*, 1915, **94**, 79.
21. H. Sharlit, *J. Biol. Chem.*, 1932–33, **99**, 537.
22. A. A. Albanese and J. E. Frankston, *J. Biol. Chem.*, 1942, **144**, 563.

UREA (IN BLOOD AND URINE)

A determination of the blood urea in cases of nephritis or Bright's disease usually constitutes the most important single test, both in regard to diagnosis and prognosis; it serves also as a

means of checking treatment and progress. The hypobromite method, as generally used in urine analysis, is not well suited for the examination of blood and in this medium urea is now almost always determined by measuring the ammonia liberated by the enzyme urease, either volumetrically or colorimetrically. By application of the latter principle, proposed by Twort and Archer[1], liberated ammonia is measured by means of a specially modified Nessler's reagent and a determination can be conducted on 0·2 ml. of the sample. The original technique has been improved by Archer and Robb[2] and this modification is described below.

For the determination of urea in urine the gasometric method, depending upon the liberation of nitrogen by the action of alkaline sodium hypobromite is usually preferred for clinical control purposes while the gravimetric procedure using xanthydrol[3–5] is employed to a limited extent. The interesting colorimetric method given here, which depends upon the catalysing effect of urea on the reaction between stannous chloride and furfuraldehyde in the presence of a mineral acid was proposed by Obermer and Milton[6] and is of value in experimental work with laboratory animals where only small samples are available.

Method for Blood[2]. The following special reagents are required :—

1. A Suspension of Urease made by grinding 1 urease tablet (equivalent to 50 mg. urea) in 5 ml. of 30 per cent. alcohol. This will remain in good condition for about 4 days at room temperature.

2. A Special Nessler's Reagent of Controlled Alkalinity. A double iodide solution is prepared by dissolving 37·5 g. of potassium iodide in 25 ml. of water, adding 50 g. of mercuric iodide and, when solution is complete, diluting with water to 250 ml., filtering and finally diluting to 500 ml. The Nessler's reagent is then prepared by adding 300 ml. of this iodide solution to 1400 ml. of a 10 per cent. w/v aqueous solution of sodium hydroxide and diluting the mixture to 2000 ml. with water. The strength of the sodium hydroxide solution must lie between 9·5 and 10·5 per cent. w/v.

The sample to be examined, 0·2 ml. measured in a blood pipette, is transferred to a non-tapered centrifuge tube containing 2 ml. of water, the pipette being washed out with the latter. To the diluted blood 0·2 ml. of the urease suspension is added and the tube immersed in a water-bath at 55° C. for 15 minutes. The

tube is removed and 0·3 ml. of a 10 per cent. aqueous solution of sodium tungstate, $Na_2WO_4.2H_2O$, added followed by 0·3 ml. of 2/3N sulphuric acid and 5 ml. of water. After mixing and allowing the protein precipitate to flocculate, the tube is centrifuged until the supernatant fluid is quite clear, then exactly 5 ml. is pipetted off, transferred to a suitable tube containing 5 ml. of water and 2 ml. of the special Nessler's solution added. The brownish-yellow colour produced is matched against standards made by diluting appropriate quantities of a 0·011 per cent. aqueous solution of ammonium sulphate to 10 ml. with water and adding 2 ml. of Nessler's reagent. Under the conditions of the test 1 ml. of the above standard solution of ammonium sulphate is equivalent to 40 mg. urea per 100 ml. of blood and colours can be matched within a range extending from 20 to 220 mg. urea per 100 ml. A series of standards may be prepared and the colour matched directly but it is more usual to make a single standard approximating to the colour of the sample and determine the exact relative intensity of colour by means of a Duboscq type colorimeter. The standard solution of ammonium sulphate should be freshly made by diluting a stock solution containing 2·200 g. of the pure dry salt per litre.

Method for Blood Using Permanent External Standards. The Lovibond Comparator has been applied for use with the above method. Two discs are available: the one, covering a range from 20 to 100 mg. urea per 100 ml. of blood in steps of 10 mg. each and the other, from 110 to 160 mg. in steps of 10 mg. and then in steps of 20 mg. to 220 mg.

Method for Urine[6]. The sample (0·2 ml.) is pipetted into a small test tube, 1 ml. of 10 per cent. solution of stannous chloride in concentrated hydrochloric acid added, followed by 0·3 ml. of a mixture made by dissolving 0·3 ml. of freshly distilled furfuraldehyde in 7 ml. of glacial acetic acid and making the volume up to 21 ml. with a 5 per cent. aqueous solution of gum Ghatti. The contents of the tube are shaken and allowed to stand for 30 minutes; if the room temperature is below 10° C. the time interval may advantageously be increased to 45 minutes while, if it is above 20° C., it should be decreased to 20 minutes. After standing thus, an intense purple colour will have developed but this is then changed through brownish-green to a golden brown by adding 4 ml. of a mixture composed of 3 vols. of a 30 per cent. aqueous solution of sodium acetate (hydrated) and 1 vol. of a 5 per cent. solution of gum Ghatti and allowing to stand for a further 30

minutes. The colour is then compared by means of a colorimeter with that of suitable standards prepared by similarly treating solutions of urea of known concentration containing, say, from 1 to 4 per cent. If much protein is present in the urine under examination it must be removed since, otherwise, a precipitate may form on adding the acid mixture. To effect this, 0·5 ml. of a 5 per cent. solution of lead subacetate and 0·5 ml. of a 10 per cent. solution of acetic acid is added to 2 ml. of the sample, the mixture centrifuged, the lead removed from 2 ml. of the clear supernatant liquid by adding 0·5 ml. of 10 per cent. aqueous solution of sodium sulphate and again centrifuging. Urea in the clear fluid is then determined as described above, the result being corrected by multiplying by the dilution factor 1·875.

Discussion. In the method for the determination of urea in blood the suspension of urease can be replaced by 0·1 g. of *fresh* soya bean meal, an extra 0·2 ml. of water being added to maintain the correct volume of the test mixture. However, it will generally be found more convenient to use the stable tablets containing the isolated enzymes. If preferred, the enzyme may be separated from 10 g. of jack bean meal by suspending in 125 ml. of water, adding 5 ml. of 0·1N hydrochloric acid and filtering after several minutes. To 50 ml. of the filtrate 1 ml. of a 10 per cent. solution of acetic acid is added, the precipitated urease is separated by centrifuging and dissolved in 5 ml. of a citrate buffer and the solution diluted to 50 ml. with water; the buffer is prepared by dissolving 60 g. of sodium citrate in about 180 ml. of water, adding 1 ml. of glacial acetic acid and diluting the mixture to 200 ml. with water.

With each set of new reagents a blank test should be performed using 0·2 ml. of water instead of blood in order to ensure that no perceptible colour is produced as a result of possible impurities. If it is preferred to remove the precipitated proteins by filtration rather than by centrifuging, high-grade specially washed filter papers should be used as the ordinary qualities may contain ammonium salts and thus lead to false results.*

The colorimetric method cannot be expected to yield precisely accurate results since blood is liable to contain substances which

* A simplified technique in which the proteins are removed by precipitation with trichloracetic acid followed by filtration was proposed by A. A. Osman and H. G. Close (*Brit. Med. J.*, 1931, i, 1064) and an assembly of the necessary apparatus and reagents for conducting the determination by this method is issued by The British Drug Houses, Ltd. It is listed as The B.D.H. Ureamometer Outfit.

produce a colour with Nessler's reagent, but for most clinical purposes celerity and convenience will generally outweigh the advantage of obtaining analytical results of high precision by the elaborate methods in which the ammonia is isolated by aspiration[8], or distillation[9, 10] and finally determined volumetrically. However, it is of interest to note that Gentzkow and Masen[11] claim to have effected an improvement in the colorimetric method by including potassium persulphate and potassium gluconate in the final reaction mixture: their stock Nessler's solution is made in accordance with the specification of Folin and Wu[12] but the working solution is made to contain 5 per cent. of sodium hydroxide instead of 7 per cent. For the determination, 5 ml. of whole oxalated blood is employed and a one-tenth aliquot part is used for the final colour reaction in which, it is stated, any tendency for haze or turbidity to develop is eliminated.

The normal concentration of urea in the blood of an adult on a full diet ranges from 15 to 40 mg. per 100 ml. but in persons over 60 years of age it may rise to 50 mg. per 100 ml. Where there is renal inefficiency as a result of kidney disorders the urea content may increase to over 200 mg. per 100 ml. of blood.

The solution of stannous chloride in concentrated hydrochloric acid used in the determination of urea in urine, is not stable and should be freshly made or stored in a refrigerator. The colour reaction is a modification of a qualitative test for hexose and pentose sugars discovered by Foulger[7]. The procedure as described is not disturbed by any of the inorganic salts likely to occur in urine or by creatinine, creatine, dextrose (up to 5 per cent.) peptone, acetone, uric acid, amino-acetic acid, cystine, tyrosine, tryptophane or bile salts. Guanidine, at a concentration of 1 per cent., gives a colour about equal to that yielded by 0·05 per cent. of urea but, since guanidine is never present in urine in higher concentration than 0·1 per cent., error from this source will be inappreciable. Highly pigmented samples of urine should be decolorised by treatment with charcoal: experiment has shown that no adsorption of urea will occur if the sample is slightly acid.

REFERENCES

1. F. W. Twort and H. E. Archer, *Lancet*, 1923, **204**, 1102.
2. H. E. Archer and G. D. Robb, *Quart. J. Med.*, 1925, **18**, 274.
3. R. Fosse, *Compt. rend.*, 1913, **157**, 948.
4. R. Fosse, A. Robyn and F. François, *Compt. rend.*, 1914, **159**, 367.
5. L. Frenkel, *Ann. Chim. anal.*, 1920, [ii], **2**, 234.
6. E. Obermer and R. F. Milton, *Analyst*, 1938, **63**, 423.

7. J. H. Foulger, *J. Biol. Chem.*, 1932, **99**, 207.
8. D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.*, 1916, **24**, 117.
9. O. Folin and A. Svedberg, *J. Biol. Chem.*, 1930, **88**, 77.
10. C. E. Redemann, *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 635.
11. C. J. Gentzkow and J. M. Masen, *J. Biol. Chem.*, 1942, **143**, 531.
12. O. Folin and H. Wu, *J. Biol. Chem.*, 1919, **38**, 81.

URIC ACID (IN BLOOD AND URINE)

The original procedures for the colorimetric determination of uric acid were developed by Folin and his collaborators[1–5], the first papers appearing during the year 1912. Originally, the method consisted in causing the uric acid to reduce phosphomolybdic acid, the reaction being facilitated by the presence of sodium cyanide. Later, a mixture of phosphomolybdic and phosphotungstic acids was used and this was soon changed in favour of phosphotungstic acid entirely free from molybdenum. In the original technique, and all the earlier modifications the uric acid was separated by precipitation as the silver salt and this principle is still sometimes used and is known as the " indirect " method : however, in 1922, Benedict[6], using arseno-phosphotungstic acid mixture as reagent, introduced the " direct " procedure in which treatment with silver lactate is omitted. This simplification was accepted by Folin who still further perfected both methods[7] and also the preparation of molybdenum-free phosphotungstic acid[8]. The " direct " method, as applied to blood on the microchemical scale, has again been slightly modified by King, Haslewood and Delory[9] and their technique is given here. For clinical purposes the determination of uric acid in urine has been almost entirely replaced by the corresponding examination as applied to blood but details of both " direct " and " indirect " procedures are given by Folin in one of his later papers[7] ; the simpler " direct " methods for both blood and urine are described below.

Method for Blood[9]. The following special reagents are required :—

1. An Aqueous Solution Containing 2 per cent. w/v of Sodium Cyanide and 6 per cent. w/v of Urea. This solution should be freshly prepared each week.

2. Folin's Uric Acid Reagent. This is made by treating 100 g. of molybdenum-free sodium tungstate gradually with a solution

of 32 ml. of phosphoric acid (s.g. 1·75) in 150 ml. of water, gently boiling the mixture under a refluxing condenser for 1 hour, adding sufficient bromine water to decolorise the liquid, boiling off the excess bromine, allowing to cool and diluting to 500 ml. In order to make the special molybdenum-free sodium tungstate a solution of 250 g. of sodium tungstate in 500 ml. of water is treated with 5N hydrochloric acid until neutral to litmus paper. The solution is saturated with hydrogen sulphide, allowed to stand for 24 hours and then treated with 400 ml. of absolute alcohol which is added gradually with constant shaking; after standing for a further 24 hours the mixture is filtered, the precipitate washed with alcohol (50 per cent.) until the filtrate is colourless, then dissolved in 375 ml. of water, 0·5 ml. of bromine added and the mixture boiled gently until the excess of bromine is dispelled. The heating is continued and a 40 per cent. w/v aqueous solution of sodium hydroxide added until the mixture is distinctly alkaline to phenolphthalein. The cooled solution, filtered if necessary, is treated with 200 ml. of absolute alcohol, allowed to stand for 24 hours, the white crystals filtered off and dried *in vacuo*.

3. Stock Standard Solution of Uric Acid. A solution of lithium carbonate is made by dissolving 0·6 g. in 150 ml. of cold water; this solution is filtered if necessary, warmed to 60° C. and added to a litre flask containing precisely 1 g. of pure uric acid. The warm mixture is shaken for 5 minutes, cooled under the tap then treated with 20 ml. of formaldehyde solution (38 per cent.) and enough water to half fill the flask. A few drops of methyl orange indicator solution are added and then gradually with shaking, 25 ml. of N sulphuric acid. The solution should turn pink when 2 to 3 ml. of acid remain to be added. The mixture is finally diluted to 1 litre and stored in a dark glass-stoppered bottle when it will keep almost indefinitely; 1 ml. of this solution contains 1 mg. of uric acid.

4. Diluted Standard Solution of Uric Acid, containing 0·004 mg. per ml., should be freshly made by diluting 2 ml. of the stock solution with water, adding 1 ml. of formaldehyde solution (38 per cent.) and making up the volume to 500 ml. with more water.

Capillary blood (0·2 ml.) is transferred to a centrifuge tube containing 3·2 ml. of a 3·0 per cent. aqueous solution of sodium sulphate, $Na_2SO_4.10H_2O$, and 0·6 ml. of Folin's uric acid reagent

added. After mixing the contents, the tube is centrifuged and 2 ml. of the clear supernatant fluid (equivalent to 0·1 ml. of the sample) is treated with 3 ml. of the cyanide-urea reagent. At the same time a mixture of 1 ml. of the diluted uric acid standard solution, 0·7 ml. of water and 0·3 ml. of Folin's uric acid reagent is treated with 3 ml. of the cyanide-urea reagent. The two tubes are plugged with cotton wool, immersed in a boiling water-bath for 5 minutes then cooled in running tap water for 5 minutes and the relative intensities of the blue colours compared by means of a suitable colorimeter. In order that the colour of the standard may closely approximate to that of the sample it may be necessary to apply the test to varying quantities of the diluted standard solution, the volume being suitably adjusted with distilled water.

Method for Urine[7]. To a 100-ml. graduated flask about half filled with water is added 1 ml. of the sample to be examined and sufficient water to bring the liquid to the mark. After mixing, 5 ml. of the diluted urine is introduced into a 25-ml. graduated test tube and to another similar tube is added 5 ml. of the diluted standard uric acid solution as described for use in the examination of blood. To each tube is added 10 ml. of the cyanide-urea solution and, after mixing, 4 ml. of Folin's uric acid reagent, both as described in the procedure for blood. After allowing the mixture to stand at room temperature for 25 minutes they are diluted to 25 ml. and the colour of the sample compared with that of the standard which is nearest to it in intensity.

Discussion. In this procedure the phosphotungstic acid reagent serves to precipitate the proteins while the excess produces a blue colour when reduced by uric acid in presence of cyanide. According to Folin[7], probably less than 10 per cent. of the colour obtained represents the direct reduction of the reagent by the urate, the other 90 per cent. (more or less) being induced reduction due to the presence of the cyanide ; the latter reaction is rather more specific for uric acid than the directly produced smaller fraction. The indirect reduction is subject to great variations in magnitude according to the quality of the uric acid reagent, the quality of the cyanide, the degree of alkalinity, the presence of carbonate, of amino compounds and so forth. Because of these obscure factors the method for the determination of uric acid probably represents the most complex reaction that has been successfully applied to colorimetric analysis.

Uric acid is normally present in blood to the extent of 1 to 4 mg. per 100 ml. In conditions of renal impairment, and in gout, high

values are found. In conducting the test it is important that the blood be not laked, as interfering substances, such as glutathione and ergothioneine, would be liberated from the cells. As originally planned, the determination of uric acid in blood was conducted on a macro-scale using 5 ml. of sample but the details are not included here as this technique offers little advantage over the micro-method and is now rarely employed.

Dependable uric acid values cannot be obtained from urines which contain much bile. Taken by itself, the problem represented by icteric urines is probably of slight importance but it does raise the question as to whether there may not be other urines in which this method of determining uric acid fails. The urines of some herbivorous animals may well be a case in point and in such circumstances the reliability, or otherwise, of the method should be carefully checked by conducting recovery experiments after adding known amounts of pure uric acid.

References

1. O. Folin and A. B. Macallum, Jr., *J. Biol. Chem.*, 1912, **11**, 265.
2. O. Folin and W. Denis, *J. Biol. Chem.*, 1912, **12**, 239.
3. O. Folin and A. B. Macallum, Jr., *J. Biol. Chem.*, 1913, **13**, 363.
4. O. Folin and W. Denis, *J. Biol. Chem.*, 1913, **13**, 469.
5. O. Folin and W. Denis, *J. Biol. Chem.*, 1913, **14**, 95.
6. S. R. Benedict, *J. Biol. Chem.*, 1922, **51**, 187.
7. O. Folin, *J. Biol. Chem.*, 1933, **101**, 111.
8. O. Folin, *J. Biol. Chem.*, 1934, **106**, 311.
9. E. J. King, G. A. D. Haslewood and G. E. Delory, *Lancet*, 1937, **232**, 886.

SECTION IV

ALKALOIDS, HORMONES AND VITAMINS

INTRODUCTION

Alkaloids. Notwithstanding the multitude of qualitative colour tests available for members of this group the development of quantitative procedures depending upon colour production has received comparatively little attention, a fact which is somewhat surprising as these substances probably respond more readily to the application of colorimetric methods than most classes of organic compounds. However, it is only in special circumstances that such procedures offer any advantage in the assay of vegetable drugs since, in general, when once the alkaloids have been separated the proportion present may better be determined by gravimetric or volumetric methods.

Isolation of Alkaloids from Viscera. Much research has been devoted to this subject but the original Stas-Otto process[1, 2] is still preferred by the majority of toxicologists, particularly in the absence of circumstantial evidence as to the probable identity of the poison present. The following is a condensed account of the procedure recommended by Frank Bamford[3] :—

> The weighed sample is cut into small pieces with scissors, the pieces covered with alcohol (95 per cent.), the liquid acidified to moistened litmus paper by the addition of tartaric acid, the mixture warmed to 50° C. and then allowed to stand overnight. The liquid is separated from the solid material by squeezing through a double thickness of muslin, the solids pressed into a hard dry cake using a mechanical screw-press, the exuded liquid being added to the initial filtrate, then the cake passed through a mincing machine and extracted twice more with strong alcohol in the same manner as before, excepting that treatment in the press is not usually necessary. The mixed extracts are allowed to stand some hours, filtered through a thick fluted paper (Schleicher and Schüll, No. 572), the filtrate partially

evaporated in a strong current of air over a warm, but not boiling, water-bath, separated fat and protein filtered off and the evaporation completed. Boiling absolute alcohol is added to the residue, the animal matter broken up with a glass rod, using sand if necessary, the mixture cooled overnight in a refrigerator, the alcoholic extract filtered and the filtrate evaporated to dryness under reduced pressure.

If the residue still contains fat it is taken up with very dilute sulphuric acid, transferred to a separator and the fat extracted by shaking gently with light petroleum. If an emulsion forms, the aqueous layer is saturated with sodium sulphate when the further addition of a little alcohol should effect separation. The lower layer is drawn off, the light petroleum washed with acidulated water, the washing added to the main aqueous liquid, the latter neutralised with sodium carbonate, evaporated to dryness at a low temperature in a current of air, the resulting cake of salt extracted with absolute alcohol, the alcoholic solution filtered and evaporated to dryness under reduced pressure. The residue is dissolved in very dilute acetic acid, a slight excess of an aqueous solution of lead acetate previously acidified with acetic acid added, the precipitate coagulated by warming, the mixture filtered, excess lead removed from the latter by cautiously adding dilute sulphuric acid and the lead sulphate filtered off. Any non-basic poisons are removed at this stage by extracting with ether, the aqueous liquid is then rendered alkaline with dilute sodium hydroxide solution and any alkaloids which may be present, except morphine, extracted by shaking out with chloroform. The aqueous liquid is acidified with sulphuric acid, then rendered alkaline with ammonia, an equal volume of methyl alcohol added, and any morphine extracted with five or six successive portions of chloroform. In each case, after evaporation of the solvent, any alkaloidal residue will generally be sufficiently pure for identification and determination. In difficult cases further purification can be effected by dissolving in dilute sulphuric acid, precipitating with Mayer's reagent, filtering, washing the precipitate with dilute acid, decomposing with hydrogen sulphide solution, filtering off the mercuric sulphide, again rendering alkaline and extracting with chloroform.

In carrying out the above process no trouble should be experienced by reason of interference from the basic nitrogenous pro-

ducts of putrefaction, collectively termed ptomaines, since almost without exception they volatilise or, being readily soluble in water, remain in the aqueous liquid. Among modern alternatives to the Stas-Otto method may be mentioned the proposal of Stewart, Chatterji and Smith[4] in which proteins are eliminated by conducting the initial extraction with trichloracetic acid and the alkaloids are isolated by adsorption on kaolin. Doubt has been expressed as to the reliability of this method and Daubney and Nickolls[5, 6] have developed a more promising process in which the sample is frozen, minced, treated with diluted acetic acid and the proteins precipitated by saturating with ammonium sulphate. However, alcohol as an initial solvent has the advantage that it will certainly extract almost all organic poisons and G. Roche Lynch has stated[7] that he never had any difficulty in isolating morphine from viscera by the ordinary Stas-Otto process while Bamford emphasises the value of lead acetate for the purification of the final extracts[8].

Hormones. Attempts to evaluate preparations containing hormones by colorimetric methods have not, so far, led to any notable results and the author has only ventured to include procedures for three members of the group. The fact that the physiologically active substances belonging to this class are nearly always accompanied by numerous relatively inert compounds of closely allied chemical composition is the fundamental difficulty which confronts the analyst, whence chemical assays, as contrasted with biological, are of limited value. It may, however, be of some interest to pass in review some of the proposals which have been made to deal with the problem by means of colorimetric reactions.

Kober[9] proposed a qualitative colour test for the œstrogens (œstrone, œstradiol and œstriol ; the two latter constituting the follicular hormone of the ovary) using phenolsulphonic acid and several investigators have attempted to apply the test quantitatively[10–14], one of the more recent procedures being due to Bachman and Pettit[15] which embodies a preliminary separation of œstrone and œstradiol from œstriol and other chromogenic substances ; but in all cases the technique for carrying out the colour test is rather involved. Alternative methods include the use of benzoyl chloride[16], α-nitroso-β-naphthol in the presence of nitric acid[17] and diazotised dianisidine[18], this latter probably being the best, although when applied to the determination of œstrone in human urine it is necessary to conduct a lengthy process of isolation in order to eliminate interfering substances. The

fact that the method given in the text for the colorimetric determination of androsterone and other 17-ketosteroids using *m*-dinitrobenzene responds equally to testosterone lends interest to the suggestion of V. L. Koenig *et al.* for the colorimetric determination of the latter using potassium guaiacol sulphonate in the presence of sulphuric acid and copper sulphate and measuring the green fluorescence produced. No serious attempts appear to have been made to apply colorimetric determinations to hormones other than those mentioned in the text.

Vitamins. While it is clear that the application of colorimetric analysis has contributed little to the elucidation of the problems connected with the hormones, it is far otherwise with the vitamins. Before the development of the spectrophotometric method for the determination of vitamin A the well-known Carr-Price antimony trichloride test provided most valuable data, materially aiding the search for rich natural sources and the preliminary work of artificial concentration, while it is still widely employed in routine work connected with the fortification of foodstuffs.

With regard to the determination of vitamin B_1 it is probably safe to assert that Jansen's thiochrome test is the method of choice and its importance is attested by the extensive literature devoted to its application. Suggestions have been made for the colorimetric determination of certain other members of the B complex but, apart from nicotinic acid and nicotinamide, the procedures have not been sufficiently well developed to justify their description in this book although it may be helpful to mention that a method dependent upon its fluorescence has been proposed[20] for the determination of vitamin B_2 (riboflavine or lactoflavine) and applied to urine[21, 22] while a promising technique for the estimation of vitamin B_6 (pyridoxine) using a modification of the Gibbs phenol test[23] has been described by Scudi *et al.*[24, 25] and adapted to the examination of various pharmaceutical and biological materials by Bird, Vandenbelt and Emmett[26].

For the determination of vitamin C the volumetric method using phenol-indo-2 : 6-dichlorophenol is nearly always preferred but a few colorimetric procedures have been proposed. Thus, Roe has recommended decomposition of ascorbic acid to furfuraldehyde by treatment with hydrochloric acid and colorimetric determination of the latter with aniline[27] but, while this obviates interference due to reducing material such as sulphydryl compounds, the results are influenced by the presence of pentoses and uronic acids. Other suggestions include reduction of phospho-

and silico-molybdates and tungstates[28 29] while another recommendation involves adding an excess of phenol-indo-2 : 6-dichlorophenol, measuring the colour of the unreduced dye after extracting it with either nitrobenzene[30] or chloroform[31] and deducting the result from the value obtained for the total quantity of dye added. Carruthers[32] claims that accurate results can be obtained directly, provided the light transmission at 605 mμ of the dye is measured at a reaction equivalent to *p*H 6·5 before and after reduction, while interference from glutathione and cysteine is minimised by the addition of mercuric chloride.

REFERENCES

1. J. S. Stas, *Bull. Acad. roy. Med. Belg.*, 1851, **11**, 304.
2. J. Otto, *Ann. Chem. Pharm.*, 1856, **100**, 39.
3. F. Bamford, " Poisons : Their Isolation and Identification," London : J. & A. Churchill, Ltd., 1940.
4. C. P. Stewart, S. K. Chatterji and S. Smith, *Brit. Med. J.*, 1937, ii, 790.
5. C. G. Daubney and L. C. Nickolls, *Analyst*, 1937, **62**, 851.
6. C. G. Daubney and L. C. Nickolls, *Analyst*, 1938, **63**, 560.
7. G. Roche Lynch, *Analyst*, 1938, **63**, 237.
8. F. Bamford, *Analyst*, 1938, **63**, 645.
9. S. Kober, *Biochem. Z.*, 1931, **239**, 209.
10. S. L. Cohen and G. F. Marrian, *Biochem. J.*, 1934, **28**, 1603.
11. G. F. Cartland, R. K. Meyer, L. C. Miller and M. H. Rutz, *J. Biol. Chem.*, 1935, **109**, 213.
12. G. Pincus, G. Wheeler, G. Young and P. Zahl, *J. Biol. Chem.*, 1936, **116**, 253.
13. C. Bachman, *J. Biol. Chem.*, 1939, **131**, 455.
14. C. Bachman, *J. Biol. Chem.*, 1939, **131**, 463.
15. C. Bachman and D. S. Pettit, *J. Biol. Chem.*, 1941, **138**, 689.
16. S. Görtz, *Biochem. Z.*, 1934, **273**, 396.
17. K. Voss, *Z. physiol. Chem.*, 1937, **250**, 218.
18. N. B. Talbot, J. K. Wolfe, E. A. MacLachlan, F. Karush and A. M. Butler, *J. Biol. Chem.*, 1940, **134**, 319.
19. V. L. Koenig, F. Melzer, C. M. Szego and L. T. Samuels, *J. Biol. Chem.*, 1941, **141**, 487.
20. A. D. Emmett, O. D. Bird, R. A. Brown, G. Peacock and J. M. Vandenbelt, *Ind. Eng. Chem., Anal. Ed.*, 1941, **13**, 219.
21. M. Swaminathan, *Indian J. Med. Res.*, 1942, **30**, 37.
22. M. Swaminathan, *Indian J. Med. Res.*, 1942, **30**, 45.
23. H. D. Gibbs, *J. Biol. Chem.*, 1927, **72**, 649.
24. J. V. Scudi, H. F. Koones and J. C. Keresztesy, *Proc. Soc. Exp. Biol. Med.*, 1940, **43**, 118.
25. J. V. Scudi, *J. Biol. Chem.*, 1941, **139**, 707.
26. O. D. Bird, J. M. Vandenbelt and A. D. Emmett, *J. Biol. Chem.*, 1942, **142**, 317.
27. J. H. Roe, *J. Biol. Chem.*, 1936, **116**, 609.
28. E. Brand and B. Kassell, *J. Biol. Chem.*, 1938, **125**, 115.
29. M. L. Isaacs, *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 948.
30. F. Bukatsch, *Z. physiol. Chem.*, 1939, **262**, 20.
31. H. F. W. Kirkpatrick, *J. Soc. Chem. Ind.*, 1943, **62**, 39.
32. C. Carruthers, *Ind. Eng. Chem., Anal. Ed.*, 1942, **14**, 826.

ATROPINE, HYOSCYAMINE AND HYOSCINE (SCOPOLAMINE)

These alkaloids can be determined colorimetrically by a quantitative application of Vitali's test[1] using acetone as solvent[2]. Atropine and hyoscyamine give precisely the same response to the test while the intensity of the colours produced by equal weights of hyoscine and hyoscyamine are inversely proportional to their molecular weights. The method has been applied to the assay of belladonna and stramonium and to galenicals made from these drugs[3], and also to hypodermic tablets and injections[4]. A colour reaction for atropine in which the alkaloid is heated with *p*-dimethylaminobenzaldehyde in the presence of sulphuric acid[5] has been recommended for quantitative purposes[6] but it is subject to the disadvantage that the colour produced varies from violet to orange according to the time of heating and, also, numerous other organic bases yield deeply coloured mixtures with the reagent.

Method[3, 4]. The solution to be examined, which may be an injection or aqueous solution of hypodermic tablets, is diluted with water so that each ml. may be expected to contain between 0·8 and 1·2 mg. of the solanaceous alkaloid, 2 ml. of this is transferred into a small separator, 0·5 ml. of dilute solution of ammonia (10 per cent. w/w NH_3) added followed by 3 ml. of chloroform. The mixture is shaken, allowed to separate, the chloroformic layer drawn off into another separator and the extraction repeated twice using 3 ml. of chloroform on each occasion. The chloroformic extracts are mixed, washed once with 2 ml. of water, transferred into a 50-ml. stoppered measuring cylinder, the separator containing the wash water is rinsed with 1 ml. of fresh chloroform and the latter run into the main chloroformic extract. Exactly 20 ml. of 6 per cent. acetic acid (made with approximately 5 per cent. alcohol) is added, the stopper inserted and the mixture shaken for about 15 seconds. After allowing to stand until the immiscible liquids have separated, about 5 ml. of the upper layer is pipetted off, filtered through a dry paper and exactly 1 ml. of the filtrate transferred to an evaporating dish of 5 cm. diameter. The dish is placed on a boiling water-bath and the liquid evaporated *just* to dryness and 0·2 ml. of fuming nitric acid (s.g. 1·5 : AnalaR grade) immediately added from a dropping pipette. After ensuring that the acid has made contact with the whole of

the alkaloidal residue it is evaporated off, the dish being left on the water-bath for a total of 3 minutes, excluding the time required to evaporate the solution of alkaloid in dilute acetic acid. The residue is dissolved in about 3 ml. of dry acetone, the solution transferred with the help of a small glass rod to a standard stoppered 10-ml. measuring cylinder, the dish washed with further small quantities of acetone and the washings transferred to the measure, until the latter contains exactly 10 ml. of solvent. After allowing to cool, should this be necessary, 0·1 ml. of a 3 per cent. solution of potassium hydroxide (AnalaR) in methyl alcohol (AnalaR), which has been prepared not more than a fortnight beforehand, is added, the stopper inserted, the cylinder inverted once and the mixture then allowed to stand for exactly 5 minutes. A portion of the purple liquid is transferred to a 1-cm. cell, the intensity of the colour immediately measured by means of a Lovibond tintometer and the value obtained for the red component correlated with the amount of alkaloid present in the proportion of acetic acid extract taken for the colour test by reference to Table XIX. The result found for the amount of

TABLE XIX. RELATION BETWEEN THE QUANTITY OF ATROPINE (OR HYOSCYAMINE) AND THE COLOUR PRODUCED

Quantity of Anhydrous Atropine (or Hyoscyamine) mg.	Colour after dilution to 10 ml. : observed in 1-cm. cell Lovibond Units		
	Red	Blue *	Brightness *
0·025	1·2	0·8	0·2
0·050	2·6	1·8	0·6
0·070	4·0	2·4	0·7
0·075	4·3	2·6	0·8
0·100	6·2	3·5	1·0
0·125	8·4	4·3	1·2
0·135	9·1	4·5	1·5
0·150	10·6	5·4	2·1

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

anhydrous atropine (or hyoscyamine) base present may be converted into content of the commonly occurring salts of these alkaloids, or into hyoscine or its hydrobromide by multiplying by the following factors : atropine sulphate ($1H_2O$), 1·201 ; hyoscyamine sulphate ($2H_2O$), 1·234 ; hyoscyamine sulphate

(anhydrous), 1·171 ; hyoscyamine hydrobromide (anhydrous), 1·280 ; hyoscine, 1·049 ; hyoscine hydrobromide ($3H_2O$), 1·515.

Application to Hypodermic Tablets, etc. Also Containing Morphine[4]. A measured quantity of the solution to be examined, which may be an injection or an aqueous solution of hypodermic tablets and which is expected to contain approximately 1 mg. of the solanaceous alkaloid, is transferred into a small separator. After diluting with water until the volume of the liquid approximates to 5 ml., 0·5 ml. of a solution of ferric chloride (5 per cent. w/v of $FeCl_3$ in water) is added, the mixture allowed to stand for 2 minutes and then 2 g. of sodium citrate added. When the latter has dissolved the mixture is rendered alkaline by adding 0·5 ml. of dilute solution of ammonia and the extraction and colorimetric determination of the solanaceous alkaloid completed as described above.

Application to Belladonna Leaf and Root[3]. A miniature percolator with an overall length of 17·5 cm. is made from glass tubing of 8 mm. bore. The tube is sharply narrowed about 3 cm. from one end while the other end is spun out to form a flange of about 2·5 cm. diameter. The sample under examination is reduced to No. 60 powder, 1 g., accurately weighed, is introduced into a 50-ml. beaker, 1 ml. of alcohol (95 per cent.) and 0·1 ml. of dilute solution of ammonia (10 per cent. w/w NH_3) added and the mixture stirred until evenly wetted. About 5 ml. of chloroform is added, heated to boiling point and as much of the drug as possible is transferred into the dry miniature percolator which has been previously plugged with about 0·02 g. of cotton-wool and suspended within a stoppered 100-ml. measuring cylinder. If necessary, the drug is gently compressed with a small glass rod so that the solvent percolates at the rate of about 1 drop a second. More chloroform is added to the beaker, the transference of the drug to the percolator is completed and the extraction continued until the volume of the percolate is 31 ml. Sufficient 6 per cent. acetic acid (made with approximately 5 per cent. alcohol) is added to the percolate to bring the level of the liquid to the 80-ml. graduation mark, the cylinder is stoppered and gently shaken for about 15 seconds. After allowing to stand until the immiscible liquids have separated, or until the upper part of the aqueous layer is free from coloured chloroform, about 5 ml. of the upper layer is pipetted off, filtered through a dry paper and exactly 1 ml. of the filtrate transferred to an evaporating dish of 5 cm. diameter. The colorimetric determination is then conducted

precisely as already described commencing with the evaporation of the liquid *just* to dryness.

Application to Stramonium Leaf[3]. The method given for belladonna leaf or root is followed but 2 ml. instead of 1 ml. of the filtered solution of alkaloid in acetic acid is taken for the colour test.

Application to Galenicals[3] (*Powdered Belladonna Leaf B.P.*). An accurately weighed portion, 1 g., is introduced into a 50-ml. beaker, 0·5 g. of sawdust (previously extracted with ether and passed through a No. 60 mesh sieve) is added. After mixing, the powders are evenly wetted with 1 ml. of alcohol (95 per cent.) and 0·1 ml. of dilute solution of ammonia and the assay then continued by percolating with chloroform as for belladonna leaf, special caution being exercised in order to obviate any tendency towards clogging due to the finely divided condition of the sample.

(*Tincture of Belladonna B.P. and Tincture of Stramonium B.P.*). The sample, 15 ml., contained in a small beaker is evaporated over a boiling water-bath until the volume is reduced to about 5 ml., then 2 ml. of alcohol (95 per cent.) is added and the evaporation continued until approximately 1 ml. of liquid remains. To the residue is added 1 g. of extracted sawdust and 0·1 ml. of dilute solution of ammonia, the mixture stirred and the assay continued by percolating with chloroform as for belladonna leaf.

(*Liniment of Belladonna B.P. and Chloroform of Belladonna B.P.C.*). To 1 ml. of the sample, contained in a small beaker, is added 0·1 ml. of dilute solution of ammonia and 0·5 g. of extracted sawdust. After stirring until a uniform mixture is produced the assay is continued by percolating with chloroform as for belladonna root.

(*Liquid Extract of Belladonna B.P.*). To 0·5 ml. of the sample, contained in a small beaker, is added 0·5 ml. of alcohol (95 per cent.) then 0·1 ml. of dilute solution of ammonia and, finally, 0·5 g. of extracted sawdust. After stirring until a uniform mixture is produced the assay is continued by percolating with chloroform as for belladonna root.

(*Liquid Extract of Stramonium B.P.*). To 2 ml. of the sample, contained in a small beaker, is added 0·1 ml. of dilute solution of ammonia and 1 g. of extracted sawdust. After stirring until a uniform mixture is produced the assay is continued by percolating with chloroform as for belladonna leaf.

(*Glycerin of Belladonna B.P.C.*). To about 1 g., accurately

weighed into a small beaker, is added 1 ml. of alcohol (50 per cent.), 0·1 ml. of dilute solution of ammonia and 1 g. of extracted sawdust. After stirring until a uniform mixture is produced the assay is continued by percolating with chloroform as for belladonna leaf.

(*Green Extract of Belladonna B.P.C. and Extract of Stramonium B.P.C.*). The assay is conducted in the same manner as for glycerin of belladonna excepting that 0·5 g. of the sample is taken and only 0·5 g. of extracted sawdust is used.

(*Dry Extract of Belladonna B.P.*). To about 0·5 g., accurately weighed into a small beaker, is added 1 ml. of alcohol (50 per cent.), the mixture stirred until uniform, then 0·1 ml. of dilute ammonia and 0·5 g. of extracted sawdust added and the assay continued by percolating with chloroform as for belladonna leaf.

(*Dry Extract of Stramonium First Addendum* 1936 *to B.P.* 1932). To about 0·5 g., accurately weighed into a small beaker, is added 0·5 g. of lard, 1 ml. of alcohol (50 per cent.) and 1 ml. of chloroform. The mixture is gently warmed and stirred until uniform then 0·1 ml. of dilute solution of ammonia and 0·5 g. of extracted sawdust added and the assay continued by percolating with chloroform as for belladonna leaf.

(*Ointment of Belladonna B.P.C.*). To about 1 g., accurately weighed into a small beaker, is added 1 ml. of alcohol (50 per cent.) and 1 ml. of chloroform. The mixture is gently warmed and the assay continued by the method described for dry extract of stramonium commencing with the addition of 0·1 ml. of dilute solution of ammonia.

Discussion. For the successful quantitative application of this colour test it is essential that the solanaceous alkaloids be converted into their acetates; attempts to apply the method directly to the bases have always led to low results[3, 4]. The colour initially produced fades appreciably at first, but after 5 minutes remains practically constant for at least another 2 minutes. As with all methods depending upon the production of transient colours the intensity should be measured in terms of an external standard, as in the Lovibond tintometer, or referred to a previously calibrated curve by means of a photometric instrument; neither direct visual comparison nor measurement with colorimeters of the balancing type is satisfactory. For the purposes of calibration, hyoscyamine alkaloid the purity of which has been checked by observations of its optical rotation, basicity and melting-point should be employed; the use of atropine as a

standard should be avoided. For the determination of each point on the curve the appropriate quantity of alkaloid is dissolved in a mixture of 30 ml. of chloroform, 1 ml. of alcohol (90 per cent.) and 0·1 ml. of dilute solution of ammonia contained in a standard-stoppered 100-ml. measuring cylinder and sufficient 6 per cent. acetic acid (made with approximately 5 per cent. alcohol) is added to bring the level of the liquid to the 80-ml. graduation mark ; after shaking and allowing to separate, a portion of the upper layer is filtered and 1 ml. of the filtrate submitted to the colour test.

The accuracy of the test is not affected by varying the amount of nitric acid used within the limits of 0·1 to 0·3 ml. but the use of fuming acid, at least 95 per cent., is essential. The volume of potassium hydroxide solution added to the acetone extract is not critical and the quantity used may lie between 0·05 and 0·2 ml. The slight haze produced by the addition of the alkali disappears before the colour is measured. It is imperative that all apparatus be free from moisture and that AnalaR grade reagents be used. The presence of other alkaloids, and other substances generally, is likely to interfere with the colour test, but in the important case of hypodermic tablets and injections containing either atropine or hyoscine in admixture with morphine the interference of the latter has been eliminated by submitting the initial solution to oxidation with ferric chloride in the manner described above.

The application of this colorimetric test to the alkaloidal assay of stramonium and European belladonna has been found to be especially useful in cases where only small samples of material are available[7]. In cases where the drug is in a finely divided state, as with the standardised powdered leaf of the Pharmacopœia, admixture with sawdust prior to percolating should overcome any tendency to clogging. With dry extract of stramonium some trouble may be encountered due to the formation of an emulsion when the chloroformic percolate is shaken with the dilute solution of acetic acid : this may be due to the starch contained in the sample but the difficulty is minimised by using lard as directed. The method is not applicable to the assay of the official hyoscyamus or its preparations since the high proportion of extractive material coupled with the low concentration of alkaloids interferes with the effective isolation of the latter. Belladonna of Indian origin (*Atropa acuminata*) contains in addition to hyoscyamine, an appreciable proportion of volatile bases which interfere with the colorimetric assay by forming a haze in the final reaction mixture

which, besides making measurement difficult, accelerates fading of the purple colour. In view of this limitation it is important to note that *Atropa acuminata* is now recognised for official use, in addition to *Atropa Belladonna*, and is included in the Fifth Addendum, 1942, to the British Pharmacopœia, 1932, the prescribed method of assay being modified to ensure removal of volatile bases prior to the volumetric determination of the isolated alkaloids.

In conclusion it may be observed that the author has obtained a positive reaction in the application of Vitali's test to ethyl tropate, which suggests that the colour may be due to the formation of a conjugated chain from two or more tropyl residues exhibiting resonance. This would account for the fact that hyoscine (epoxytropine tropate) gives a positive reaction while homatropine (tropine mandelate) does not.

REFERENCES

1. D. Vitali, *Z. anal. Chem.*, 1881, **20**, 563.
2. C. Morin, *J. Pharm. Chim.*, 1936, **23**, 545.
3. N. L. Allport and E. S. Wilson, *Quart. J. Pharm.*, 1939, **12**, 399.
4. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1942, **15**, 238.
5. R. Wasicky, *Z. anal. Chem.*, 1915, **54**, 393.
6. R. P. Daroga, *J. Indian Chem. Soc.*, 1941, **18**, 579.
7. G. M. Watson, C. R. C. Heard and W. O. James, *Quart. J. Pharm.*, 1941, **14**, 253.

DIAMORPHINE (HEROIN)

Denigès' colour test for morphine and its derivatives using very dilute copper sulphate in association with ammonia and hydrogen peroxide[1] has been recommended for the determination of diamorphine[2] but the yellowish-red colour produced does not exhibit a sufficient degree of gradation to warrant its quantitative application. The reaction mentioned by Miller[3] using sulphuric acid and formaldehyde is also unsatisfactory. The method described here[4] depends upon preliminary hydrolysis of the alkaloid with boiling dilute hydrochloric acid and determination of the morphine so produced.

Method[4]. A measured quantity of the solution to be examined, expected to contain between 0·3 and 0·9 mg. of the alkaloid, is diluted to 10 ml. with water and 10 ml. of dilute hydrochloric acid (10 per cent. HCl) added. The mixture is boiled under a reflux

condenser for 10 minutes, allowed to cool, transferred to a Nessler glass and the determination completed by the general method given for morphine (see p. 310). The colour produced is conveniently matched against standards prepared from a 0·002 per cent. solution of anhydrous morphine in 0·1N hydrochloric acid whence the indicated morphine content is multiplied by 1·486 in order to convert the result into terms of diamorphine hydrochloride ($1H_2O$) or by 1·295 if the result is to be calculated as diamorphine base.

Discussion. Neither codeine nor ethylmorphine (the hydrochloride of which is known under the trade name Dionin) give any colour with nitrite and ammonia, irrespective of whether or not they have been treated with boiling dilute hydrochloric acid. Apomorphine in dilute acid solution gives a deep reddish-brown colour with nitrite alone which is intensified by the addition of excess ammonia, hence its presence will interfere with the determination of morphine and diamorphine ; but apomorphine may be differentiated from both by reason of the deep colour produced in acid solution on addition of sodium nitrite. This latter reaction might well serve for the colorimetric determination of apomorphine.

REFERENCES

1. G. Denigès, *Compt. rend.*, 1910, **151**, 1062.
2. F. Bamford, *Analyst*, 1931, **56**, 586.
3. R. Miller, *Amer. J. Pharm.*, 1915, **87**, 248.
4. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1942, **15**, 238.

EPHEDRINE

The colour reaction due to Chen and Kao[1], and included in the British Pharmacopœia as an identity test for ephedrine hydrochloride has been modified in order to render it quantitative[2]. Essentially, the original test consists in adding copper sulphate to an aqueous solution of the alkaloidal salt, rendering alkaline with sodium hydroxide and shaking with ether, the presence of ephedrine being indicated by a purple colour imparted to the ethereal layer. This procedure is quite unsuitable for colorimetric work but by using *cyclo*hexane in place of ether and adjusting the proportions of copper sulphate and sodium hydroxide a test has been evolved which is applicable to the assay of tablets of ephedrine hydrochloride or ephedrine sulphate.

Method[2]. A measured quantity (5 ml.) of an aqueous solution of the tablets, expected to contain the equivalent of 1 to 1·5 mg. of ephedrine base, is transferred to a small separator, 1 ml. of dilute solution of ammonia (10 per cent. w/w NH_3) added followed by 2 ml. of methylene chloride. After shaking and allowing to separate, the lower layer is drawn off into another separator, washed by shaking with 2 ml. of a saturated aqueous solution of sodium chloride then run off into a dry stoppered weighing bottle. The ammoniacal mixture in the first separator is extracted with two further successive 2-ml. portions of methylene chloride, each extract is washed in turn with the same saturated solution of sodium chloride already in the second separator and transferred to the weighing bottle. To the mixed extracts is added 1 ml. of a 1 per cent. solution of benzoic acid in methylene chloride and the whole evaporated to dryness over a boiling water-bath. To the residue is added exactly 1 ml. of water, the mixture gently warmed until solution is effected and, after allowing to cool, 0·1 ml. of a 10 per cent. aqueous solution of copper sulphate, $CuSO_4.5H_2O$, and 2 ml. of a 20 per cent. aqueous solution of sodium hydroxide (both of which should have been recently prepared) are added. After mixing, 3 ml. of *cyclo*hexane is introduced, the whole vigorously shaken and transferred to a dry test tube. The intensity of the stable purple colour of the separated upper layer is matched against standards similarly prepared from ephedrine hydrochloride satisfying the requirements of the British Pharmacopœia and which, in addition, contains total chlorine, present as chloride, equivalent to 100 per cent. of the alkaloidal salt. Alternatively, the colour may be measured by means of a Lovibond tintometer and the value obtained for the red component correlated with the amount of ephedrine present in the 5 ml. of the solution taken for the assay by reference to Table XX. The result found for the amount of anhydrous ephedrine base present may be converted into content of anhydrous salts by multiplying by the following factors: hydrochloride 1·223; sulphate 1·298.

In order to determine ephedrine in injections the sample is diluted with water so that each ml. may be expected to contain between 4 and 8 mg. of base and the colour test is applied to 1 ml. commencing with the addition of 0·1 ml. of copper sulphate solution.

Discussion. This colour reaction is unusually insensitive since 2 mg. or less of ephedrine base does not give any response although

TABLE XX. RELATION BETWEEN THE QUANTITY OF EPHEDRINE AND THE COLOUR PRODUCED

Quantity of Ephedrine (anhydrous) mg.	Colour of the *cyclo*hexane layer : observed in 1-cm. cell Lovibond Units		
	Red	Blue *	Brightness *
3·0	0·8	0·6	0·2
4·0	1·6	0·7	0·3
5·0	2·9	1·1	0·4
5·5	3·7	1·2	0·4
6·0	4·0	1·3	0·6
6·5	4·8	1·6	0·7
7·0	5·8	2·0	0·8
7·5	6·9	2·3	0·8
8·0	7·6	2·5	0·9
8·5	8·6	2·8	1·0
9·0	9·0	3·1	1·0
10·0	11·5	3·6	1·4

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

with quantities between 3 and 10 mg. the gradation is quite satisfactory. Both pseudo-ephedrine and synthetic ephedrine give precisely the same colour in the test as the natural alkaloid. Lactose interferes by reason of its reducing action on copper sulphate, hence, when applying the method to tablets it is necessary to isolate the alkaloid and, since ephedrine base is volatile, provision has to be made to prevent loss by adding benzoic acid. Since lactose is slightly soluble in chloroform, methylene chloride, in which the sugar is insoluble, is used for extracting the alkaloid. The colorimetric determination is not disturbed by the presence of procaine but the effect of other basic substances has not been investigated.

REFERENCES

1. K. K. Chen and C. H. Kao, *J. Amer. Pharm. Assoc.*, 1926, **15**, 625.
2. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1942, **15**, 238.

ERGOMETRINE AND ERGOTOXINE

Several alkaloids have been isolated from ergot of which the most important is the water-soluble base ergometrine discovered in 1935 by H. W. Dudley and J. Chassar Moir[1] in this country

and, almost contemporaneously, by a team of investigators in the United States[2] who termed the substance ergotocin.* Ergotoxine which is insoluble in water, was first isolated and characterised by G. Barger and F. H. Carr[3] and, prior to the discovery of ergometrine, was regarded as the most important active principle. Associated with these, are two physiologically inert alkaloids, namely, the water-insoluble base ergotinine and ergometrinine which is isomeric with ergometrine and is water-soluble. Ergotinine, the first crystalline alkaloid to be isolated[4], is isomeric with ergotoxine and can be converted into the phosphate of the latter by treating it with boiling absolute ethyl alcohol containing a little phosphoric acid[5]. In ergot of Festuca grass another alkaloid, called ergotamine, is predominant ; it is similar to, but not chemically identical with, ergotoxine and associated with it is a physiologically inactive counterpart termed ergotaminine.

A qualitative colour test for ergotinine using acetic acid and strong sulphuric acid was described by Tanret[4]. This reaction, which is also given by ergotoxine[3], was adapted to yield roughly quantitative results by Evers[6], who measured the blue-violet colours produced by means of a Lovibond tintometer. Subsequently, van Urk[7] discovered that *p*-dimethylaminobenzaldehyde in the presence of mineral acid constituted a much more satisfactory reagent and this new test was developed for quantitative use by Maurice I. Smith[8] whose procedure was exhaustively examined and slightly modified for official use in this country by a Sub-Committee to the British Pharmacopœia Commission[9]. As described in the B.P., 1932, it is necessary to develop the colour by exposure to bright light for at least 30 minutes but further investigation showed that by including in the reagent a certain optimum concentration of ferric chloride the maximum, stable, blue-violet colour is developed in 5 minutes, whether exposed to bright light or not[10]. It has also been shown[10] that many aldehydes, besides *p*-dimethylaminobenzaldehyde, yield colours with ergot alkaloids in the presence of strong mineral acids as, for example, vanillin, piperonal, paraldehyde, *p*-acetylaminobenzaldehyde, and this fact suggests that the mechanism of Tanret's original test may be associated with the formation of traces of acetaldehyde by the action of the strong mineral acid on acetic acid. Following the discovery of ergometrine it was found that

* In the United States Pharmacopœia XII (1942) the maleate of this alkaloid is official under the name Ergonovine Maleate but the name ergometrine is sometimes used in American literature. In Continental Europe it is known as ergobasine.

this alkaloid also yielded a colour by the same test, spectroscopically identical with that produced by ergotoxine but 1·78 times more intense[11]. The modified reagent containing ferric chloride as catalyst was included in the First Addendum, 1936, to the B.P., 1932.

From the clinical viewpoint ergometrine is much more valuable then ergotoxine, hence the method of assay published in the B.P., 1932, which fails to take account of the water-soluble alkaloid, has given place to a procedure developed by C. H. Hampshire and G. R. Page[12–14] and now included in the Sixth Addendum, 1943. A description of the colorimetric test when using solutions of the purified alkaloids is given below together with its application to the crude drug and to the official Liquid Extract of Ergot.

Method. The sample of alkaloidal base or salt is dissolved in a 1 per cent. aqueous solution of tartaric acid the strength being adjusted so that 1 ml. may be expected to contain about 0·05 mg. of the pure base. To 1 ml. of this solution is added 2 ml. of a 0·125 per cent. w/v solution of *p*-dimethylaminobenzaldehyde in sulphuric acid (65 per cent. v/v) containing 0·005 per cent. w/v of ferric chloride, $FeCl_3$. After the mixture has stood for 5 minutes the intensity of the blue-violet colour is measured by means of a Lovibond tintometer, using a 1-cm. cell. Considering only the blue component of the colour, the amount of alkaloid present in the quantity of solution submitted to the test is calculated on the basis that 0·05 mg. of solvent-free ergometrine produces a colour equal to 7·6 blue units or that the value for the same weight of ergotoxine is 4·1 units. Ergotoxine ethanesulphonate contains approximately 83·6 per cent. of ergotoxine whence the weight of base found multiplied by 1·196 will give the amount of salt. Alternatively, the intensity of the colour due to the sample may be matched in a colorimeter against that produced by applying the test to a standard solution freshly prepared by dissolving 0·012 g. of ergotoxine ethanesulphonate in about 5 ml. of warm water containing 1 g. of tartaric acid, allowing to cool and diluting with water to 100 ml. This solution, which may be preserved from mould growth by the inclusion of 0·1 ml. of saturated aqueous solution of mercuric chloride, contains 0·1 mg. of ergotoxine per ml. If this method of measurement be employed the strength of the test solution should be so adjusted that the colour produced does not differ by more than 20 per cent. from that given by the standard solution.

Application to Ergot (*Ergometrine by Difference*)[12–14]. A repre-

sentative sample is ground to a moderately fine powder and 10 g. transferred to a continuous extraction apparatus of the Soxhlet type, or preferably of the design illustrated in Fig. 14, and the fat removed using light petroleum (b.pt. 40° to 50° C.). The fat-free drug is dried at a temperature not exceeding 40° C., transferred to a porcelain dish, sufficient peroxide-free ether (Anæsthetic

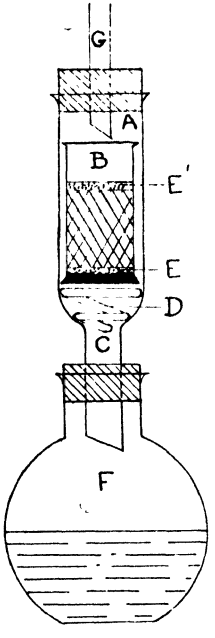


FIG. 14. A CONTINUOUS EXTRACTION APPARATUS FOR VEGETABLE DRUGS

The wider part of the outer tube of stout glass is about 18 cm. in length, and is 4·8 to 5 cm. in bore while the lower end C is about 5 cm. in length and has an external diameter of about 1·6 cm. The tube B, 9 cm. in length with an external diameter of 3·8 cm., is open at both ends, the lower being flanged and covered with calico attached by means of thin string, and supported on the coil of iron wire or bent glass rod D. The powdered drug is introduced into B between two pads of cotton wool E and E′ and the extractor attached to the flask F, containing the solvent, and to the tube of a reflux condenser G. (After P. A. W. Self and C. E. Corfield, *Quart. J. Pharm.*, 1930, **3**, 408.)

Ether, B.P.) added to form a semi-liquid mass, then 8 ml. of dilute solution of ammonia (10 per cent. w/w NH_3) incorporated while the mixture is stirred with a glass rod. Most of the ether is allowed to evaporate, the residue returned to the continuous extraction apparatus and the total alkaloids removed by extracting with about 100 ml. of peroxide-free ether during 5 hours. The ethereal solution is filtered through a plug of cotton wool, the

flask and filter washed with more of the same solvent until about 110 ml. of filtrate is obtained. After adding 20 ml. of acetone the liquid is transferred to a separator and extracted, first with 20 ml. of a 1 per cent. aqueous solution of tartaric acid and then with three further portions each of 10 ml. of the same acid solution. The mixed acid extracts are transferred to an open dish and the dissolved solvents removed by submitting to reduced pressure for about an hour. The solution of the total alkaloids is diluted to 50 ml., or other suitable known volume, 1 ml. is submitted to the colorimetric test already described and the result is calculated in terms of ergotoxine base.

Half the acid extract of the total alkaloids prepared as above (representing 5 g. of the original drug) is transferred to a separator, rendered alkaline with dilute solution of ammonia and the total alkaloids removed by shaking with successive quantities of 40, 30, 30 and 20 ml. of peroxide-free ether. The ethereal layers are mixed and the water-soluble alkaloids (ergometrine and ergometrinine) are removed by extracting with five successive quantities, each of 40 ml., of water which has been made faintly alkaline to litmus with dilute solution of ammonia and saturated with peroxide-free ether. These alkaline aqueous extracts are discarded. The water-insoluble alkaloids remaining in the ethereal solution are then extracted with successive quantities of 10, 5, 5 and 5 ml. of a 1 per cent. aqueous solution of tartaric acid, the mixed acid extracts transferred to an open dish and the dissolved solvents removed by submitting to reduced pressure for about an hour. The solution of the water-insoluble alkaloids is diluted to 25 ml. or other suitable known volume, 1 ml. is submitted to the colorimetric test and the result is calculated in terms of ergotoxine base. The figure thus obtained is subtracted from the percentage of total alkaloids as previously determined and the difference multiplied by the factor 0·538 to effect conversion to ergometrine.

(*Ergometrine Directly*)[15]. Into a percolator of about 2 cm. bore is introduced a plug of cotton wool weighing approximately 0·25 g. then 1 g. of ivory nut meal of commerce (in about No. 20 powder) is added. Upon this is introduced a second pledget of cotton wool, then a further layer of ivory nut meal and finally a third portion of cotton wool. The percolator is then attached to a suction filtering flask.

A representative sample of the ergot to be assayed is reduced to No. 60 powder, 5 g. transferred to a stoppered conical flask, 50 ml. of acetone and 0·7 ml. of dilute solution of ammonia (10 per cent.

w/w NH_3) added and the mixture shaken at intervals during 20 minutes. After allowing to settle the supernatant liquid is decanted into the prepared percolator, as much of the drug as possible being left behind in the flask. Gentle suction is applied to the percolator, the drug in the flask is washed with two 10-ml. portions of acetone, the washings being poured through the percolator. When the ergot in the latter is dry it is transferred back into the flask and to it is added 50 ml. of peroxide-free ether and 0·25 g. of light magnesium oxide diffused in 5 ml. of water, the mixture shaken at intervals during 20 minutes then 2 g. of anhydrous sodium sulphate added, the mixture vigorously shaken and the whole transferred into the percolator and washed through with 90 ml. of peroxide-free ether. The percolate is transferred to a separator, the filtering flask being washed with a further 10 ml. of ether : 10 ml. of a 1 per cent. aqueous solution of tartaric acid is added, the mixture shaken then allowed to separate and the lower layer transferred to an evaporating dish. The extraction of the total alkaloids is continued with four separate 10-ml. portions of tartaric acid solution. The last extract is tested for alkaloids by mixing 1 ml. with 2 ml. of *p*-dimethylaminobenzaldehyde reagent and if any appreciable colour is produced additional extractions are made. The dissolved solvents from the combined acid extracts are removed by submitting to reduced pressure for about an hour then the liquid is diluted with water to 70 ml. and 10 ml. transferred into a 25-ml. graduated flask and diluted to the mark with water. The concentration of total alkaloids in this second dilution is determined by means of the colorimetric test and the result calculated in terms of ergotoxine base.

The reaction of the remaining 60 ml. of the first dilution is adjusted to *p*H 5·5 by adding methyl red indicator solution and a sufficient quantity (between 20 and 30 ml.) of a 3 per cent. w/v aqueous solution of disodium hydrogen phosphate, Na_2HPO_4, to render the colour of the mixture equal to that produced by adding methyl red indicator to a buffer solution consisting of a 3 per cent. w/v solution of disodium hydrogen phosphate, Na_2HPO_4, in a 1·42 per cent. w/v aqueous solution of tartaric acid. The liquid is transferred to a separator, and the ergotoxine is extracted by shaking, first with 40 ml. of peroxide-free ether, then with three separate portions each of 25 ml. The ether layers are combined, then shaken with 10 ml. of the *p*H 5·5 buffer solution and the latter added to the extracted aqueous liquid which now contains all the ergometrine (with the ergometrinine), while the ether

contains the ergotoxine (together with other water-insoluble alkaloids), the respective alkaloids in each being derived from six-sevenths of the amount of ergot originally taken for the assay.

In order to determine the ergotoxine the ethereal solution is extracted with four 10-ml. portions of aqueous tartaric acid solution, the dissolved ether removed from the combined extracts by gently warming in a current of air, the cooled liquid diluted to 100 ml. with water and the colorimetric test applied.

To determine the ergometrine, 7 ml. of a 20 per cent. w/v solution of sodium hydroxide is added to the aqueous liquid from the above separation and this is followed by sufficient sodium chloride (about 35 g.) to produce a saturated solution and to leave a small proportion undissolved. The alkaloidal material is extracted by shaking, first with 40-ml., then with three 25-ml. portions of peroxide-free ether. The separated and combined ethereal layers are then extracted with four portions each of 5 ml. of tartaric acid solution, the dissolved ether removed from the combined aqueous liquids by gently warming in a current of air, the cooled liquid diluted to 25 ml., or the smallest convenient volume, and the colorimetric test applied.

Application to Liquid Extract of Ergot[15]. A measured volume (10 ml.) of the sample is transferred to a separator, 7 ml. of a 20 per cent. w/v aqueous solution of sodium hydroxide and 50 ml. of a saturated aqueous solution of sodium chloride added followed by 50 ml. of a mixture of 3 vols. of ether and 1 vol. of acetone. After shaking and allowing to separate, the aqueous layer is drawn off and the extraction of the alkaloids completed by shaking with further 40-ml. portions of the mixed solvent. The last extract is tested for alkaloids by evaporating about 5 ml. to dryness, dissolving any residue in 1 ml. of tartaric acid solution and adding 2 ml. of *p*-dimethylaminobenzaldehyde reagent. The ether-acetone extracts are combined and the alkaloids extracted by shaking with successive 10-ml. portions of a 1 per cent. aqueous solution of tartaric acid, 1 ml. of the fifth extract being tested with the *p*-dimethylaminobenzaldehyde reagent and, if necessary, additional extractions made. The dissolved solvents from the combined acid extracts are removed by submitting to reduced pressure for about an hour then the liquid is diluted with water to 70 ml. The concentration of total alkaloids, in terms of ergotoxine, is determined by diluting 10 ml. of this acid solution to 25 ml. with water and applying the colorimetric test and the

proportion of water-soluble and water-insoluble alkaloids is directly determined by operating on the remaining 60 ml. in the manner already described above for ergot itself.

Discussion. Although the intensity of the blue-violet colour due to the action of the *p*-dimethylaminobenzaldehyde reagent on ergometrine was originally reported[11] as being 1·78 times that given by ergotoxine under the same conditions, subsequent work suggested that the true value is more nearly 1·85, hence when the Lovibond tintometer is used it is customary to assume that 0·05 mg. of ergotoxine base will produce a colour in a 1-cm. cell with a blue component equal to 4·1 units and that the corresponding value for solvent-free ergometrine is 7·6. The intensity of the Lovibond red colour which it is necessary to add in order to match the tint produced by the test amounts to approximately a third that of the blue. During the extraction of the alkaloids from the fat-free drug by the method of Hampshire and Page using ether it has been shown that only about 2 per cent. of the alkaloids are decomposed[16]. The degree of comminution described as a " moderately fine powder " is defined in the British Pharmacopœia, 1932, as a " Powder of which all the particles pass through a No. 44 sieve and not more than 40 per cent. through a No. 85 sieve." The use of anæsthetic quality ether is essential: if ether containing the slightest trace of peroxide is employed at any stage in the assay the true intensity of colour in the final test is not attained.

In the second method for the assay of ergot given above preliminary removal of fat with light petroleum is not necessary. The method adopted for the extraction of the alkaloids is a combination of the ammoniacal acetone process advocated by Daglish and Wokes[17] and the original proposal of C. C. Keller[18], while the separation of the ergotoxine group of alkaloids from the water-soluble pair is based upon the observation of Schou and his collaborators[19, 20] who showed that if the mixed alkaloids be dissolved in an aqueous medium of acid reaction between *p*H 4 and 6 the ergotoxine, to the exclusion of the ergometrine and ergometrinine, can be extracted by shaking with ether. In the procedure described above it is necessary to transfer the ergometrine left in the buffer solution into ether and then take it back again into tartaric acid in order, as far as possible, to minimise the volume of the final solution, since the amount of water-soluble alkaloids present in ergot is small compared with the quantity of ergotoxine. The preliminary determination of the total alkaloids

in terms of ergotoxine serves as a check upon the accuracy of the subsequent separation since the sum of the actual ergotoxine found and the ergometrine, calculated into terms of ergotoxine, should agree closely with the first value. An analytical method for the separation of the two water-soluble alkaloids has been proposed by D. C. Grove which depends upon removal of ergometrine, to the exclusion of ergometrinine, from a slightly ammoniacal aqueous solution by shaking with successive portions of ether[21]. A graphical procedure has also been suggested depending upon the known partition of any solute between two immiscible solvents where the molecular weight of the solute is the same in each[22].

Ergotoxine occurs to the extent of about 0·18 per cent. in good specimens of Spanish, Portuguese or Bulgarian ergot, but Russian, Polish and Hungarian material rarely contains more than 0·01 per cent. The amount of ergometrine present in ergot is much less, being only about 0·01 per cent. in the drug derived from southern Europe while the other varieties which are low in ergotoxine content are correspondingly poor sources of ergotmetrine. Ergot of Festuca grass from New Zealand has been found to contain upwards of 0·5 per cent. of water-insoluble alkaloids including a high proportion of ergotamine and ergotaminine while the water-soluble fraction is also higher than in ergot of rye but is thought by some investigators to consist largely of the physiologically inactive ergometrinine. In order to conform to the requirements of the Sixth Addendum, 1943, of the British Pharmacopœia, 1932, ergot must contain not less than 0·2 per cent. of total alkaloids calculated as ergotoxine, of which not less than 15 per cent. consists of water-soluble alkaloids, calculated as ergometrine: the latter figure, of course, includes ergometrinine. Ergot is often administered direct in the powdered form and for this purpose it is necessary to remove the fat, which occurs to the extent of about 30 per cent., to prevent deterioration of alkaloidal strength during storage. Such material is included in the Sixth Addendum under the name Prepared Ergot and is required to contain between 0·19 and 0·21 per cent. of total alkaloids calculated as ergotoxine, of which not less than 15 per cent. consists of water-soluble alkaloids calculated as ergometrine. Liquid Extract of Ergot is official in the B.P., 1932, and is required to contain, when freshly prepared, 0·06 per cent. of total alkaloids calculated as ergotoxine and not less than 0·04 per cent. after storage.

REFERENCES

1. H. W. Dudley and J. Chassar Moir, *Brit. Med. J.*, 1935, i, 520.
2. M. E. Davis, F. L. Adair, G. Rogers, M. S. Kharasch and R. R. Legault, *Amer. J. Obst. Gyn.*, 1935, **27**, 155.
3. G. Barger and F. H. Carr, *J. Chem. Soc.*, 1907, **91**, 337.
4. C. Tanret, *Compt. rend.*, 1875, **81**, 896.
5. S. Smith and G. M. Timmis, *J. Chem. Soc.*, 1931, 1888.
6. N. Evers, *Yearb. Pharm.*, 1927, 415.
7. H. W. van Urk, *Pharm. Weekblad*, 1929, **66**, 473.
8. M. I. Smith, *U.S. Public Health Reports*, 1930, **45**, 1466.
9. Pharmacopœia Commission, Reports of Sub-Committees, No. 8, 1931.
10. N. L. Allport and T. T. Cocking, *Quart. J. Pharm.*, 1932, **5**, 341.
11. N. L. Allport and S. K. Crews, *Quart. J. Pharm.*, 1935, **8**, 447.
12. C. H. Hampshire and G. R. Page, *Quart. J. Pharm.*, 1936, **9**, 60.
13. C. H. Hampshire and G. R. Page, *Quart. J. Pharm.*, 1938, **11**, 57.
14. C. H. Hampshire and G. R. Page, *Quart. J. Pharm.*, 1939, **12**, 594.
15. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1941, **14**, 106.
16. C. H. Hampshire and M. W. Partridge, *Quart. J. Pharm.*, 1941, **14**, 116.
17. C. Daglish and F. Wokes, *Quart. J. Pharm.*, 1939, **12**, 451.
18. C. C. Keller, *Schweiz. Woschr. Chem. Pharm.*, 1894, **32**, 121 and 133.
19. S. A. Schou and I. Bennekou, *Dansk Tidss. Farm.*, 1938, **12**, 257.
20. S. A. Schou and M. Tønnesen, *Dansk Tidss. Farm.*, 1938, **12**, 272.
21. D. C. Grove, *J. Amer. Pharm. Assoc.*, 1941, **30**, 260.
22. L. F. Knudsen and D. C. Grove, *Ind. Eng. Chem., Anal., Ed.*, 1942, **14**, 556.

MORPHINE

Of the numerous colour reactions for this alkaloid two have been successfully developed for quantitative work, the one depending upon the capacity of morphine to liberate iodine from iodic acid[1] and the other, originated by D. Radulescu, based upon the formation of a brown-coloured nitroso derivative due to the action of nitrous acid[2]. Of these, the latter is to be preferred, both on grounds of reliability and sensitivity and it has been officially adopted for the morphinometric assay of Camphorated Tincture of Opium (Paregoric) and as a test for the limit of morphine present as impurity in Codeine B.P., Codeine Phosphate B.P. and Diamorphine Hydrochloride B.P. The same colour reaction has been applied by D. C. Garratt[3] to the determination of morphine in Aromatic Powder of Chalk with Opium B.P. Powder of Ipecacuanha and Opium B.P. (Dover's Powder), Gall and Opium Ointment B.P.C. and Tincture of Chloroform and Morphine B.P.C. The method is also of value for the assay of hypodermic tablets and injections containing morphine[4].

Method. A quantity of an acidic solution expected to contain

between 0·2 and 0·6 mg. of the alkaloid contained in a Nessler glass is diluted to about 20 ml. with 0·1N mineral acid (hydrochloric or sulphuric serve equally well), 8 ml. of a freshly prepared 1 per cent. aqueous solution of sodium nitrite is added then the mixture rendered alkaline by the addition of a 10 per cent. aqueous solution of ammonia (12 ml. or sufficient to render the mixture distinctly alkaline) and the liquid diluted to 50 ml. with water. The stable yellowish-brown colour is matched against standards similarly prepared from a 0·002 per cent. solution of anhydrous morphine in 0·1N hydrochloric acid.

Application to Camphorated Tincture of Opium B.P. A measured quantity of the sample (10 ml.) is transferred to a dish, evaporated just to dryness over a boiling water-bath and 5 ml. of lime water added. The mixture is well stirred, filtered into a separator and the dish and filter washed with a further 10 ml. of lime water. The filtrate is extracted with two successive 10-ml. portions of ether, the mixed ethereal extracts washed with 5 ml. of lime water followed by 5 ml. of water, these washings added to the main aqueous liquid and the ether rejected. To the mixed alkaline liquids is added 0·15 g. of ammonium sulphate, sufficient water to produce about 30 ml., then 30 ml. of alcohol (95 per cent.) and 30 ml. of chloroform. After shaking and allowing to separate the lower layer is run off into another separator, washed by shaking with a mixture consisting of 5 ml. of alcohol (95 per cent.) and 10 ml. of water and, after separation, is transferred to a flask. The liquid in the first separator is further extracted with two successive quantities of a mixture of 15 ml. of alcohol (95 per cent.) and 30 ml. of chloroform, each extract being successively washed in the second separator with the same alcohol-water mixture previously used for washing the first extract and then transferred to the flask. The mixed alcohol-chloroform solution is evaporated to dryness, the alkaloidal residue dissolved in 10 ml. of N hydrochloric acid and the solution diluted to 100 ml. with water. To 8 ml. of this solution, representing 0·8 ml. of the camphorated tincture of opium being assayed, is added 8 ml. of a 1 per cent. aqueous solution of sodium nitrite followed by 12 ml. of a 10 per cent. aqueous solution of ammonia and the mixture diluted with water to 50 ml. In the same manner, 8 ml. of a 0·005 per cent. solution of anhydrous morphine in 0·1N hydrochloric acid is treated with 8 ml. of the sodium nitrite solution and 12 ml. of dilute solution of ammonia and then 8 ml. of the acid solution of the alkaloidal residue, representing 0·8 ml. of the origi-

nal tincture, is added and the volume of the mixture adjusted to 50 ml. with water. The strength of the sample is then determined by ascertaining the volume of the darker solution which, when diluted with water to 50 ml., will produce a depth of colour equal to that of 50 ml. of the lighter solution. The official tincture should contain between 0·045 and 0·055 per cent. w/v of anhydrous morphine.

Application to Aromatic Powder of Chalk with Opium B.P.[3]. An intimate mixture of 4 g. of the sample and 0·5 g. of calcium hydroxide is made into a paste which is then transferred to a 100-ml. graduated flask with about 90 ml. of water and shaken occasionally during 30 minutes. The mixture is then made up to volume, filtered, 25 ml. of the filtrate transferred to a separator, 0·15 g. of ammonium sulphate added and the liquid extracted with three successive portions of ether, the mixed ethereal extracts washed with 5 ml. of water, the washing added to the main aqueous layer and the ether rejected. The morphine is extracted from the aqueous liquid using a mixed solvent of alcohol and chloroform in the manner described above for the assay of the Camphorated Tincture of Opium. The alkaloidal residue is dissolved in 5 ml. of N hydrochloric acid, the solution diluted to 25 ml. with water and the colorimetric determination conducted on 5 ml. using as the standard 5 ml. of a 0·01 per cent. solution of anhydrous morphine in 0·2N hydrochloric acid which has been treated with the nitrite and ammonia reagents and then with 5 ml. of the acid solution of the alkaloidal residue prepared from the sample. The official powder is required to contain between 0·235 and 0·265 per cent. of anhydrous morphine.

Application to Powder of Ipecacuanha and Opium B.P.[3] The procedure given for Aromatic Powder of Chalk with Opium is followed excepting that 1 g. of the sample is taken and the 25 ml. of filtrate is extracted with ether before adding the ammonium sulphate. The official powder is required to contain between 0·95 and 1·05 per cent. of anhydrous morphine.

Application to Gall and Opium Ointment B.P.C.[3] The fat is removed from 3 g. of the sample by extraction during 3 hours with light petroleum using a Soxhlet apparatus. The residue is dried and the morphine determined in the manner indicated above for Powder of Ipecacuanha and Opium. The theoretical content of anhydrous morphine in the B.P.C., 1934, preparation is 0·75 per cent.

Application to Tincture of Chloroform and Morphine B.P.C.[3] To

10 ml. of the sample contained in a separator is added 5 ml. of water followed by 0·5 ml. of strong ammonia (s.g. 0·880), 15 ml. of alcohol (95 per cent.) and 15 ml. of chloroform. The mixture is shaken, allowed to separate, the lower layer drawn off and the extraction repeated twice using each time 8 ml. of alcohol and 15 ml. of chloroform. Each alcohol-chloroform extract is separately washed with the same portion of 15 ml. of a mixture of 1 vol. of alcohol and 2 vols. of water. The alcohol-chloroform extracts are mixed, the solvent removed by distillation the residue dissolved in 75 ml. of N hydrochloric acid and 2 ml. taken for the colorimetric determination which is conducted in the manner as already described for Aromatic Powder of Chalk with Opium. The B.P.C., 1934, preparation should contain 0·229 per cent. of morphine hydrochloride, the factor for converting the result in terms of anhydrous morphine into the hydrated salt ($3H_2O$) being 1·317.

Application to Hypodermic Tablets and Injections[4]. A hypodermic tablet is dissolved in N hydrochloric acid and the solution diluted to 100 ml. with the same solvent (or 1 ml. of injection is diluted to 100 ml. with N hydrochloric acid). A quantity of this solution, expected to contain between 0·8 and 1·3 mg. of morphine, is transferred to a 25-ml. stoppered measuring cylinder, 5 ml. of a freshly prepared 0·5 per cent. aqueous solution of sodium nitrite is added, the liquid mixed by gentle shaking, 2 ml. of dilute solution of ammonia (10 per cent. w/w) added, the liquid again shaken and finally diluted with water to 25 ml. The intensity of the yellowish-brown colour is conveniently measured by means of a Lovibond tintometer and the value obtained for the yellow component correlated with the amount of morphine present in the 25 ml. of reaction mixture by reference to Table XXI. The result found for the content of anhydrous morphine base may be converted into content of hydrated salts by multiplying by the following factors : hydrochloride ($3H_2O$), 1·317 ; sulphate ($5H_2O$), 1·330 ; tartrate ($3H_2O$), 1·357.

Discussion. There are probably a number of substances besides morphine which give colours under the conditions of Radulescu's test and it is possible that in some cases yellowish-brown tints are produced. However, with the exception of apomorphine (*cf.* p. 299) most of the important vegetable alkaloids are without effect on the determination and in this connection it may be noted that morphine present in hypodermic tablets and injections which also contain salts of atropine or hyoscine may be assayed by the

TABLE XXI. RELATION BETWEEN THE QUANTITY OF ANHYDROUS MORPHINE AND THE COLOUR PRODUCED

Quantity of Morphine (anhydrous) mg.	Colour after dilution to 25 ml. : observed in 1-cm. cell Lovibond Units		
	Yellow	Red *	Brightness *
0·2	0·6	0·4	0·3
0·4	1·1	0·8	0·5
0·6	1·7	1·2	0·6
0·8	2·3	1·4	0·7
1·0	2·8	1·6	0·7
1·2	3·4	2·0	0·7
1·4	3·9	2·3	0·7
1·6	4·5	2·6	0·7
1·8	5·0	3·1	0·7
2·0	5·6	3·2	0·7

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the yellow component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

method described without modification. Codeine, diamorphine and ethylmorphine do not give any colour. It has been stated[5] that psychotrine, a minor alkaloid of ipecacuanha, gives Georges' iodate reaction similarly to morphine but there does not appear to be any information recorded in the literature regarding its response to Radulescu's test; but, even if a colour is produced, Garratt's compensating technique described above will effectually eliminate any difficulty which might otherwise arise. The green colour formed by the action of nitrous acid on phenazone (antipyrine) is changed to blue on rendering alkaline with ammonia and, of course, the presence of this compound would vitiate the determination of morphine. Both phenolic and non-phenolic constituents of Aromatic Powder of Chalk with Opium give coloured derivatives with nitrite and ammonia closely resembling the tint of nitroso-morphine and in the procedure given above this difficulty is overcome by extracting with ether after making the lime solution ammoniacal with ammonium sulphate ; the morphine remains in the aqueous phase and is extracted as usual with chloroform in the presence of alcohol.

The procedure given here for the colorimetric assay of Camphorated Tincture of Opium is taken from Report No. 12 of the Committees of the British Pharmacopœia Commission (1939) and is based on Garratt's modification[3] of the official method which embodies a technique to compensate for the tint of the alkaloidal

extract due to the colouring matter of the opium. An alternative method of allowing for the natural colour of the extracts consists in rendering alkaline with ammonia a similar quantity of the alkaloidal solution prepared from the sample, then matching the colour of the unknown by adding a standard morphine solution in which the colour has been developed with nitrite and ammonia prior to its dilution to volume[6].

REFERENCES

1. L. Georges, *J. Pharm. Chim.*, 1906, **23,** 513.
2. D. Radulescu, *Bul. Soc. Sci. Bucuresci*, 1905, **14,** 602.
3. D. C. Garratt, *Quart. J. Pharm.*, 1937, **10,** 466.
4. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1942, **15,** 238.
5. F. J. Todd, *Yearb. Pharm.*, 1927, 509.
6. D. C. Garratt, Private Communication.

PILOCARPINE

The Pharmacopœial identity test, originated by H. Helch[1], has been modified for quantitative work by Shupe[2] and also applied to the assay of hypodermic tablets and injections[3].

Method[2, 3]. The solution to be examined, which may be an injection or solution of hypodermic tablets, is diluted with water so that it may be expected to contain between 0·08 and 0·15 mg. of pilocarpine base per ml. and 10 ml. is transferred to a small separator. To this is added precisely 1 ml. of a 10 per cent. w/v aqueous solution of acetic acid followed by exactly 5 ml. of chloroform, 1 ml. of a 5 per cent. aqueous solution of potassium chromate and 1 ml. of approximately 3 per cent. w/v solution of hydrogen peroxide. The mixture is shaken for 30 seconds and, after allowing to separate, the chloroformic layer is filtered and the intensity of the stable violet colour compared with that of standards similarly prepared from pilocarpine nitrate satisfying the specification of the British Pharmacopœia. Alternatively, the colour may be measured by means of a Lovibond tintometer and the value obtained for the blue component correlated with the amount of pilocarpine present in the 10 ml. of diluted solution taken for the test by reference to Table XXII. The result found for the amount of anhydrous pilocarpine base present may be converted into content of anhydrous salts by multiplying by the following factors : nitrate 1·339 ; hydrochloride, 1·177.

TABLE XXII. RELATION BETWEEN THE QUANTITY OF PILOCARPINE AND THE COLOUR PRODUCED

Quantity of Pilocarpine (anhydrous) mg.	Colour of the chloroformic extract: observed in 1-cm. cell Lovibond Units		
	Blue	Red *	Brightness *
0·2	0·9	0·6	0·1
0·5	2·1	1·1	0·1
0·8	3·4	2·3	0·1
1·0	4·2	3·1	0·1
1·2	4·9	3·7	0·1
1·5	6·1	4·7	0·2
2·0	8·3	4·9	0·2

* These values are subject to slight variation: they are included as guides to accurate matching, but only the figures for the blue component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

Discussion. Notwithstanding that the violet colour produced in this test contains a large proportion of red, thus detracting from the predominance of the blue component, the tintometer method of measurement works well in practice. Provided it is screened from bright daylight the colour does not appreciably fade during 2 hours. The acidity of the reaction mixture exercises considerable influence upon the intensity of colour produced and, therefore, instead of acidifying with dilute sulphuric acid as specified for the qualitative test, a precise quantity of acetic acid is employed. Apomorphine gives a similar colour reaction[4].

REFERENCES

1. H. Helch, *Pharm. Post*, 1902, **35**, 289.
2. I. S. Shupe, *J. Assoc. Off. Agric. Chem.*, 1940, **24**, 757.
3. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1942, **15**, 238.
4. A. Wangerin, *Pharm. Zeit.*, 1903, **47**, 739.

PROCAINE (ETHOCAINE)

A modification of a diazo reaction proposed by H. Willstaedt[1] in which procaine is coupled with 1-amino-8-naphthol-3 : 6-disulphonic acid (H acid) has proved to be quite satisfactory for the colorimetric determination of this base[2]. The test depending upon the yellow colour produced when a solution in dilute hydro-

chloric acid is treated with *p*-dimethylaminobenzaldehyde,[3] although a sensitive reaction, is not suitable for quantitative work[2].

Method[2]. The neutral solution to be examined, which may be an injection or solution of hypodermic tablets, and which is expected to contain between 0·02 and 0·05 mg. of procaine base, is transferred into a 10-ml. stoppered measuring cylinder. If necessary, it is diluted with water to 5 ml. then 1 ml. of N hydrochloric acid and 0·5 ml. of a freshly prepared 1 per cent. aqueous solution of sodium nitrite is added. After mixing and allowing to stand for 30 seconds, 1 ml. of a 15 per cent. aqueous solution of ammonium sulphamate, $NH_2SO_3NH_4$, is added, the mixture vigorously shaken and allowed to stand for 30 seconds. Finally, 1 ml. of a freshly prepared 1 per cent. solution of the refined acid sodium salt of 1-amino-8-naphthol-3 : 6-disulphonic acid in 20 per cent. w/v aqueous sodium hydroxide solution is added and the mixture diluted to 10 ml. with water. The intensity of the stable red colour is compared with that of standards similarly prepared from procaine hydrochloride satisfying the requirements of the British Pharmacopœia and which, in addition, yields anhydrous procaine base of the correct melting point (58° to 60° C.) and contains total chlorine, present as chloride, equivalent to 100 per cent. of the alkaloidal salt. Alternatively, the colour may be measured by means of a Lovibond tintometer and the value obtained for the red component correlated with the amount of procaine present in the 10 ml. of reaction mixture by reference to Table XXIII. The indicated content of anhydrous procaine base is multiplied by 1·155 in order to convert the result into terms of procaine hydrochloride (anhydrous) or by 1·890 if the result is to be calculated as procaine borate (Borocaine).

Discussion. This colour reaction is not specific as, of course, there may well be many organic bases which would yield a red colour closely resembling that produced by procaine just as there are numerous basic compounds which give a yellow colour with an acid solution of *p*-dimethylaminobenzaldehyde. However, determinations may be conducted in the presence of many of the substances ordinarily dispensed with procaine, including adrenaline, ephedrine, boric acid, chlorbutol, sodium sulphite, ammonium chloride, sodium chloride and sodium phosphate, but it should be noted that the method is not applicable to solutions preserved with cresols or other phenols.

In several of the foreign pharmacopœias procaine hydrochloride

TABLE XXIII. RELATION BETWEEN THE QUANTITY OF PROCAINE AND THE COLOUR PRODUCED

Quantity of Procaine (anhydrous) mg.	Colour after dilution to 10 ml. : observed in 1-cm. cell Lovibond Units		
	Red	Yellow *	Blue *
0·010	1·1	0·2	—
0·015	1·8	0·2	—
0·020	2·4	0·2	—
0·025	3·1	0·2	—
0·030	3·7	0·2	—
0·035	4·3	—	0·1
0·040	5·0	—	0·1
0·045	5·7	—	0·1
0·050	6·3	—	0·2

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results.

is termed *p*-aminobenzoyldiethylamino-ethanol hydrochloride while it is also frequently referred to by various trade names such as Allocaine, Kerocain, Neocaine, Novocain, Planocaine, Sevicaine and Syncaine.

REFERENCES

1. H. Willstaedt, *Biochem. Z.*, 1934, **269**, 182.
2. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1942, **15**, 238.
3. S. N. Chakravarti and M. B. Roy, *Analyst*, 1937, **62**, 603.

QUININE

Suggestions for the colorimetric determination of this alkaloid include isolation of the phosphotungstic acid precipitate followed by its solution in alkali and formation of the blue reduction complex with titanous chloride[1] ; oxidation of quinine with bromine water, followed by potassium ferricyanide then excess ammonia and extraction of the red erythroquinine with chloroform[2] ; and coupling with diazotised *p*-nitraniline after hydrolysis with sulphuric acid[3]. Besides involving separation of a precipitate, the first method fails to effect any distinction from large numbers of other alkaloids ; the erythroquinine reaction is difficult to apply quantitatively since the proportion of bromine required is a critical factor dependent upon the amount of quinine present ;

and, lastly, the author has not been able to confirm the efficacy of the coupling method. More satisfactory, is the method depending upon the reaction between quinine and eosin to produce a red compound which can be extracted with chloroform, and a modification of Prudhomme's original procedure[4] is described below.

Method. Two special reagents are required :—

1. A Buffer Solution prepared by mixing 30 ml. of a 4·539 per cent. aqueous solution of potassium dihydrogen phosphate, KH_2PO_4, with 70 ml. of a 5·937 per cent. aqueous solution of disodium hydrogen phosphate, $Na_2HPO_4.2H_2O$.
2. A Solution of Eosin prepared not more than a week before use by dissolving 0·2 g. of the yellow shade dye (sodium salt of tri-bromofluorescein) in about 70 ml. of water, extracting three times, each with 50 ml. of chloroform, or until the last extract is colourless, and then diluting to 100 ml. with water.

To about 10 ml. of the solution to be tested, expected to contain between 0·1 and 0·25 mg. of quinine is added 2 ml. of the buffer solution followed by 1 ml. of eosin solution. The mixture is transferred to a separator and extracted by vigorously shaking with three 2·5-ml. portions of chloroform, 2 minutes being allowed for the immiscible liquids to separate and each chloroformic layer being washed with the same 6 ml. of washing liquor prepared by mixing 1 ml. of the buffer solution with 5 ml. of water. The extracts are transferred to a 10-ml. graduated flask, diluted to the mark with alcohol (95 per cent.) and the intensity of the stable pink colour compared with that of standards similarly prepared from quinine acid sulphate of known alkaloidal content. Alternatively, the colour may be measured by means of a Lovibond tintometer and the value obtained for the red component correlated with the amount of quinine present in the solution taken for the test by reference to Table XXIV. The result found for the amount of anhydrous quinine base present may be converted into content of hydrated quinine ($3H_2O$) by multiplying by the factor 1·167. The content of the commonly occurring salts of the alkaloid may be derived by applying the following factors : sulphate ($7\frac{1}{2}H_2O$), 1·360 ; acid sulphate ($7H_2O$), 1·692 ; hydrochloride ($2H_2O$), 1·224 ; acid hydrochloride (anhydrous), 1·225 ; hydrobromide ($2H_2O$), 1·361 ; acid hydrobromide ($3H_2O$), 1·666

Discussion. The above technique has been developed by J. Allen, working in the author's laboratory, the essential alterations to Prudhomme's original method consisting in the washing of the

TABLE XXIV. RELATION BETWEEN THE QUANTITY OF QUININE AND THE COLOUR PRODUCED

Quantity of Quinine (anhydrous) mg.	Colour of the chloroform layer : observed in 1-cm. cell Lovibond Units		
	Red	Blue *	Brightness *
0·050	0·6	—	—
0·075	1·2	—	—
0·100	2·0	—	0·3
0·125	2·9	—	0·6
0·150	3·8	0·1	0·8
0·175	4·7	0·1	0·8
0·200	5·6	0·2	1·0
0·225	6·5	0·2	1·0
0·250	7·3	0·2	1·0
0·275	7·9	0·2	1·0
0·300	8·3	0·2	1·0

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results. As the colour produced is sometimes brighter than the Lovibond glasses it may be necessary to dull it down by means of the neutral tints.

eosin reagent with chloroform to remove colouring matter soluble in the organic solvent and the washing of the chloroform extracts containing the quinine-eosin complex. Prudhomme states that the test also responds to physostigmine, pilocarpine, atropine and ephedrine but the modified procedure is not disturbed by any of these alkaloids when present in quantities of the same order as the useful range for quinine. When present in similar amounts, strychnine, brucine, hyoscine, morphine, diamorphine and codeine do not interfere with the determination of quinine while relatively large quantities of urea, urethane, phenazone, amidopyrine and caffeine have no effect. The test is equally sensitive to the other common cinchona alkaloids while emetine gives a response approximately one-tenth that given by quinine.

REFERENCES

1. C. A. Rojahn and R. Seifert, *Arch. Pharm.*, 1930, **268**, 499.
2. R. Monnet, *J. Pharm. Chim.*, 1933, **18**, 59.
3. J. A. Sanchez, *J. Pharm. Chim.*, 1935, **21**, 24.
4. R.O. Prudhomme, *J. Pharm. Chim.*, 1940, **26**, 8.

STRYCHNINE

Denigès modification[1] of Malaquin's colour test[2] can be applied to quantitative work for determining the strength of simple solutions of strychnine salts[3] and for the colorimetric assay of nux vomica and its preparations[4].

Method. The solution to be examined, which may be an injection or solution of hypodermic tablets, is diluted with hydrochloric acid (10 per cent. w/w HCl) so that it may be expected to contain between 0·03 and 0·05 mg. of strychnine in 5 ml. and this amount is introduced into a dry test tube containing 0·2 g. of zinc amalgam (40 per cent. Hg) which has recently been momentarily immersed in 5 per cent. aqueous solution of mercuric chloride and washed with distilled water. The tube is placed in a boiling water-bath for 7 minutes, then cooled under the tap, 0·05 ml. of a freshly prepared 0·1 per cent. aqueous solution of sodium nitrite added and the intensity of the stable pink colour compared with that of standards similarly prepared from strychnine hydrochloride satisfying the specification of the British Pharmacopœia. Alternatively, the colour may be measured by means of a Lovibond tintometer and the value obtained for the red component correlated with the amount of strychnine present in the 5 ml. of solution taken for the test by reference to Table XXV. The

TABLE XXV. RELATION BETWEEN THE QUANTITY OF STRYCHNINE AND THE COLOUR PRODUCED

(Test Applied to Alkaloidal Base, Hydrochloride or Sulphate)

Quantity of Strychnine (anhydrous) mg.	Colour of the reaction mixture : observed in 1-cm. cell Lovibond Units		
	Red	Yellow *	Brightness *
0·020	2·1	0·5	0·2
0·025	2·7	0·5	0·4
0·030	3·3	0·6	0·5
0·035	3·9	0·6	0·5
0·040	4·5	0·6	0·5
0·045	5·0	0·7	0·6
0·050	5·7	0·8	0·7
0·055	6·3	0·9	0·8
0·060	6·9	0·9	0·8

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

result found for the amount of anhydrous strychnine base present may be converted into content of the commonly occurring salts by multiplying by the following factors: hydrochloride ($2H_2O$), 1·217; sulphate ($5H_2O$), 1·281.

Application to Nux Vomica Seeds[4]. A miniature percolator with an overall length of 16 cm. is made from glass tubing of 8 mm. bore. The tube is sharply narrowed about 3 cm. from one end while the other end is spun out to form a flange of about 2·5 cm. diameter. The sample under examination is reduced to about No. 60 powder, 1 g., accurately weighed, is introduced into a conical flask of about 100 ml. capacity and 3 ml. of alcohol (95 per cent.) added. After heating the mixture on a boiling water-bath until most of the alcohol has evaporated, 10 ml. of tetrachlorethylene (technical grade) is added followed by 0·6 g. of diethylenediamine (Piperazine B.P.C., hydrated), the flask is connected to a reflux condenser and the contents heated so that the solvent boils vigorously for 15 minutes. After allowing to cool slightly, as much as possible of the solvent and powdered drug is transferred into the dry miniature percolator which has been previously plugged with about 0·02 g. of cotton-wool and suspended within a stoppered 100-ml. measuring cylinder. If necessary, the drug is gently compressed with a small glass rod so that the solvent percolates at the rate of about 1 drop a second. More hot tetrachlorethylene is added to the flask, the transference of the drug to the percolator is completed and the extraction continued until the volume of the percolate is 42 ml. Sufficient 0·125N sulphuric acid is added to the percolate to bring the level of the liquid to the 90-ml. graduation mark, the cylinder is stoppered and shaken for about 15 seconds. After allowing to stand until the immiscible liquids have separated, about 10 ml. of the upper layer is pipetted off and filtered through dry paper. Exactly 5 ml. of the filtrate is transferred to a conical centrifuge tube of 15 ml. capacity, 1·5 ml. of a 9 per cent. w/v aqueous solution of oxalic acid and 1 ml. of a 5 per cent. w/v solution of potassium ferrocyanide in 0·2 per cent. w/v aqueous solution of sodium carbonate (Na_2CO_3) is added. After allowing to stand at room temperature for 15 minutes the tube is immersed in a freezing mixture, agitated until the contained liquid freezes, then removed and gently warmed until the contents just melt. About 0·1 g. of kieselguhr (acid washed) is added and the mixture centrifuged. The clear supernatant liquid is poured off and discarded, about 2 ml. of a 0·1 per cent. w/v aqueous solution of sulphuric acid containing 1 per cent.

of oxalic acid added and the precipitate dislodged with a thin glass rod. The latter is rinsed free from adherent particles by adding about 8 ml. more of the washing liquid to the tube and the mixture is again centrifuged. In the same manner the washing of the precipitate is repeated once with the mixed acid solution and the clear supernatant liquid decanted as completely as possible. The precipitate of strychnine ferrocyanide is dissolved in 1 ml. of concentrated hydrochloric acid by gently boiling over a small luminous flame until the kieselguhr is dispersed throughout the solution. About 10 ml. of dilute hydrochloric acid (10 per cent. w/w HCl) is added, the mixture transferred to a 100-ml. graduated flask, the centrifuge tube rinsed out and the flask filled to the mark using dilute hydrochloric acid throughout. A portion of this solution is filtered and the colorimetric test applied to 5 ml. of the filtrate in the manner described above, the intensity of the pink colour produced being compared with that of standards prepared by treating known quantities of strychnine hydrochloride with potassium ferrocyanide, separating the precipitate and dissolving it in hydrochloric acid in the same way as the acid extract of the total alkaloids of nux vomica. Alternatively, the colour may be measured by means of a Lovibond tintometer and the value obtained for the red component correlated with the amount of strychnine present in the 5 ml. of solution taken for the test by reference to Table XXVI.

TABLE XXVI. RELATION BETWEEN THE QUANTITY OF STRYCHNINE AND THE COLOUR PRODUCED

(Test Applied to Strychnine Ferrocyanide)

Quantity of Strychnine (anhydrous) mg.	Colour of the reaction mixture : observed in 1-cm. cell Lovibond Units		
	Red	Yellow *	Brightness *
0·02	1·5	0·2	0·2
0·03	2·5 (2·45)	0·4	0·3
0·04	3·4	0·5	0·3
0·05	4·4 (4·35)	0·5	0·4
0·06	5·3	0·6	0·5
0·07	6·3	0·7	0·6
0·08	7·3 (7·25)	0·9	0·7
0·09	8·2	0·9	0·8
0·10	9·2	1·1	1·0

* These values are subject to slight variation : they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

Application to Galenicals[4] (*Powdered Nux Vomica B.P.*). The assay is conducted as described for nux vomica seed but before transferring the mixture of solvent and powdered drug to the percolator, 0·5 g. of sawdust is introduced into the flask. The sawdust used for this purpose is prepared by extracting it with ether until all resin and fat is removed and then passing through a No. 60 sieve.

(*Liquid Extract of Nux Vomica B.P.*). To 1 ml. contained in a small beaker is added 2 ml. of approximately N alcoholic potassium hydroxide, 5 ml. of chloroform and 1 g. of extracted sawdust. After stirring, as much as possible of the mixture is transferred into a dry miniature percolator which has been previously plugged with about 0·02 g. of cotton-wool and suspended within a stoppered 100-ml. measuring cylinder. After packing down fairly tightly with a small glass rod the mixture is percolated with boiling chloroform in the manner described for nux vomica seed until the volume of the cooled percolate is 43 ml. Sufficient dilute sulphuric acid (N acid diluted 1 to 14) is added to the percolate to bring the level of the liquid to the 90-ml. graduation mark, the cylinder is stoppered and shaken for about 15 seconds. The assay is then continued as described above for nux vomica seed commencing with the filtration of a portion of the upper layer and the precipitation of strychnine as ferrocyanide in exactly 5 ml. of the filtrate.

(*Tincture of Nux Vomica B.P.*). The sample, 10 ml., contained in a small beaker is evaporated over a boiling water-bath until the volume is reduced to about 1 ml. The assay is then continued with the addition of alcoholic potassium hydroxide, chloroform and extracted sawdust as described for liquid extract of nux vomica.

(*Dry Extract of Nux Vomica B.P.*). About 0·2 g., accurately weighed, is introduced into a centrifuge tube containing 10 ml. of alcohol (50 per cent.) and the tube is warmed so that the dilute spirit boils gently for 1 minute. The mixture is centrifuged, the clear supernatant liquid decanted into a beaker, any calcium phosphate, or other insoluble residue, remaining in the tube is washed with two separate 5-ml. portions of alcohol (50 per cent.) each washing being centrifuged until the supernatant liquid is clear and then added to the initial extract in the beaker. If difficulty is experienced in obtaining a clear supernatant liquid in the last washing a little sodium chloride should be added to the dilute alcohol. The combined alcoholic extracts are evaporated

over a boiling water-bath until the volume is reduced to about 1 ml. and the assay then continued with the addition of alcoholic potassium hydroxide, chloroform and extracted sawdust as described for liquid extract of nux vomica.

Discussion. It has been observed that in respect of its sensitivity in this colour test zinc amalgam deteriorates during storage and it is therefore important that its activity be restored just prior to use by momentary immersion in mercuric chloride solution. It should be especially noted that the colour produced by a given weight of strychnine present as ferrocyanide is not quite so intense as that due to the same weight of alkaloid present as hydrochloride or sulphate, hence the need for a separate table correlating colour intensities with Lovibond units in the application of the test to the assay of nux vomica and its preparations. If the method is applied to other salts of strychnine consideration should be given to the possible influence of the acid radicle on the intensity of colour produced.

The utilisation of this test for the assay of nux vomica was first suggested by François who applied the colour reaction directly to an extract of the total alkaloids.[5] Since the character of the colour produced is greatly altered by the presence of brucine, which itself produces an orange tint, it is evident that a satisfactory procedure must include separation of the strychnine. The precipitation of strychnine ferrocyanide, as originally recommended by Dunstan and Short[6], is facilitated and rendered strictly quantitative by the inclusion of oxalic acid. Hibbard has reported[7] that the use of this reagent renders possible the micro-chemical separation of zinc as ferrocyanide and attributes the suggestion to unpublished work by P. R. Stout. The influence of oxalic acid in assisting the precipitation of strychnine ferrocyanide is considerable since, in its presence, a fraction of a milligram of strychnine will separate as the crystalline ferrocyanide in a minute or two whereas, without the acid, nothing will show even after several hours. Precipitation takes place most readily when potassium ferrocyanide is added to an acid solution of strychnine which is approximately 0·072N with respect to sulphuric acid and 0·33N to oxalic acid; it is less perfect when hydrochloric acid replaces sulphuric acid. The solution of potassium ferrocyanide employed is stabilised by the inclusion of 0·2 per cent. of sodium carbonate[8], thus obviating the possibility of errors due to the partial decomposition which may occur in the absence of alkali.

Owing to the abundance of hard horny endosperm in nux

vomica seed, the expeditious extraction of the alkaloids demands a solvent having a fairly high boiling point together with a strong organic base which is partly dissolved by the extracting liquid. These conditions are satisfied by tetrachlorethylene (b. pt. 119° to 122° C.) in association with diethylene-diamine. Since, under the conditions of the assay as described above, about one-third of the base passes into the organic solvent the percolate is shaken with 0·125N sulphuric acid in order that the final concentration of free acid after neutralising the dissolved diethylene-diamine shall approximate to 0·072N.

Extended experience has demonstrated that the colorimetric procedure as applied to nux vomica seed is quite satisfactory and, owing to the rapidity with which the alkaloids can be extracted, it is more quickly completed than the official assay. In the case of galenicals, however, the results obtained have been erratic and some more efficient method of extracting the alkaloids would seem to be required ; thus, it might be better to add sawdust, evaporate the sample to dryness and then to extract the residue using the procedure described for the seed.

REFERENCES

1. G. Denigès, *Bull. Soc. chim.*, 1911, **9**, 537.
2. P. Malaquin, *J. Pharm. Chim.*, 1909, **30**, 546.
3. N. L. Allport and N. R. Jones, *Quart. J. Pharm.*, 1942, **15**, 238.
4. J. Allen and N. L. Allport, *Quart. J. Pharm.*, 1940, **13**, 252.
5. A. François, *Bull. Soc. pharm. Bordeaux*, 1930, **68**, 158,
6. W. R. Dunstan and F. W. Short, *Yearb. Pharm.*, 1883, 469.
7. P. L. Hibbard, *Ind. Eng. Chem., Anal. Ed.*, 1934, **6**, 423.
8. I. M. Kolthoff and E. A. Pearson, *Ind. Eng. Chem., Anal. Ed.*, 1931, **3**, 381.

ADRENALINE

Many tests have been proposed for the colorimetric determination of this substance but few give satisfaction in practice. Two methods will be described here, the one, suitable for gland extracts dependent upon the formation of a red oxidation product with potassium persulphate as originally proposed by A. J. Ewins[1] and modified by Barker, Eastland and Evers[2] and the other, which is more sensitive and is applicable to the examination of tissues, based upon the production of a blue colour when arsenomolybdic acid is reduced by adrenaline. The technical details of

the latter test are due to F. H. Shaw[3] who has modified and greatly improved the original procedure of Whitehorn[4]. Arsenomolybdic acid is reduced by a number of substances besides phenols, such as ascorbic acid, reduced glutathione, various polyhydroxy and thiol compounds and in general $\Delta^{1:2}$-diols. To eliminate interference by such substances the adrenaline is adsorbed on a specially prepared aluminium hydroxide which is afterwards dissolved in the reagents of the test. The optimum reaction for the adsorption of adrenaline is between *p*H 8·0 and 8·5 ; on the acid side of *p*H 7·0 no adsorption takes place. Hence, in order to remove glutathione, and possibly other interfering substances, the procedure advocated by Shaw includes a treatment with aluminium hydroxide at *p*H 4 which is followed by a second contact with the adsorbent after adjustment of the reaction to between *p*H 8·0 and 8·5. Ascorbic acid is not adsorbed from either acid or alkaline media.

Method Using Persulphate[2]. The adrenaline is converted into a red oxidation product by treatment with the following reagent :—

Potassium Persulphate	0·2 g.
Sodium Chloride	1·0 g.
Disodium Hydrogen Phosphate ($Na_2HPO_4.12H_2O$) . . .	0·239 g.
Sodium Dihydrogen Phosphate ($NaH_2PO_4.2H_2O$) . . .	0·937 g.
Water	to produce 100 ml.

For the examination of gland extracts the reaction of the sample is adjusted to about *p*H 5·4 (using methyl red as external indicator) and 1 ml. is mixed with 1 ml. of the persulphate reagent. The mixture is transferred to a suitable cell, its colour immediately measured, then the cell is placed in a thermostat at 22° C. and, after 30 minutes when the colour due to adrenaline will have developed, the intensity of the colour is again determined. The difference in the colour before and after standing for 30 minutes is compared with that given by a standard solution of adrenaline treated in a similar manner. For the measurement of the colour the originators of this procedure employed a Lovibond tintometer: they found that, with a 1-cm. cell, 0·1 mg. of adrenaline gave a final colour equivalent to 9·5 red plus 3·4 yellow Lovibond units and that the values for smaller quantities showed a linear relation, the lines for both colours meeting the abscissa at a point corresponding to 0·005 mg. of adrenaline thus indicating the limit of

sensitivity of the test. If alcohol is present in the sample under examination it must be removed by evaporation *in vacuo* ; even 1 per cent. will disturb the development of the colour. Provided the reaction of the substance being tested is rendered more acid than *p*H 5 and the temperature is not allowed to rise above 30° C., alcohol can be removed without incurring loss of adrenaline : after this treatment the reaction of the residue should be adjusted to *p*H 5·4 and the original volume of the liquid restored by the addition of water.

Discussion. The reaction of the persulphate reagent should be equivalent to *p*H 5·5 ; it is stable for about a month if stored in a cool place away from light. Aged material should be regarded as unsatisfactory since, in spite of the buffer present, the reagent becomes unduly acid owing to the slow decomposition of the persulphate. The sodium chloride is incorporated into the reagent because of its stabilising effect on the colour produced by adrenaline. The reaction of the mixture exercises a profound influence on the intensity and rate of production of the colour and Barker, Eastland and Evers found that in the absence of a buffer the acidity of the mixture increased as the colour due to adrenaline developed ; in order to eliminate this irregularity buffer salts were introduced into the reagent. Copper was observed to have a catalytic effect on the test but, although in its presence the colour develops in about 30 seconds, it is unstable. Other heavy metals, such as mercury, cobalt, nickel and manganese also possess some catalytic influence but not to the same degree as copper. Iron, on the other hand, has a slight inhibitory effect.

Method Using Arsenomolybdic Acid[3]**.** The following reagents are required :—

1. The Arsenomolybdic Acid Solution prepared by dissolving 60 g. of crystalline sodium molybdate and 10 g. of sodium arsenate in 250 ml. of water, filtering, washing the filter, adding to the combined filtrate and washings 5 ml. of bromine water and diluting to 500 ml. with water. For use 100 ml. of this solution is mixed with 8 ml. of concentrated sulphuric acid.

2. A Mixture of Equal Volumes of Concentrated Sulphuric Acid and Water.

3. A Solution of 10 g. of Sodium Sulphite, $Na_2SO_3.7H_2O$, in 50 ml. of Water which must be less than 2 days old. Not more than 30 minutes before use, 2 ml. of this solution is added to 14 ml. of Reagent No. 2.

4. A Solution of Phenolphthalein not more than 3 days old made by dissolving 0·1 g. in 100 ml. of 0·01N sodium hydroxide.

5. A Suspension of Aluminium Hydroxide made by dissolving 25 g. of potassium alum in about 200 ml. of hot water, cooling to 20° C. and slowly adding a solution of 5 g. of sodium hydroxide in 20 ml. of water, filtering off the precipitate, washing with several portions of water and then suspending it in 100 ml. of water. This suspension should dissolve in a 4 per cent. solution of sodium hydroxide.

6. Standard Adrenaline : a dilution of 1 in 10,000 is prepared in approximately 0·01N sulphuric acid. This will keep for several days. It is diluted to 1 in 10,000,000, etc., with 0·01N sulphuric acid as required. The diluted standard will keep for several hours : the stability is improved by the addition of a little amino-acetic acid.

The solution to be tested, containing between 0·1 and 0·5 μg. of adrenaline (though 0·04 μg. can be detected), should be neutral or faintly acid. Blood should be run into an equal volume of a 10 per cent. aqueous solution of trichloracetic acid as rapidly as possible after removal from the body ; if it is kept for only a few minutes a substance is formed which is indistinguishable in this test from adrenaline. Other tissues are immersed in trichloracetic acid (at least 1 ml. per g. of tissue) and then cut up finely : after 30 minutes the mixture is filtered and the residue well washed with trichloracetic acid solution. The filtrate, or a suitable aliquot part, is transferred to a centrifuge tube, 2 drops of the phenolphthalein reagent added, the mixture carefully neutralised with a 4 per cent. aqueous solution of sodium hydroxide, 1 drop of N sulphuric acid added, to render the reaction equivalent to approximately *p*H 4, followed by 2 ml. of the aluminium hydroxide suspension. The mixture is shaken, centrifuged for 2 minutes, the supernatant fluid transferred to another tube, then 1 ml. of the aluminium hydroxide suspension for each 5 ml. of solution and 1 drop of phenolphthalein reagent added. The reaction is adjusted to *p*H 8·5 by adding solution of sodium hydroxide until a faint pink colour is produced and the mixture centrifuged as before. The supernatant fluid is discarded, and about 3 ml. of water, made just alkaline to phenolphthalein with sodium hydroxide, is poured on to the precipitate ; it is centrifuged, the fluid discarded, 2 ml. of water and 0·35 ml. of a 4 per cent. solution of sodium hydroxide added. The aluminium hydroxide at the bottom of the tube,

upon which any adrenaline which was originally present will be adsorbed, should now go into solution. After 2 minutes, 2 ml. of the sulphurous acid reagent (No. 3) is added, the mixture poured into a tube containing 0·7 ml. of the arsenomolybdic acid solution which has been immersed in a boiling water-bath for 5 minutes, and the heating is continued. After exactly 5 minutes the tube is removed and placed in a beaker of cold water. The volume is made up to 5·5 ml. and the colour either measured by means of a colorimeter or matched against standards. If the former method be adopted a blank should be prepared by taking the same volume of aluminium hydroxide suspension as has been used in the test, adding 2 drops of phenolphthalein reagent, making just alkaline with 4 per cent. solution of sodium hydroxide and centrifuging. The supernatant liquid is then discarded, 2 ml. of water and 0·35 ml. of a 4 per cent. solution of sodium hydroxide added to the aluminium hydroxide followed by the sulphurous acid reagent and the mixture added to arsenomolybdic acid solution as before.

A standard blue colour is prepared by subjecting 2 ml. of an appropriate solution of adrenaline to the second, but not necessarily to the first, adsorption with aluminium hydroxide and subsequent treatment with alkali, sulphurous acid and arsenomolybdic acid as described above. If a calibration curve is to be prepared for use with a colorimeter it is advisable to subtract the colour of the blank both from that due to the unknown and that due to the standard. However, the use of a curve is not to be recommended since, although constant results can be obtained on any one day, unexplained variations in the position of the curve may occur when the test is repeated on different days; hence it is advisable to prepare standards from adrenaline solution of known strength.

Discussion. Shaw discovered that brief preliminary treatment of adrenaline with alkali in the presence of oxygen considerably increases the intensity of the colour which it will produce in the above test. If the treatment with alkali is prolonged the colour is decreased. Adrenaline is known to be oxidised under these conditions and apparently one of the products of oxidation is a more active reducing agent than adrenaline itself; this product is destroyed by longer exposure to alkali. A series of substances allied to adrenaline, including adrenalone, was tested, but none is the product in question since none produced as much colour as adrenaline. *p*-Sympatol was the only substance which resembled adrenaline in giving increased colour after alkali treatment and

it is the only substance among those examined whose side chain is identical with that of adrenaline. These facts suggest that this particular property of the side chain of adrenaline is highly specific and that if the reducing power of a tissue extract be increased by treatment with alkali the observation can be taken as evidence that the sample contains adrenaline or some other phenol with the same side chain.

In order to apply this test of specificity, the solution to be examined is divided into two, and one half treated as described above and the other similarly, except that 0·35 ml. of the 4 per cent. solution of sodium hydroxide which is used to dissolve the aluminium hydroxide after the second adsorption is replaced by 0·35 ml. of water containing 1 drop of N sulphuric acid. When the reduction is due to 0·04 μg. or more of adrenaline the ratio of the colour given by the alkali-treated half of the solution to that given by the untreated half is from 2 to 3·5. With smaller quantities the ratio falls.

Table XXVII, which is reproduced from F. H. Shaw's paper, " shows the concentration of a number of substances required to give a total colour equivalent to that given by adrenaline (1 μg. per ml.). These estimates were prepared by testing the solutions of the substance directly without adsorption. The proportional

TABLE XXVII. RESPONSE OF ADRENALINE AND ALLIED SUBSTANCES TO THE ARSENOMOLYBDIC ACID TEST (After F. H. Shaw, *Biochem. J.*, 1938, **32**, 19)

Substance	Chemical Formula	Concentration Giving Equivalent Colour after Alkali Treatment g. per ml.	Proportional Increase of Colour Due to Alkali
Adrenaline	$(OH)_2C_6H_3CH(OH)CH_2NHCH_3$	1	5
Corbasil	$(OH)_2C_6H_3CH(OH)CH(CH_3)NH_2$	12	No change
Dopa	$(OH)_2C_6H_3CH_2CH(COOH)NH_2$	12	,,
Epinine	$(OH)_2C_6H_3CH_2CH_2NHCH_3$	13	,,
nor-Adrenaline	$(OH)_2C_6H_3CH(OH)CH_2NH_2$	16	,,
Adrenalone	$(OH)_2C_6H_3COCH_2NHCH_3$	30	,,
Catechol	$(OH)_2C_6H_4$	100	Diminishes
p-Sympatol	$(OH)C_6H_4CH(OH)CH_2NHCH_3$	2000	5
Tyramine	$(OH)C_6H_4CH_2CH_2NH_2$	No Colour	—
Ephedrine	$C_6H_5CH(OH)CH(CH_3)NHCH_3$	,,	—
Glutathione		500	No change
Ascorbic acid		500	Diminishes
Glyceraldehyde		1000	,,

increase of colour when adrenaline was tested with alkali was found to be 5 whereas the ratio was only 3·5 when the two fractions of adrenaline were both adsorbed by the method described above. This difference is apparently due to the fact that the small amount of alkali necessary for adsorption changes the adrenaline slightly so as to increase the colour, thus reducing the apparent effect of the larger quantity of alkali." Table XXVII " shows that catechol derivatives are active in less concentrations and that small changes in the side change abolish the effect of alkali. Sympatol was the only other substance tested which contains the same side chain as adrenaline and the ratio was about the same as that for adrenaline, though it was necessary to employ high concentrations to obtain any appreciable colour. All other substances tested in the series gave ratios of 1 or less. On the other hand, the small amount of colour given in blank tests was found to be increased by alkali in the ratio of 1·1 to 1·5. Ratios of 2 or over can therefore be used as evidence for the presence of adrenalines." In summarising his method Shaw states that adrenaline can be satisfactorily estimated in tissues provided that the concentration exceeds 10^{-7}; if less is present the test gives a result which must be interpreted with caution.

It has been stated that Shaw's method is unreliable[5] and Bloor and Bullen have encountered difficulties[6]. They found that in the first aluminium hydroxide adsorption of the blood filtrate 1 drop of N sulphuric acid was not sufficient to prevent adsorption of adrenaline, some 16 or 18 drops being necessary whence much of the aluminium hydroxide was dissolved. Again, in the special enhancing treatment described by Shaw in which 0·35 ml. of 4 per cent. sodium hydroxide solution and 2 ml. of water with 2 minutes' heating are employed, the aluminium hydroxide did not dissolve completely and the resulting increase of colour was small and uncertain. Bloor and Bullen propose that both these steps be omitted and that the heating time for the development of the colour be extended to 10 minutes. Their procedure is essentially as follows :—

Blood is drawn from a vein and 5 ml. at once run into 20 ml. of a 10 per cent. aqueous solution of trichloracetic acid contained in a 50-ml. conical centrifuge tube. After thorough mixing, the material is centrifuged for 5 minutes at about 1000 r.p.m. and the clear supernatant fluid poured through a filter into a test tube. For the determination, 1 ml. of the filtrate (which may contain about 0·4 μg. of adrenaline) is measured into a small centrifuge

tube (16×105 mm.), 1 ml. of 0·4N sulphuric acid and 2 drops of phenolphthalein solution added, the mixture titrated with N sodium hydroxide until a faint permanent pink colour is produced then 2 ml. of the suspension of aluminium hydroxide added, the mixture again neutralised to a faint pink colour and, after well shaking and allowing to stand for 5 minutes, centrifuged for 5 minutes at about 2000 r.p.m. The supernatant fluid is poured off, the tubes drained by inversion over absorbent paper, 2 ml. of the arsenomolybdic acid solution added and the tubes immersed in boiling water for 5 minutes then 3 ml. of the sulphurous acid reagent (No. 3) added, the heating of the mixture in the boiling water-bath continued for a further 10 minutes after which it is cooled by allowing the tube to stand in ice-water for 15 minutes. The solution is then diluted to 40 ml. and the colour matched against standards.

It is pointed out that variability of the blank tests remains a major difficulty and the method cannot be regarded as capable of giving precise results. Bloor and Bullen also question the specificity of the test: they obtained colours corresponding to values of 0·2 to 0·5 μg. per ml. of adrenaline in the venous blood of dogs and humans but this material was found to be stable to alkali under conditions in which adrenaline added to blood, or blood filtrates, was completely destroyed. Blood filtrates to which adrenaline had been added, when treated with alkali, gave the same or slightly higher values than corresponding filtrates containing no added adrenaline. Hence, judging from the results of these experiments, there seems to be considerable doubt as to whether there is any adrenaline in venous blood and it would not appear that the arsenomolybdate method for its colorimetric determination is sufficiently specific to settle the problem.

References

1. A. J. Ewins, *J. Physiol.*, 1910, **40**, 317.
2. J. H. Barker, C. J. Eastland and N. Evers, *Biochem. J.*, 1932, **26**, 2129.
3. F. H. Shaw, *Biochem. J.*, 1938, **32**, 19.
4. J. C. Whitehorn, *J. Biol. Chem.*, 1935, **108**, 633.
5. E. B. Tietz, H. Dornheggen and D. Goldman, *Endocrinology*, 1940, **26**, 641.
6. W. R. Bloor and S. S. Bullen, *J. Biol. Chem.*, 1941, **138**, 727.

ANDROSTERONE (AND 17-KETOSTEROIDS)

A method for the colorimetric determination of this hormone was proposed by W. Zimmermann[1] and has received the attention of several other investigators[2–6].

Method[5]. To 0·2 ml. of the extract to be tested contained in a tube graduated at 10 ml., is added 0·2 ml. of a 2 per cent. solution of *m*-dinitrobenzene in 95 per cent. ethyl alcohol (this reagent solution should not be more than 10 days old) and 0·2 ml. of an exactly 5N aqueous solution of potassium hydroxide (as free as possible from carbonate). The tube is closed with a glass or rubber stopper, the contents gently shaken, maintained at 25° ±0·2° C. for 45 minutes, diluted to 10 ml. with 95 per cent. ethyl alcohol and the pink colour measured. A blank using 0·2 ml. of the 95 per cent. alcohol in place of the extract under test should be nearly colourless. Standards can be prepared from solutions of androsterone, dehydro-*iso*-androsterone or œstrone which should contain 0·2 mg. per ml. of one of these pure substances.

Discussion. It is necessary to standardise each new supply of solid potassium hydroxide ; also, there should be no precipitation of carbonate when 10·4 ml. of 95 per cent. redistilled ethyl alcohol is mixed with 0·2 ml. of 5N aqueous solution.

The test is not specific. However, according to Holtorff and Koch[5] the following substances which might be present do not interfere : stilbœstrol, pregnanediol, benzoic acid, Δ^5-cholestene-3 : 4-dione, α-cholesterol oxide, Δ^5 - 7 - keto - 3 - hydroxycholenic ethyl ester, Δ^5-3 : 4-cholestenedione-4-enol acetate, Δ^5-3-acetoxy-7 - keto - cholenic ethyl ester, Δ^5 - 3 - acetoxy - 5 - hydroxy - 6 - ketocholenic ethyl ester, cholesterol, digitonin, β-hydroxybutyric acid, ergosterol, œstriol and 7-oxocholesterol. Interfering substances include acetone, androstenedione, androstenetrione, testosterone, 6-oxocholesterol, Δ^4-3 : 6-cholestenedione-6-enol ethyl ether, cholestanone, cholestenone, cholestenone acetate, *epi-allo*-pregnanolone, 3-keto-12-hydroxycholanic acid and its ethyl ester, acetoacetic ester, 6 - oxo - 17-acetotestosterone, dehydrocholic acid, dehydrohyodeoxycholic acid, deoxycorticosterone and progesterone.

As already indicated, dehydro-*iso*-androsterone and œstrone react similarly to androsterone. However, according to Friedgood and Whidden[6] the presence or absence of water influences the colour reaction with androsterone and dehydro-*iso*-androsterone,

the latter yielding about 25 per cent. more colour when the test is conducted as described above. These investigators find that when the colour reaction is carried out in absolute alcohol these two androgens give the same intensity of colour per unit weight and hence, in this circumstance, mixtures can be more satisfactorily treated as simple solutions of one or the other substance from a chromogenic viewpoint. It is reported that the results obtained by the use of the aqueous solution of potassium hydroxide are higher in individual instances than those for corresponding groups when alcoholic potash is employed but the figures for both series lie within the same range and the differences are not clinically significant. In a recent collaborative investigation[7] the results of over 1500 urinary 17-ketosteroid assays on a series of patients suffering from various endocrine and related disorders are presented.

REFERENCES

1. W. Zimmermann, *Z. physiol. Chem.*, 1935, **233**, 257.
2. H. Wu and C. Y. Chon, *Chinese J. Physiol.*, 1937, **11**, 413.
3. W. Zimmermann, *Z. physiol. Chem.*, 1936, **245**, 47.
4. N. H. Callow, R. K. Callow and C. W. Emmens, *Biochem. J.*, 1938, **32**, 1312.
5. A. F. Holtorff and F. C. Koch, *J. Biol. Chem.*, 1940, **135**, 377.
6. H. B. Friedgood and H. L. Whidden, *Endocrinology*, 1941, **28**, 237.
7. R. W. Fraser, A. P. Forbes, F. Albright, H. Sulkowitch and E. C. Reifenstein, Jr., *J. Clin. Endocrinol.*, 1941, **1**, 234.

STILBŒSTROL

The extensive employment of 4 : 4′-dihydroxy-α : β-diethylstilbene, or stilbœstrol, as a synthetic substitute for the naturally occurring ovarian hormones has stimulated investigations into possible methods for its determination. Two colorimetric procedures have been proposed, Dechene[1] applying the " xanthoproteic " reaction while Tubis and Bloom[2] recommend a method depending upon the reduction of the reagent of Folin and Ciocalteu[3] by the two phenolic groups of the stilbœstrol molecule. The latter method, with its application to the assay of tablets is described below and, in addition, a modification suited to the assay of stilbœstrol dipropionate in oil, which has been proposed by Dracass and Foster[4], is also given.

Method for Stilbœstrol[2]. The sample, 0·2 to 0·9 mg., is dissolved

in 10 ml. of alcohol (30 per cent.) contained in a 100-ml. graduated flask, the solution diluted with 50 ml. of water and 5 ml. of Folin and Ciocalteu's reagent (1 vol. of stock diluted with 2 vols. of water ; see p. 414) added. After mixing, 15 ml. of a 20 per cent. aqueous solution of sodium carbonate, Na_2CO_3, is added, the resulting liquid diluted to the graduation mark with water and allowed to stand at room temperature for 1 hour. The stable blue colour produced is compared with that of standards similarly prepared from pure stilbœstrol.

Application to Tablets[2]. An accurately weighed sample of the ground tablets, expected to contain about 5 mg. of stilbœstrol, is treated with 30 ml. of boiling alcohol (95 per cent.) under a reflux condenser for 15 minutes. After allowing to cool, the mixture is diluted to 100 ml. with water, then filtered and the colour test described above applied to 10 ml. of the filtrate. If the tablets under examination are coloured with water-soluble dyestuffs, a quantity of the powdered material, expected to contain 5 mg. of stilbœstrol, is transferred to a separator containing 50 ml. of water and the resulting solution extracted with four separate portions of ether. The combined ethereal extracts are evaporated to dryness, the residue is dissolved in 30 ml. of alcohol (95 per cent.), the solution diluted to 100 ml. with water then filtered and the colorimetric test applied to 10 ml. of the filtrate.

Application to Stilbœstrol Dipropionate in Oil[4]. To 1 ml. of the sample, containing between 1 and 10 mg. of stilbœstrol dipropionate per ml., is added 10 ml. of alcohol (95 per cent.) containing 2 to 3 drops of concentrated sulphuric acid and the mixture is boiled under a reflux condenser for 2 hours. After allowing to cool, the reaction mixture is washed into a separator with about 50 ml. of ether and the solution extracted three times with 10-ml. portions of N sodium hydroxide, care being taken to ensure that an excess of alkali is present during the first shake. The combined alkaline extracts are washed with about 25 ml. of ether and, after separating, the ethereal layer is shaken with 5 ml. of N sodium hydroxide. The separated ether is discarded, the alkaline liquids mixed, acidified with dilute sulphuric acid and the stilbœstrol extracted with two portions of ether. The separated ethereal extracts are successively washed with the same 5-ml. portion of water, then transferred to a flask and the ether removed by evaporation. The residue is dissolved in sufficient 0·4 per cent. aqueous solution of sodium hydroxide to make a solution expected to contain approximately 10 mg. of stilbœstrol dipropionate in

100 ml. To 5 ml. of this solution is added first, 3 ml. of Folin and Ciocalteu's reagent (1 vol. of the stock diluted with 2 vols. of water: see p. 414), then 2 ml. of a 20 per cent. aqueous solution of sodium carbonate, Na_2CO_3. The mixture is heated in a boiling water-bath for 5 minutes, allowed to cool, diluted with water to 25 ml. and filtered or centrifuged. The intensity of the blue colour thus produced is compared by means of a colorimeter with a standard made by dissolving 7 mg. of stilbœstrol (equivalent to 10 mg. of stilbœstrol dipropionate) in 2 ml. of 20 per cent. aqueous solution of sodium hydroxide, diluting to 100 ml. with water and treating 5 ml. of this solution with Folin and Ciocalteu's reagent and sodium carbonate similarly to the solution derived from the sample.

Discussion. The value of this method is limited, since many other substances possessing reducing properties will react with the complex phosphomolybdic-phosphotungstic acid solution of Folin and Ciocalteu forming a blue coloration. In applying the method to stilbœstrol dipropionate in oil the residue from the initial extraction process should be dissolved in the appropriate quantity of 20 per cent. sodium hydroxide and the resulting solution diluted to volume with water. With samples of oil containing 1 mg. of the dipropionate per ml. it is advisable to take 5 ml. for the extraction instead of 1 ml. Sesame oil contains sufficient naturally occurring phenolic bodies to interfere with the determination of stilbœstrol dipropionate and, if this oil has been used to dissolve the ester, a correction must be applied for the colour due to the solvent.

A striking colour test for stilbœstrol which, however, is only approximately quantitative has been described by T. T. Cocking[5]. If 1 mg. of stilbœstrol be dissolved by gentle warming in 4 ml. of glacial acetic acid, 1 ml. of a 1 per cent. v/v solution of bromine in the same solvent added and the mixture warmed on a water-bath for a few minutes an orange solution is produced containing the equivalent of 0·02 mg. of stilbœstrol in 0·1 ml. which may be kept as an approximate standard. For producing the colour, 0·1 ml. of this solution is mixed in a small test tube with 0·1 ml. of absolute alcohol and then with 1 ml. of water. The resulting violet colour, which dissolves in organic solvents to give a red solution, may be used for purposes of comparison or it may be transferred to chloroform by vigorously shaking with 1 ml. of the solvent and allowing to separate. The volume of chloroform used may be varied to assist matching but if it is separated from

the aqueous layer the colour quickly fades. The colour given by 0·04 mg. of stilbœstrol extracted by 5 ml. of chloroform, rapidly separated, dried and measured in a 1-cm. cell is approximately equivalent to 5·0 red +1·0 yellow Lovibond units. If to a solution of stilbœstrol in glacial acetic acid a much smaller quantity of bromine be added (approximately in the proportion of one quarter of an atom of bromine per molecule of stilbœstrol) and the mixture warmed in a water-bath a fine emerald green colour is produced.

REFERENCES

1. E. B. Dechene, *J. Amer. Pharm. Assoc.*, 1941, **30,** 208.
2. M. Tubis and A. Bloom, *Ind. Eng. Chem., Anal. Ed.*, 1942, **14,** 309.
3. O. Folin and V. Ciocalteu, *J. Biol. Chem.*, 1927, **73,** 627.
4. W. R. Dracass and G. E. Foster, *Analyst*, 1943, **68,** 181.
5. T. T. Cocking, *Analyst*, 1943, **68,** 144.

β-CAROTENE

Green leaves and other vegetable products contain α- and β-carotene and a mixture of hydroxylated carotoids collectively termed " xanthophyll." Only those carotenoids which possess an intact half of the β-carotene molecule can act as precursors of vitamin A hence, from a commercial point of view, α- and β-carotenes are the only substances of this class whose determination is usually required. Indeed, it has been usual to include α-, β- and γ-carotenes as one substance under the name of " carotene " and, in fact, in many leafy materials β-carotene is, for all practical purposes, the only one present. For a lucid account of the chemical constitution and physiological significance of the carotenoids a paper by R. A. Morton[1] should be consulted. The following procedure for the determination of β-carotene in grass products has been recommended by W. M. Seaber[2] and adopted as a tentative official method by the Grass Driers' Association[3].

Method. From 0·25 to 0·5 g. of the dried grass, according to the richness of the sample, is ground in a mortar with about 20 times its weight of No. 60 mesh silver sand and the whole transferred to an extraction thimble lined with filter paper, the mortar being rubbed out with a little more sand which is added to the ground powder. A plug of cotton wool is placed in the top of the thimble and the sample is extracted in a continuous-drip type of

extractor with a mixture of 15 ml. of acetone and 45 ml. of light petroleum (b. pt. 40° to 60° C.) for at least an hour, or until no more colour is removed. In some cases it may be advisable to re-grind the mixture and to submit it to the extraction process a second time. The cooled extract is transferred to a separator, the flask rinsed, first with 3 ml. and then with 2 ml. of light petroleum, and 5 ml. of a colourless 30 per cent. solution of potassium hydroxide in methyl alcohol added. The separator is thoroughly shaken for 2 minutes, 200 ml. of water introduced and the separator gently inverted once or twice. The aqueous layer is run off and the light petroleum layer vigorously shaken with a further 200-ml. portion of water. In this way chlorophyll and flavones are removed. Xanthophylls are then extracted from the petroleum ether by shaking successively with 30-, 15- and 15-ml. portions of diluted methyl alcohol (s.g. 0·830 at 15°/15° C. made by mixing 90 vols. of absolute methyl alcohol with 10 vols. of water separately measured). The light petroleum solution is made up to 50 ml., or to such other volume as is convenient for conducting a colorimetric comparison. If necessary the solution may be brightened by shaking with about a gram of anhydrous sodium sulphate.

The colour of the light petroleum solution prepared as above is compared by means of a colorimeter with that of a 0·025 per cent. aqueous solution of potassium dichromate solution which a Committee of Chemists of the Grass Driers' Association has agreed shall be considered as having the same colour intensity as a light petroleum solution containing 0·158 mg. of β-carotene per 100 ml. It is generally accepted that within reasonable limits the graph expressing the relation between carotene and potassium dichromate obtained by the use of various colorimeters is a straight line.

Discussion. The strength of carotene solution given above is extremely close to that of 0·160 mg. per 100 ml. found quite independently by Connor[4]. Seaber[2], using a Spekker photoelectric absorptiometer, obtained the slightly higher figure of 0·165 mg. per 100 ml. as the equivalent of a 0·025 per cent. aqueous solution of potassium dichromate and it is specifically stated by the Grass Driers' Association in the description of their tentative official method that the value of 0·158 mg. per 100 ml. does not apply to photo-absorptiometers and if such instruments are used they must be calibrated with pure carotene. If a spectrophotometer is employed, about twice as much sample as is needed for the other methods should be taken, the extinction coefficient, $E_{1\%}^{1\,cm.}$,

is calculated for $\lambda=450$ mμ in light petroleum solution or for $\lambda=463$ mμ in chloroform, and referred to 2500 and 2200 respectively as the values accepted for pure β-carotene in these solvents.

Ferguson[5], using β-carotene, m. pt. 180° to 182° C., isolated from cocksfoot grass (*Dactylis glomerata*), has expressed the colour of its solution in light petroleum in terms of Lovibond units : these values are shown in Table XXVIII.

TABLE XXVIII. THE COLOUR OF CAROTENE SOLUTION IN LIGHT PETROLEUM EXPRESSED IN LOVIBOND UNITS (W. S. Ferguson, *Analyst*, 1935, **60**, 680)

β-Carotene per 100 ml. mg.	Colour (observed in 1-cm. cell) Lovibond Yellow Units	β-Carotene per 100 ml. mg.	Colour (observed in 1-cm. cell) Lovibond Yellow Units
0·0609	0·95	0·2436	4·55
0·0812	1·30	0·2639	5·00
0·1015	1·60	0·2842	5·70
0·1218	2·05	0·3045	6·10
0·1421	2·30	0·3248	6·90
0·1624	2·80	0·3451	7·35
0·1827	3·20	0·3654	8·05
0·2030	3·70	0·3857	8·75
0·2233	4·05	—	—

In making these observations it was necessary to employ the red slides of the tintometer in amounts varying from 0·2 unit in the more dilute solutions to 0·5 unit for the deeper colours.

Ferguson also compared the colour of carotene solutions in light petroleum against that of a 0·1 per cent. aqueous solution of potassium dichromate using a Klett colorimeter. The carotene solutions were set at 20 mm. on the scale and the matchings were made by varying the height of the potassium dichromate column. The data thus obtained are presented in Table XXIX. By either method the matching of the colours is easier and more accurate at the lower concentrations. On converting the above values for the Klett colorimeter into terms of a 0·025 per cent. solution of potassium dichromate the equivalent carotene solution works out to 0·150 mg. per 100 ml.

The light petroleum solution obtained by the above procedure contains some yellow colouring matter apart from carotene, the nature of which has not yet been satisfactorily elucidated. In

TABLE XXIX. HEIGHT OF A 0·1 PER CENT. AQUEOUS SOLUTION OF POTASSIUM DICHROMATE MATCHING 20 MM. COLUMNS OF CAROTENE SOLUTIONS IN LIGHT PETROLEUM (W. S. Ferguson, *Analyst*, 1935, **60**, 680)

β-Carotene per 100 ml. mg.	Depth of Potassium Dichromate Column mm.	β-Carotene per 100 ml. mg.	Depth of Potassium Dichromate Column mm.
0·0483	1·60	0·1800	6·04
0·0676	2·26	0·2029	6·85
0·0966	3·21	0·2318	7·65
0·1256	4·19	0·2705	8·75
0·1546	5·16	0·3280	10·50

order to determine true carotene, Seaber[2] recommends adding 3 per cent. of acetone to the light petroleum solution and passing the mixture through a column of activated alumina about 6 or 7 cm. long and 4 mm. in diameter. In this way the carotene is separated from other colouring material since the latter is adsorbed. The alumina column should be washed with a 3 per cent. solution of acetone in light petroleum and the percolate diluted to volume, its colour measured and converted to carotene in accordance with the data presented above. If the original grass or other leafy sample is in its natural state and has not been dried, plain acetone should first be used to extract the specimen and remove the water, after which the material may be ground with sand and extracted with light petroleum.

The main procedure as described is applicable to flour and cereals, generally, and to carrots and tomatoes. When the chromatographic purification is applied to the latter, the lycopene, which is the principal pigment of tomatoes, is held by the alumina as a purple-red layer while the β-carotene passes through.

In the examination of palm oil and similar fatty substances, extraction will coincide with saponification. The above cold process works satisfactorily using 30 per cent. solution of potassium hydroxide in methylated alcohol at the rate of 15 ml. for each gram of oil, then 50 ml. of light petroleum, diluting with water, removing the aqueous layer and repeating the treatment with 7·5 ml. of potash solution. This same method is applicable to butter.

Seaber disclaims priority for the introduction of the above cold saponification process which originated in the laboratory of Messrs.

John Hughes, Official Analysts to the Grass Driers' Association. A similar procedure based upon the work of Guilbert[6] has been described by Peterson, Hughes and Freeman[7]. For grasses the ratio between the pure carotene as obtained by the chromatographic treatment and the crude carotene as originally extracted by the light petroleum varies from 0·7 to 0·8, though the proportion may fall still lower. Some interesting results obtained by Seaber[2] of the carotene content of various types of forage and other food are given in Table XXX.

TABLE XXX. RESULTS OF CAROTENE DETERMINATIONS APPLIED TO FORAGE AND OTHER FOODS (After W. M. Seaber, *Analyst*, 1940, **65**, 266)

Commodity	Content of Carotene (calculated on dried sample)	
	Without Chromatographic Treatment p.p.m.	Purified by Chromatographic Treatment p.p.m.
Grass meal	160	125
„ „	275	220
„ „	440	325
„ „	425	315
„ „	320	225
Carrot	720	—
Carrot meal, dried	500	—
Grass meal and molasses	145	115
„ „ „ „	400	310
Alfalfa, Canadian	175	160
Hay	90	—
Palm Oil	920	—
Silage, red clover	65	45
„ white clover	130	102
Tomato, green	55	—
„ pink	110	—
„ red	220	—
Wheat flour, bleached	1	—
Grass meal	250	220
„ „	535	410
Spinach	600	—
Barley shoots	155	—
Lucerne leaf	310	275
„ stalk	205	193
„ meal	220	190
Turnip leaf	550	—
„ stalk	40	—

Instead of removing associated pigments by the above phase separation, T. Barton Mann[8] prefers to remove the solvent from

the initial acetone-light petroleum extract, dissolve the residue in light petroleum and pass the solution through fat-free bone meal which adsorbs all pigments except β-carotene ; after washing the column with light petroleum until no further colour is removed, the determination is completed in the usual manner by measuring the colour of the eluate. By this procedure the use of alkali to remove chlorophyll is obviated.

REFERENCES

1. R. A. Morton, *Chem. & Ind.*, 1940, **59**, 301. Abstracted *Nature*, 1940, **145**, 286 ; and *Analyst*, 1940, **65**, 263.
2. W. M. Scaber, *Analyst*, 1940, **65**, 266.
3. *Analyst*, 1941, **66**, 334.
4. C. L. Conner, *J. Biol. Chem.*, 1928, **77**, 619.
5. W. S. Ferguson, *Analyst*, 1935, **60**, 680.
6. H. R. Guilbert, *Ind. Eng. Chem., Anal. Ed.*, 1934, **6**, 452.
7. W. J. Peterson, J. S. Hughes and H. F. Freeman, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 71.
8. T. Barton Mann, *Analyst*, 1944, **69**, 34.

NICOTINIC ACID, NICOTINAMIDE AND TRIGONELLINE

Nicotinic acid and its derivatives are of importance owing to their curative value in deficiency diseases such as pellagra and other nutritional disorders. Two reactions have been applied to their colorimetric determination ; one, depending upon the reddish-purple colour produced by interaction with dinitrochlorbenzene[1] has been developed by Vilter *et al.*[2] while the other is based upon the yellow colour which results from the addition of cyanogen bromide and an amine to derivatives of pyridine[3]. Methods based upon the latter reaction have found more favour and two modifications will be described, the one being applied to liver extracts while the other is especially designed for the examination of urine. Nicotinamide occurs in association with nicotinic acid and since both compounds are therapeutically active a process of hydrolysis is included in the method of determination ; by omitting this step the proportion of each is readily ascertained.

Method for Liver Extracts: Nicotinic Acid and Nicotinamide[4]. A test solution is prepared by adding 2 ml. of the sample to 18 ml. of alcohol (redistilled from concentrated sulphuric acid), the mix-

ture heated to 50° C. and maintained at that temperature for 5 minutes and then filtered through a Whatman No. 1 paper. Nicotinamide present in this test solution is hydrolysed by evaporating 10 ml. just to dryness on a water-bath, adding 4 ml. of N hydrochloric acid and heating on a boiling water-bath for 10 minutes. After allowing to cool, the acid solution is neutralised by adding 4 ml. of N sodium hydroxide and the mixture diluted to 10 ml. with water. An aliquot part of this hydrolysed solution is taken and diluted to 3 ml. so that the proportion of alcohol and water is as 1 : 2, and 6 ml. of cyanogen bromide solution (freshly made by titrating a saturated aqueous solution of bromine with a 10 per cent. aqueous solution of potassium cyanide until the yellow colour is just destroyed) added followed by 1 ml. of a 4 per cent. by volume solution of redistilled aniline in alcohol (redistilled from concentrated sulphuric acid). After allowing to stand for 5 minutes the intensity of the colour is measured by means of a Lovibond tintometer employing a 1-cm. cell and the intensity of the yellow component correlated with the amount of nicotinic acid in accordance with the values given in Table XXXI. In compiling these figures an approxi-

TABLE XXXI. RELATION BETWEEN THE QUANTITY OF NICOTINIC ACID AND THE COLOUR PRODUCED (G. E. Shaw and C. A. Macdonald, *Quart. J. Pharm.*, 1938, **11**, 380)

Quantity of Nicotinic Acid mg.	Colour observed in 1-cm. cell : Lovibond Yellow Units
0·01	0·8
0·02	2·3
0·03	3·6
0·04	5·2
0·05	6·7
0·06	8·4
0·07	10·0

mate allowance has been made for the residual colour of the treated liver extracts. If any other system of colour measurement is employed a similar adjustment should be made. A determination of the nicotinic acid, a distinct from the acid and amide taken together, can be made by applying the colour test to a suitable proportion of the filtrate which has not been evaporated and heated with acid.

Discussion. The above method, due to G. E. Shaw and C. A.

Macdonald[4], is based upon the work of M. Swaminathan[5] and has proved to be a convenient means for evaluating commercial liver extracts with reference to their content of nicotinic acid. The final colour produced in the test attains its maximum intensity in 5 minutes then slowly fades. It should be understood that the colour reaction will respond to other pyridine compounds which may be present in samples and hence the results obtained must be deemed to be maximum values and in some cases higher than the true content of nicotinic acid; however, apart from low-grade liver extracts, the error from this source will not be significant.

According to Daroga[6] nicotinic acid can be quantitatively isolated from foodstuffs by suspending the sample in 250 ml. of water, adding 3 g. of calcium oxide and 20 g. of sodium chloride then steam distilling until 400 ml. of distillate has been collected in a receiver containing 20 ml. of 6N hydrochloric acid. This liquid may be evaporated to a volume of about 2 ml. without losing nicotinic acid and the latter might then be determined by the method given above, due allowance being made for any colour inherent in the evaporated filtrate.

Method for Urine: Nicotinic Acid and Nicotinamide[7]. The sample is rendered just neutral to phenolphthalein and 20 ml. evaporated over a boiling water-bath to a thick syrup; this is transferred to a flask with the aid of 4 ml. of water and 6 ml. of concentrated hydrochloric acid, 0·25 ml. of concentrated nitric acid added and the mixture gently boiled for 1 hour under a refluxing condenser, chips of silica being added to maintain steady ebullition. After cooling, the mixture is diluted to 20 ml. with water then 15 ml. transferred to a beaker, partially neutralised by the addition of 2 ml. of 12N potassium hydroxide, cooled and the reaction adjusted to *p*H 1·0, the measurement being conducted by means of a glass electrode or methyl violet as external indicator. The liquid is then transferred to a test tube (175 $\times$22 mm.) containing 2 g. of Lloyd's reagent (fuller's earth for adsorption purposes) the beaker being washed with 0·2N sulphuric acid which is added to the main solution. The test tube should bear a graduation mark to indicate the level attained by a mixture of 15 ml. of liquid and 2 g. of Lloyd's reagent. The tube is closed with a rubber stopper, shaken, the gas pressure released and the tube shaken for at least a minute. The stopper and sides of the tube are washed down with 0·2N sulphuric acid, the mixture centrifuged and the clear supernatant fluid decanted and dis-

carded. The sediment of Lloyd's reagent is washed twice by shaking with 0·2N sulphuric acid and centrifuging. After the last washing has been decanted, 8 ml. of 0·5N potassium hydroxide is added, the mixture shaken for 1 minute, water added to the graduation mark, the contents mixed and centrifuged. The coloured supernatant fluid is decanted into another test tube, 0·6 g. of powdered lead nitrate added and the contents shaken; if the supernatant solution is highly coloured, 2 drops of ammonia (s.g. 0·880) are added and the tube shaken and centrifuged. The excess of lead nitrate is removed by adding 0·2 g. of tribasic potassium phosphate, allowing to stand for 10 minutes and centrifuging. The supernatant liquid should now be a light yellow or amber colour.

Two separate 5-ml. portions of this supernatant liquid are transferred to tubes graduated at 20 ml. and the reaction of each adjusted to *p*H 4·5 by the addition of 5N phosphoric acid, using either a glass electrode or an external indicator in conjunction with a standard buffer solution. One of the tubes is immersed in a water-bath at 75° to 80° C. for 5 minutes then 1 ml. of a solution of cyanogen bromide (freshly made by titrating a saturated aqueous solution of bromine with a 10 per cent. aqueous solution of potassium cyanide until the yellow colour is just discharged) is added and the tube kept in the water-bath for 5 minutes longer. After cooling by immersing the tube in ice-water, 10 ml. of a saturated aqueous solution of *p*-methylamino-phenol sulphate (metol) is added, the mixture diluted to 20 ml. with water, placed in a dark cupboard for 1 hour and the orange colour measured. The other tube containing 5 ml. of the original supernatant liquid adjusted to *p*H 4·5 is diluted to 20 ml. with water and the slight colour measured and deducted from that of the treated sample. Standards are prepared by submitting solutions of nicotinic acid to the colour test, quantities up to about 0·05 mg. offering a convenient range.

Discussion. Perlzweig, Levy and Sarett[7], who proposed the above procedure, found that decolorisation of urine without loss of nicotinic acid presented a difficult problem. By combining an acid hydrolysis with hydrochloric acid containing a little nitric acid and treatment with Lloyd's reagent, final test solutions are obtained which are only slightly yellow. The use of *p*-methylamino-phenol sulphate in place of aniline was suggested by Bandier and Hald[8] because it is less sensitive to other pyridine derivatives, such as nicotinuric acid and nicotine and the final

colour is more stable.* Perlzweig and his collaborators[7] suggest a method for determining trigonelline (the betaine of nicotinic acid, $CH_3.\overline{NC_5H_4COO}$) based upon their observation[9] that, although unaffected by acids, it is hydrolysed by strong alkali. The procedure consists in adding 1 ml. of the hydrochloric-nitric acid hydrolysate to a test tube containing 0·3 g. of urea and 1·3 ml. of 12N potassium hydroxide maintained at a temperature of 75° to 80° C. After 45 minutes the mixture is washed into a beaker with 8 to 10 ml. of 0·2N sulphuric acid the reaction adjusted to *p*H 1·0 with 1·5 to 2 ml. of concentrated hydrochloric acid and the adsorption on, and elution from, Lloyd's reagent and all subsequent manipulations continued as already described for the determination of nicotinic acid. The colour values obtained include the nicotinic acid derivatives together with 70 per cent. of the nicotinic acid colour yielded by the trigonelline : after the acid-hydrolysable nicotinic acid is subtracted, the remainder is multiplied by the factor 1·43 to give trigonelline ; the initial acid hydrolysis appears to be necessary.

According to the investigators cited above, normal human adults were found to excrete daily 1 to 3 mg. of nicotinic acid derivatives, exclusive of trigonelline. On a diet free from coffee the same subjects excreted 20 to 29 mg. of trigonelline each day and when ingesting coffee the figure rose considerably, the highest recorded being 204 mg.

An alternative technique for nicotinic acid using aniline as the amine has been described by D. Melnick and his collaborators[10, 11] and the same investigators have undertaken detailed studies upon the urinary excretion of nicotinic acid and its derivatives[12] and their concentration and distribution in the blood.[13] Dann and Handler have discussed[14] the application of the method described above for urine to the determination of nicotinic acid in tissues.

A procedure for the determination of trigonelline which is

* An alternative procedure for the determination of nicotinic acid in urine has been suggested by Y. L. Wang and E. Kodicek (*Biochem. J.*, 1943, **37**, 530), in which the natural colour of the sample is removed by rendering alkaline with sodium hydroxide, heating on a water-bath, allowing to cool, acidifying with hydrochloric acid, extracting part of the pigment with *iso*-butyl alcohol and oxidising the remainder with potassium permanganate. The cyanogen bromide method is then applied using a modification of the procedure recommended by L. J. Harris and W. D. Raymond (*Biochem. J.*, 1939, **33**, 2037), in which *p*-aminoacetophenone is employed as aromatic amine. It is claimed that this method of decolorisation is superior to that used by Perlzweig *et al*, being more expeditious and free from dependence upon the various physical properties of the adsorbing agent, although it is still found necessary to employ a blank.

thought to be almost specific has been proposed by Kodicek and Wang[15]. This is based upon the observation that when trigonelline is hydrolysed with alkali in alcoholic solutions methylamine is split off, whence the resulting product, now an open ring, can be combined with aromatic amines to give coloured compounds. Benzidine, which was found to be the most satisfactory amine, gives an orange-red derivative with the hydrolysed product. Pyridoxine (vitamin B_6), nicotinic acid, inpecotic acid and nicotine do not interfere and, so far as the originators of the method are aware, the only compound reacting similarly is N-methyl pyridinium hydroxide. It is claimed that 0·001 mg. of trigonelline per ml. of urine can be quantitatively determined and that recovery of the compound when added to urine approximated to 95 per cent. In typical samples of coffee and tea 7·5 mg. per g. and 0·13 mg. per g. of trigonelline, respectively, were found. The excretion of trigonelline by normal individuals on a coffee-free diet ranged from 10 to 16 mg. per day. After administering nicotinic acid the increased output of trigonelline varied from 10 to 28 per cent. of the ingested dose, depending upon the amount taken and the body weight of the subjects.

Method for Urine : Trigonelline[15]. To 10 ml. of the sample is added 0·5 ml. of a 40 per cent. aqueous solution of sodium hydroxide and the mixture heated on a boiling water-bath for 20 minutes. After allowing to cool, 1 ml. of concentrated hydrochloric acid, 0·4 ml. of a 10 per cent. aqueous solution of barium chloride, 40 ml. of alcohol (95 per cent.) and 0·5 g. of decolorising charcoal (norite) are successively added and the mixture shaken for 1 minute. After filtering, two 20-ml. portions of filtrate are separately introduced into two 50-ml. conical flasks ; to one flask is added 0·5 ml. of a standard trigonelline solution containing 50 mg. per ml. and to both mixtures 4·2 ml. of 40 per cent. solution of sodium hydroxide. The contents of the flasks are heated for 30 minutes on a boiling water-bath, then cooled, cautiously neutralised with hydrochloric acid and each liquid diluted to a volume of 30 ml. After centrifuging, separate 10-ml. portions of the liquid free from added trigonelline are each transferred to test tubes, one for the blank and the other for the unknown : the former tube should contain 1 ml. of 5 per cent. hydrochloric acid and the latter 1 ml. of a 1 per cent. solution of benzidine in 5 per cent. hydrochloric acid. After standing for 1 hour the intensity of the fully developed colour is measured by means of a suitable colorimeter or preferably with a Pulfrich photometer

using filter S50. The instrument is calibrated by similarly applying the test to the centrifuged liquid containing the known amount of added trigonelline.

Method for Food : Trigonelline[15]. The preliminary digestion with sodium hydroxide is omitted and 10 ml. of an extract of the material is treated with hydrochloric acid and barium chloride and the procedure conducted as described above.

REFERENCES

1. E. Vougerichten, *Ber.*, 1899, **32**, 2571.
2. S. P. Vilter, T. D. Spies and A. P. Mathews, *J. Biol. Chem.*, 1938, **125**, 85.
3. W. König, *J. prakt. Chem.*, 1904, **69**, 105.
4. G. E. Shaw and C. A. Macdonald, *Quart. J. Pharm.*, 1938, **11**, 380.
5. M. Swaminathan, *Nature*, 1938, **141**, 830.
6. R. P. Daroga, *J. Soc. Chem. Ind.*, 1941, **60**, 263.
7. W. A. Perlzweig, E. D. Levy and H. P. Sarett, *J. Biol. Chem.*, 1940, **136**, 729.
8. E. Bandier and J. Hald, *Biochem. J.*, 1939, **33**, 264.
9. H. P. Sarett, W. A. Perlzweig and E. D. Levy, *J. Biol. Chem.*, 1940, **135**, 483.
10. D. Melnick and H. Field, Jr., *J. Biol. Chem.*, 1940, **134**, 1.
11. D. Melnick and H. Field, Jr., *J. Biol. Chem.*, 1940, **135**, 53.
12. D. Melnick, W. D. Robinson and H. Field, Jr., *J. Biol. Chem.*, 1940, **136**, 131 and 145.
13. D. Melnick, W. D. Robinson and H. Field, Jr., *J. Biol. Chem.*, 1940, **136**, 157.
14. W. J. Dann and P. Handler, *J. Biol. Chem.*, 1941, **140**, 201.
15. E. Kodicek and Y. L. Wang, *Nature*, 1941, **148**, 23.

VITAMIN A

It has been known for many years that cod-liver oil contains a substance which gives a violet coloration with concentrated sulphuric acid and this colour reaction was included among the characteristic tests in the British Pharmacopœias of 1867, 1885 and 1898. In 1922 Drummond and Watson suggested that this colour probably indicates the presence of vitamin A and that the depth of colour is roughly proportional to the vitamin content as found by feeding experiments on rats[1]. The sulphuric acid test is, nevertheless, unsuitable for quantitative work as the colour produced is evanescent and liable to be masked by a variable amount of charring due to other constituents of the oil. In

searching for alternative condensing agents, Rosenheim and Drummond[2] found that arsenic trichloride produced a brilliant blue colour of an intensity approximately proportional to the values indicated by feeding tests. Shortly afterwards, F. H. Carr and E. A. Price proposed the use of antimony trichloride[3] which is soluble in chloroform and therefore offers a considerable advantage over the arsenic compound. This test has been widely employed and has given rise to an extremely extensive literature ; it was officially adopted in the British Pharmacopœia, 1932, but in recent years it has been partially supplanted by spectrophotometric measurement of the absorption at 325 mμ in the ultra-violet region of the spectrum and the latter has now become official, having been included in the First Addendum, 1936, to the B.P., 1932. However, although it has been established that substances besides vitamin A which give a blue tint in the antimony trichloride test may be present in liver oils and that other constituents may inhibit the full development of colour, the determination of "blue values" still provides useful information, readily differentiating between samples rich in vitamin A and those containing little or none. The test also serves for the preliminary determination of the strength of samples thus affording information as to the amount required to be taken in order that the absorption in the ultra-violet region may be satisfactorily photographed. Again, the colorimetric procedure is serviceable in laboratories lacking a quartz spectrograph and the other expensive equipment needed for taking spectrograms. It has long been known that vitamin A resides in the unsaponifiable portion of oils[4] and, except in the case of concentrates, it is generally necessary to apply the colour to the unsaponifiable portion of the material. The colour test has been adapted by Andersen and Nightingale to the determination of vitamin A naturally present in butter and that added to margarine : they express the results by application of a "dilution test" which takes the form of diluting the chloroformic solution of the unsaponifiable matter until the blue colour with antimony trichloride is only just visible.[5] Edisbury has proposed to deal with the same problem by applying the ordinary colour test to the unsaponifiable portion and measuring the light absorption of the blue reaction liquid at 620 mμ by means of a visual spectrometer with photometer[6]. The procedures given below comprise the application of the test to the examination of liver oils and concentrates ; slight modifications of the processes recommended by Andersen and Nightingale

and by Edisbury for the examination of butter and margarine.; and a description of the method recommended by Raynes and McLellan for assessing the vitamin A content of cocoa and chocolate[7].

The chemical constitution of vitamin A has been established by Karrer, Morf and Schopp[8]. In the animal kingdom it is formed by the breakdown of certain members of the carotenoid class, of which the most important is β-carotene. Symmetrical addition of two molecules of water at the central double bond and subsequent fission leads theoretically to the formation of two molecules of vitamin A from one of β-carotene. Unlike the latter, most carotenoids are unsymmetrical, but any molecule of this kind having at one end of the aliphatic chain a β-ionone grouping attached to the two necessary isoprene residues in the chain may, on similar hydrolysis, give rise to one molecule of vitamin A. On theoretical grounds such provitamins A have only half the biological activity of β-carotene but from a comparison of the spectrographic and biological results it appears, for some reason so far unexplained, that β-carotene itself is only half as active in animal metabolism as it would be if it were broken down into two molecules of vitamin A[9]. In any case, a biological claim to vitamin A activity may be based on the presence of carotenoids as well as on the vitamin itself[10]. The International Unit for vitamin A is equal to the biological activity of 0·6 microgram of the standard β-carotene kept for the Health Organisation of the League of Nations by the National Institute for Medical Research, Hampstead, London. This is issued as a solution in coconut oil, containing 500 units (300 μg.) per g. The first crystalline form of vitamin A to be produced was the β-naphthoate ester[11, 12] while vitamin A itself was prepared independently by Holmes and Corbet[13] and by T. H. Mead[12], the crystals containing combined methyl alcohol ; crystals of the actual vitamin, free from methyl alcohol of crystallisation and melting at 63° to 64° C., were first isolated by Baxter and Robeson[14, 15]. The factor to be adopted in order to convert physical or chemical results into International Units is the subject of some controversy. British investigators multiply observed extinction coefficients at 325 mμ ($E^{1\,cm.}_{1\%}$) by 1600 in order to convert to I.U. per g. while workers in the United States employ the factor 2000. The corresponding British factor for converting " blue values " as determined by the method below is 32. Baxter and Robeson[15] found an extinction coefficient of 1750 (in ethyl alcohol) for their crystalline vitamin A which, on

the basis of the computation current in the United States, corresponds to a conversion factor of 2460*.

Method (*for Liver Oils*). A solution of antimony trichloride in pure dry chloroform, saturated at 20° C., is required. To prepare this, ordinary chloroform, containing 1 to 2 per cent. of alcohol added as preservative, is washed three times with an equal volume of water and dried over anhydrous potassium carbonate. The liquid is then poured off from the solid alkali and, while being screened from light, is distilled, the first 10 per cent. of distillate being rejected. Antimony trichloride is then washed with some of the distilled chloroform until the washings are clear and is then used to prepare a solution containing not less than 21 per cent. w/v and not more than 23 per cent. w/v $SbCl_3$. The solution should be stored in a colourless glass bottle covered externally with opaque paint or paper. In order to ascertain that the concentration of the solution is correct 1 ml. is mixed with a solution of 2 g. of sodium potassium tartrate in 20 ml. of water, 2 g. of sodium bicarbonate added and the mixture titrated with 0·1N iodine. Each millilitre of 0·1N iodine is equivalent to 0·01141 g. $SbCl_3$.

To an accurately weighed quantity (about 1 g.) of the oil to be examined is added 20 ml. of alcohol (95 per cent.) and 4·4 ml. of a 60 per cent. w/w aqueous solution of potassium hydroxide and the mixture boiled for 5 minutes or sufficiently long to produce a clear liquid. The resulting soap solution is transferred to a separator, the flask rinsed with 40 ml. of peroxide-free ether (preferably freshly distilled from sodium) and the rinsings added to the alcoholic solution of soap which is then diluted with 60 ml.

* The factor 1600 was accepted in 1934 by the International Conference on Vitamin Standardisation (League of Nations Health Organisation). Notwithstanding this, many workers in the United States have favoured the factor 2000 and in an attempt to settle the question three collaborative investigations have been carried out. The first, on a halibut-liver oil, gave no grounds for departing from the figure of 1600 while the second, using the United States Pharmacopœia reference cod-liver oil, suggested the higher value of 1820. The third, based upon an examination of vitamin A β-naphthoate (*Nature*, 1943, **151**, 535) gave a figure of 1770; whence the average of the three figures is 1740. The discrepancy between the factor found in Great Britain and that found in the United States is probably due to the fact that most determinations in America are made by comparison with the United States Pharmacopœia original reference cod-liver oil of accepted value 3000 I.U. or U.S.P. Units per g. The value found for this oil by the second of the above-mentioned investigations was, however, only 2619, the ratio between this and its reputed value of 3000 being approximately equal to the ratio between 1740 and 2000. The value 2619 was obtained by comparison with the 1934 standard β-carotene whereas the higher figure was referable to the impure β-carotene of the 1931 International Standard. It seems probable that the confusion arose because the biological values of the two standards were at the time imperfectly related.

of water. A further 40 ml. of ether is added, the mixture shaken, the ether allowed to separate, the aqueous layer drawn off into another separator and extracted with two further portions of ether, each of 40 ml. The three separate ether layers are mixed, any additional soap solution which may have separated is drawn off, then the ethereal solution washed four times with four separate 50-ml. portions of water at 30° C. After separating as much water as possible from the ethereal liquid the latter is dried by the addition of a little anhydrous sodium sulphate then filtered into a distilling flask and the ether removed by distillation in an atmosphere of carbon dioxide or nitrogen. The use of porous material to promote steady boiling is not admissible. The unsaponifiable residue, while still being maintained in an inert atmosphere is dissolved in chloroform (ordinary B.P. quality) and diluted to an appropriate volume in a graduated flask, say, 25 or 50 ml.

In order to conduct the colour test, 0·2 ml. of this chloroformic solution is transferred to a 1-cm. all-glass cell, the latter placed on the platform of a Lovibond tintometer (The British Drug Houses pattern) and 2 ml. of the antimony trichloride reagent rapidly added. The blue colour, which quickly develops and then at once begins to fade, is matched at its maximum intensity using, besides the blue, the yellow or the red glasses and, if necessary, diminishing the transparency of the test colour by interposing neutral slides. Having made an approximation of the value of the maximum colour produced in terms of Lovibond units the test is repeated, several times if necessary, the volume of the chloroformic solution of the sample being so diluted that the tintometer reading lies between 4·0 and 6·0 blue. If 0·2 ml. of the solution under examination gives a colour less intense than 4·0 Lovibond blue units the test is repeated from the beginning using a larger sample of oil. The vitamin A potency is expressed as the number of Lovibond blue units given by 0·04 g. of the original sample in 0·2 ml. of solvent, the other components required for matching the actual observed colour being ignored. This Carr-Price blue value may be converted into International Units per g. (British computation) by multiplying by 32, but for refined whale products it is considered that the factor 23 gives figures more in line with the biological results[6].

(*for Concentrates*). It will generally be possible to apply the colour test direct without going through the process of isolating the unsaponifiable portion, the concentration of the chloroformic solution being so arranged that when 0·2 ml. is mixed with 2 ml.

of antimony trichloride reagent the intensity of the colour produced lies between 4·0 and 6·0 Lovibond blue units.

(*for Butter and Margarine : Dilution Test*)[5]. A weighed quantity of about 10 g. of the sample is saponified precisely in the manner described above for the examination of liver oils. The resulting alcoholic soap solution is diluted with water, cooled, made up to 100 ml. with more water and 25 ml. of the resulting liquid transferred to a separator. The unsaponifiable matter is isolated following the technique already given for the examination of liver oils, with the addition that the ether extract must be decolorised by treatment with charcoal; the dry residue is then dissolved in 2·5, 5, or 10 ml. of chloroform, according to the expected strength of the reaction. A preliminary colour test is applied by adding 1 ml. of the antimony trichloride reagent to 1 ml. of the prepared chloroformic solution. The latter is then diluted with more chloroform in successive stages until a solution is produced of which 1 ml. gives only a barely perceptible colour reaction when mixed with 1 ml. of the antimony reagent. The " dilution strength " is expressed as the volume in ml. to which the chloroformic solution of the unsaponifiable matter derived from 1 g. of sample must be diluted in order that the colour reaction may be only just visible. In order to convert " dilution strengths " into International Units per g. the results may be multiplied by 0·3 to 0·5 for preparations derived from fish liver oils or by 0·24 to 0·36 for refined whale products[6].

(*for Margarine : Using Spectrometer*)[6]. A weighed quantity of about 10 g. of the sample is saponified, the unsaponifiable material isolated and dissolved in chloroform exactly as described for the evaluation of liver oils except that the final chloroformic solution is diluted to 2·5 ml. The colour test is then conducted in a 1-cm. cell by adding to 0·4 ml. of the chloroformic solution 0·05 ml. of acetic anhydride and 4 ml. of the antimony trichloride reagent. The extinction coefficient at 620 mμ of the resulting colour is measured at the moment of its maximum intensity, the reaction mixture is then poured into a small tube and its colour allowed to fade during 25 minutes and then the extinction coefficient again determined at the same wavelength as before. The result is calculated in the manner illustrated by the following example :—

The unsaponifiable matter from 8 g. of vitaminised margarine was dissolved in chloroform, the solution diluted to 2·5 ml. and the colour test applied to 0·4 ml., the volume of the coloured

reaction mixture thus being made up to 4·4 ml. whence the original 400 per cent. solution of margarine is diluted eleven times.

Observed reading at maximum colour intensity : 1·20.
Observed reading after colour had faded : 0·25.
Whence nett $E^{1\text{ cm.}}_{620\ m\mu}$

$$=\frac{(1\cdot20-0\cdot25)\times 11}{400\times 1}=0\cdot0261 \text{ due to vitamin A.}$$

And multiplying this figure by 550 (Edisbury's original factor of 500 now being regarded as somewhat too low) gives a final result of approximately 14 International Units per g.

(*for Cocoa and Chocolate*)[7]. A weighed sample (5 g.) of the cocoa or chopped chocolate is treated in a beaker with 30 ml. of peroxide-free ether (preferably freshly distilled from sodium) and warmed for a few minutes until it can be disintegrated with a glass rod. About 2 g. of acid-washed kieselguhr is added, the mixture filtered by gentle suction through a sintered glass Gooch crucible (1G) previously prepared with a pad of asbestos about $\frac{1}{8}$ in. thick and covered with an equal thickness of kieselguhr. The beaker and crucible are washed with two 10-ml. portions of ether, the kieselguhr in the crucible is transferred to the beaker by means of a spatula, the asbestos being left intact, then more ether added to the beaker and the mixture filtered through the same crucible. A glass bead is added to the filtrate, the solvent distilled off, 15 ml. of 0·5N alcoholic potassium hydroxide added, the liquid gently boiled under a reflux condenser for 20 minutes then 15 ml. of water added and the mixture transferred to a separator using 20 ml. of water for rinsing the flask. After allowing to cool, about 50 ml. of peroxide-free ether is added to the flask then transferred to the separator, the latter shaken and, after separation has occurred, the ether transferred to another separator using a Röse-Gottlieb syphon-tube arrangement in conjunction with a blow-ball. The extraction is repeated twice using 40 ml. of ether on each occasion. The united ethereal extracts are washed with 15-ml. portions of water until the ether is clear and the aqueous layer neutral to phenolphthalein. The separated ether is filtered through a dry 7-cm. paper (Whatman No. 41) into a dry flask containing a glass bead, the separator and filter are washed with ether which is added to the main filtrate, the solvent removed by distillation and the interior of the flask then dried by continuing the gentle heating under reduced pres-

sure; while the latter is still maintained, the flask is allowed to cool, the unsaponifiable matter dissolved in chloroform (for a chocolate expected to contain 3000 I.U. per oz. 20 ml. of solvent should be used) and the colour test applied to 0·2 ml. of the solution as already described on p. 353.

Discussion. In conducting the Carr-Price test it is most important that all trace of moisture be excluded. The antimony trichloride reagent is hygroscopic, besides being somewhat corrosive, and it is therefore an advantage to use a storage and delivery apparatus such as that designed by G. Middleton[16] and illustrated in Fig. 15.* In measuring the colour by means of the tintometer either daylight or an artificial source of light may be used but, in either case, it is important that a difference of 0·1 blue unit should be perceptible. In order to avoid ocular fatigue when taking readings it is an advantage to use each eye alternately and it is advisable to avoid looking down the eyepiece of the tintometer when the reagent is being poured into the cell since the deeper blue colour initially produced may make it difficult to effect a final match. It may be noted that the British Pharmacopœia, 1932, originally specified that cod-liver oil should be examined for vitamin A by applying the colour test direct without preliminary isolation of the unsaponifiable matter and stated that when so tested 0·04 g. should give a blue colour not less saturated than that of a blue glass standardised to have the following properties on the system of colour measurement adopted at the National Physical Laboratory, Teddington :—

Colour Quality : 0·137 R+0·271 G+0·592 B.
Photometric Transmission : 34·0 per cent.

Where R, G and B respectively denote the colours of monochromatic radiations of wavelengths 700 mμ, 546 mμ and 436 mμ, the measurements being made with the N.P.L. standard white light.

Such a blue glass would correspond to 6·0 units on the Lovibond scale. In the First Addendum, 1936, the vitamin A in cod-liver oil (which should contain not less than 600 International Units per g.) is directed to be determined by application of the spectrophotometric method to the unsaponifiable portion or, alternatively, by a biological method.

* An apparatus, made to a slightly modified and improved design (Reg. No. 756296), is marketed by The British Drug Houses Ltd., under the name, The B.D.H. Automatic Pipette.

In a study of the relation between the results of the Carr-Price test and the absorption coefficients of vitamin A preparations,

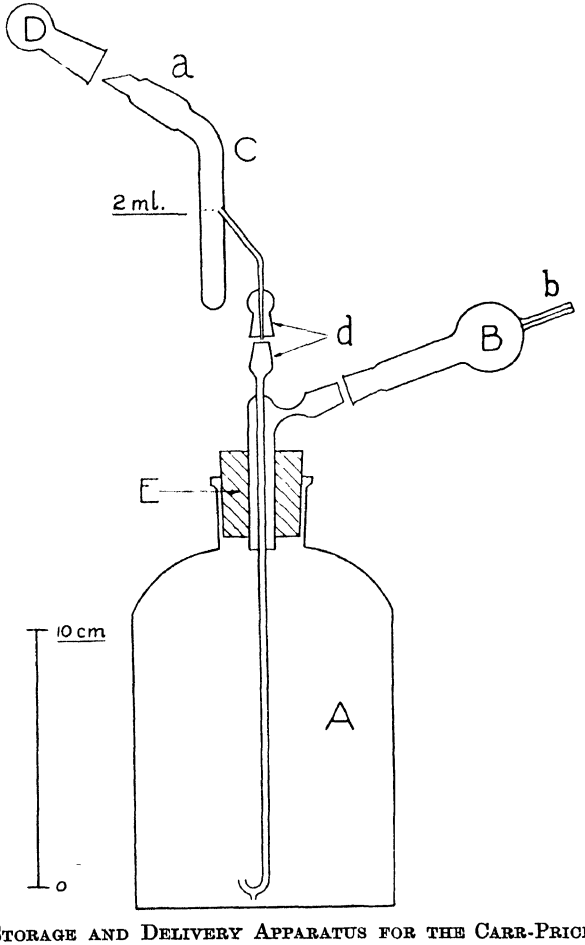


FIG. 15. STORAGE AND DELIVERY APPARATUS FOR THE CARR-PRICE REAGENT

The tube B is filled with anhydrous calcium chloride and the 600-ml. paper-covered bottle A with reagent. When required for use, the cap D is removed from the ground taper, *a*, and the measuring tube C filled by blowing air through B by means of a rubber bulb attached at *b*. The excess of liquid in C runs back on releasing the pressure : the measuring tube is then detached at the ground joint *d* and the 2 ml. of reagent poured out. When not in use the cap D should be replaced and the rubber bulb detached. If the ground joints become cemented together by action of the reagent they should be freed by soaking in hydrochloric acid but if the apparatus is carefully handled this will not be necessary. The bung E consists of a closely fitting cork and should not be composed of rubber. (After G. Middleton, *Analyst*, 1931, **56**, 236.)

S. K. Crews and S. J. Cox quote direct blue values for medicinal cod-liver oils varying from 7·2 (15·0 when conducted on the un-

saponifiable matter) to 32·0 (46·0 on unsaponifiable)[17]. While a comparison of the blue value with the results of spectrophotometric and biological tests showed considerable variation with cod-liver oils, this did not apply to purified preparations of vitamin A. Remarkable changes were observed to occur on storage : a freshly prepared oil may have a negligible blue value which then rises, at first quickly, then more gradually to a peak value and, finally, with age, generally falls again. Although other chromogenic substances which give a blue colour by the Carr-Price test are often present in cod-liver oils, these investigators never found an oil of which the blue value was above what might be expected from the absorption at 325 to 328 mμ and they suggest that the inhibiting substances present cause a greater depression of the blue colour than any increase due to chromogenic substances other than vitamin A. From this it follows that the figures given by the colorimetric method indicate the minimum content of vitamin A.

In an examination of a hundred samples of medicinal cod-liver oil Andersen and Nightingale[5] found the average " dilution strength " to be 1950, the corresponding value for the unsaponifiable part of the same oils ranging up to 1 million while for butter it was found to lie between 25 and 60.

Of his spectrometer method for the evaluation of vitaminised margarines Edisbury[6] writes : " At its best, the test amounts in practice to a determination of the final dilution required to produce a standard effect, and proportionality is not stretched too far. In this respect the method resembles the ' dilution test,' but has the advantage that bleaching with charcoal can be avoided. We have found it exceedingly difficult to prevent a loss of 10 to 15 per cent. of the total vitamin A as a result of even the most cautious bleaching. The reaction colour of the unbleached material will probably not be blue, owing to the antimony trichloride reacting with the dye to give a relatively stable red complex ; but as a rule the normal 620 mμ band is sufficiently separated from the other absorption to make estimation to about ±15 per cent. possible ; carotenoids (up to more than the vitamin content) can be ignored."

In order to convert the results of the Carr-Price test as applied to cocoa and chocolate into International Units, Raynes and McLellan[7] convert readings into blue units per g. and multiply by 1·41 which is equivalent to multiplying the " blue value " by 35·25.

REFERENCES

1. J. C. Drummond and A. F. Watson, *Analyst*, 1922, **47**, 341.
2. O. Rosenheim and J. C. Drummond, *Biochem. J.*, 1925, **19**, 753.
3. F. H. Carr and E. A. Price, *Biochem. J.*, 1926, **20**, 497.
4. T. T. Cocking and E. A. Price, *Yearb. Pharm.*, 1926, 522.
5. A. Andersen and E. Nightingale, *J. Soc. Chem. Ind.*, 1929, **48**, 139T.
6. J. R. Edisbury, *Analyst*, 1940, **65**, 484.
7. E. G. Raynes and B. G. McLellan, *Analyst*, 1943, **68**, 109.
8. P. Karrer, R. Morf and K. Schoop, *Helv. Chim. Acta*, 1931, **41**, 1036 and 1431.
9. R. A. Morton, *Chem. & Ind.*, 1940, **59**, 301.
10. H. E. Monk, *Analyst*, 1942, **67**, 83.
11. S. Hamano, *Sci. Pap. Inst. Phys. Chem. Res., Tokyo*, 1935, **28**, 69.
12. T. H. Mead, *Biochem. J.*, 1939, **33**, 589.
13. H. B. Holmes and R. E. Corbet, *J. Amer. Chem. Soc.*, 1937, **59**, 2042.
14. J. G. Baxter and C. D. Robeson, *Science*, 1940, **92**, 203.
15. J. G. Baxter and C. D. Robeson, *J. Amer. Chem. Soc.*, 1942, **64**, 2411.
16. G. Middleton, *Analyst*, 1931, **56**, 236.
17. S. K. Crews and S. J. Cox, *Analyst*, 1934, **59**, 85.

VITAMIN B_1 (ANEURINE OR THIAMIN)

The systematic chemical name for this vitamin is 3-(4′-amino-2′-methyl - pyrimidyl - 5′ - methyl) - 4 - methyl - 5 - β - hydroxyethyl-thiazolium chloride hydrochloride. It is officially referred to in Britain as Aneurine Hydrochloride (British Pharmacopœia, 1932, Third Addendum, 1941) but is also known as aneurine chloride hydrochloride or simply as aneurine. In the United States the several terms thiamin chloride hydrochloride, thiamin hydrochloride and thiamin are used. The colorimetric determination has been extensively developed and certain of the more recent procedures are fairly satisfactory. The earliest proposal due to Kinnersley and Peters[1, 2], depended upon the formation of a pink colour on treatment of the vitamin with an alkaline solution of benzenediazonium chloride *p*-sulphonic acid in the presence of formaldehyde. Another suggestion depended upon dye formation using 2 : 4-dichlorbenzenediazonium chloride[3] but the most successful method of this type was proposed by Prebluda and McCollum[4, 5] who used diazotised *p*-amino-acetophenone and extracted the red dyestuff with xylene. This procedure, which has been further perfected by Melnick and Field[6], is not sufficiently sensitive for most purposes connected with the examination of food, although it is valuable for the determination of vitamin B_1

in tablets and solutions intended for injection[7]. An alkaline solution of ferricyanide converts the vitamin into the highly fluorescent compound thiochrome[8] and Jansen[9] recommended the application of this reaction for analytical purposes. This suggestion has been widely adopted and a large number of investigators have published modified procedures with their special application to particular purposes : many of these have included isolation of the vitamin by adsorption on fuller's earth or Franconite while Cerecedo and his collaborators recommended separation by application of the principle of base exchange using synthetic zeolite[10–12]. Latterly, it has been found that, in the case of foodstuffs, the inclusion of these expedients is inclined to lead to low results and that it is better to remove interfering proteins by preliminary treatment, either with pepsin[13] or papain[14]. Besides being present as aneurine hydrochloride, vitamin B_1 occurs as cocarboxylase which Lohmann and Schuster proved to be a pyrophosphoric ester of the parent substance[15, 16]. Since, physiologically, cocarboxylase behaves similarly to aneurine, provision for its determination is made by applying a preliminary hydrolysis, most usually with taka-diastase[2], although Hennessy and Cerecedo[17] used an enzyme preparation derived from beef kidney and Conner and Straub favour the use of clarase[18].

The descriptions which follow include the diazotisation technique[6] as applied by Auerbach[7] to the determination of vitamin B_1 in tablets and solutions for parenteral administration : the thiochrome method as developed by Harris and Wang[14, 19] for yeast, yeast extracts, vegetables and milk products ; by Dawson and Martin[20, 21] for bread ; and by Egaña and Meiklejohn[22] for urine. In addition, a procedure which has been published at the instance of the Ministry of Food for the assay of fortified white flour[23] is given and also an account of its application to National wheatmeal[24].

Diazotisation Method (*for Injections and Tablets*)[7]. The diazo reagent is made by mixing 10 ml. of a 0·03 per cent. solution of *p*-amino-acetophenone in 0·2N hydrochloric acid with 2 ml. of a freshly prepared 0·1 per cent. aqueous solution of sodium nitrite, cooling the mixture in ice-water for 3 minutes and, just before use, adding 3 ml. of 2·5N sodium hydroxide. The injection solution to be tested is diluted with acidulated water (1 drop of dilute hydrochloric acid in 100 ml.) so that the dilution may be expected to contain about 0·07 mg. per ml. of vitamin B_1, 1 ml. is mixed with 2 ml. of ethyl alcohol (50 per cent. by volume) and

the tube containing the mixture immersed in a water-bath at 60° C. After warming for a minute, 1 ml. of the diazo reagent is added, the mixture warmed for a further 3 minutes then allowed to cool, acidified with dilute hydrochloric acid and shaken with 10 ml. of toluene. A portion of the red-coloured toluene layer is poured off into a dry test tube and, after shaking with a little anhydrous sodium sulphate, the tint is compared with that produced by standards similarly produced from crystalline aneurine hydrochloride or, alternatively, the colour of the toluene layer may be measured by means of a Lovibond tintometer using a 1-cm. cell and the amount of vitamin present in 1 ml. of the diluted injection solution correlated with the red component by reference to a graph constructed in accordance with the data presented in Table XXXII.

TABLE XXXII. RELATION BETWEEN THE QUANTITY OF VITAMIN B_1 AND THE COLOUR PRODUCED (DIAZOTISATION METHOD)

Quantity of Vitamin B_1 mg.	Colour observed in 1-cm. cell: Lovibond Units		
	Red	Yellow *	Brightness *
0·02	0·7	0·1	—
0·03	1·1	0·2	0·1
0·04	1·5	0·3	0·2
0·05	2·1	0·3	0·2
0·06	2·6	0·3	0·3
0·07	3·0	0·1	0·1
0·08	3·5	0·4	0·4
0·09	4·1	0·5	0·6
0·10	4·5	0·7	0·7

* These values are subject to slight variation: they are included as guides to accurate matching, but only the figures for the red component are considered in the calculation of results. As the colour produced is brighter than the Lovibond glasses it is necessary to dull it down by means of the neutral tints.

Tablets may be similarly assayed by first triturating with warm ethyl alcohol (50 per cent. by volume) and diluting the mixture with the same solvent so that 1 ml. may be expected to contain about 0·07 mg. per ml. The extract thus produced is cleared by centrifuging, 1 ml. mixed with 2 ml. of ethyl alcohol (25 per cent. by volume) and the assay continued as described for the injection solution.

Thiochrome Method (*for Yeast and Related Products*)[14, 19]. The sample is ground to as fine a state as possible and 1 or 2 g. is

heated with 15 ml. of acidulated water (4 drops of concentrated hydrochloric acid per 100 ml.) at 100° C. for 10 minutes with continual stirring. To the suspension, 5 ml. of 0·1M sodium acetate-acetic acid buffer solution of *p*H 4·0 is added and the mixture allowed to cool to 40° C. The reaction of the suspension is checked with bromocresol green as external indicator and, if necessary, adjusted to *p*H 4·0, then 0·1 g. each of papain and taka-diastase added, followed by 0·5 ml. of toluene and the mixture maintained for 16 to 18 hours at a temperature of 40° to 45° C. After the digestion, the mixture is diluted with water to a suitable volume (say, 50 ml. for a specimen of dried brewers' yeast of an average potency of 20 International Units per g.) then centrifuged, 5 ml. of the clear supernatant liquor transferred to another centrifuge tube containing 5 drops of a 6 per cent. solution of hydrogen peroxide, 5 ml. of *iso*-butyl alcohol added, the mixture shaken, centrifuged and the separated *iso*-butyl alcohol discarded. Two portions (1 or 1·5 ml.) of the aqueous layer are transferred to 25-ml. glass-stoppered cylinders and water is added to a volume of 1·5 ml. if necessary. In a third cylinder, 1 ml. of standard vitamin B_1 solution is diluted to 1·5 ml. with water. Into each cylinder is introduced 2 ml. of methyl alcohol and 1 ml. of a 20 per cent. w/v aqueous solution of sodium hydroxide, and after quickly mixing, a freshly prepared 2 per cent. aqueous solution of potassium ferricyanide is added immediately, drop by drop, to one of the cylinders containing an extract of the sample and to the cylinder containing the standard, until the yellow colour of the ferricyanide persists for more than 30 seconds. The mixtures are then each shaken with a 10-ml. portion of *iso*-butyl alcohol for 2 minutes, allowed to separate, the aqueous layers removed and discarded and 3 ml. of water introduced into each cylinder and the contents shaken. The three *iso*-butyl alcohol layers are transferred to dry conical flasks, to each of them is added 2 ml. of ethyl alcohol (95 per cent.) and 10 ml. of the alcoholic mixture derived from the sample under test and 10 ml. of that from the blank are transferred to separate test tubes and the bluish fluorescence of the sample matched visually in ultra-violet light (filtered through a nickel oxide glass screen) by adding to the blank by means of a burette the alcoholic mixture derived from the standard. In this way the free aneurine together with the cocarboxylase is measured.

In order to determine aneurine and its phosphorated derivative separately, an extract of the sample is prepared as above, the

digestion being omitted and the solution heated in a boiling water-bath for 5 minutes in order to inactivate phosphatases. One portion of this extract is assayed to give the free aneurine and the other portion is digested with taka-diastase and papain as before to estimate the total free and combined vitamin.

A standard stock solution is made containing 20 μg. of aneurine hydrochloride per ml. using water acidified with a drop of hydrochloric acid : a portion of this is diluted as required to a concentration of 4 μg per ml. and 1 ml. is used to prepare the standard fluorescent *iso*-butyl alcohol for each test. The stock solution will keep in good condition for a few days provided it is protected from light and stored in a refrigerator. Aneurine hydrochloride conforming to the Third Addendum, 1941, of the British Pharmacopœia, 1932, contains 320,000 International Units per g.

(*for Yeast Extracts*)[14, 19]. The extract is diluted until the concentration of vitamin B_1 is approximately 0·5 to 1·0 μg. per ml., the reaction is adjusted to *p*H 4·0 with acetate buffer or hydrochloric acid and 1 ml. of this material is diluted with water and examined by the method already described for yeast commencing with the addition of 0·1 g. each of papain and taka-diastase.

(*for Vegetables and Plant Tissues Generally*)[14, 19]. About 10 g. of the sample is ground to a fine pulp in a mortar with 2 to 4 drops of concentrated hydrochloric acid, water is added until the total volume lies between 30 and 40 ml., the suspension transferred to a conical flask and the latter immersed in boiling water for 10 minutes. After allowing to cool the reaction is adjusted to *p*H 4·0 and the determination continued as already described, commencing with the addition of 0·1 g. each of papain and taka-diastase.

(*for Milk, Dried Milk and Egg Products*)[14, 19]. Dried milk is first diluted by mixing 5 g. with about 30 ml. of water while, for milk itself, from 20 to 30 ml. is taken for the test without preliminary dilution. In either case the reaction is adjusted to *p*H 4·0 and the determination continued as above.

(*for Bread*)[20, 21]. Wherever possible six loaves should be cut into six equal pieces and a composite loaf withdrawn which is made up of a different portion from each of the original loaves. This composite loaf is weighed, cut into approximately half-inch cubes, air-dried, re-weighed, and ground in a small laboratory grinding mill. The drying operation may be accelerated by a small fan if necessary, but care must be exercised in order to avoid

loss of bread during the period between the weighing of the original six pieces of bread and the semi-dry cubes, so that the determined loss in weight represents only loss of moisture. When prepared in this manner, powdered bread will be found to contain about 12 per cent. of moisture and reference samples can be stored in stoppered bottles for at least 3 months. To 100 ml. of a 0·1 per cent. solution of pepsin in dilute hydrochloric acid (3·3 ml. of concentrated acid per litre) is added 20 g. of the dried bread meal and the suspension maintained at a temperature of 37° C. The reaction is adjusted to *p*H 2·0 after 2 hours, the incubation continued for a total of at least 8 hours and then 10 ml. of 2M sodium acetate-acetic acid buffer solution (*p*H 4·6) and 0·1 g. of taka-diastase added and incubation at 37° C. continued for a further 4 hours. The suspension is then diluted with water to 150 ml., centrifuged and 1·5 ml. of the separated liquid submitted to the thiochrome test as described above.

(*for White Flour*)[23]. A weighed quantity of the flour (10 g.) is made into a cream with 40 ml. of dilute hydrochloric acid (the strength may lie between 0·33 per cent. and 2·5 per cent.) and allowed to stand with frequent shaking for at least 30 minutes. A portion of the mixture is centrifuged until the supernatant liquor is no more than cloudy and contains only a negligible amount of suspended matter. Into each of two 25-ml. graduated cylinders is pipetted 3 ml. of the extract as cleared by centrifuging followed by 2 ml. of methyl alcohol and, with constant agitation, 2 ml. of 30 per cent. w/v aqueous solution of sodium hydroxide. To one of the cylinders is added immediately 2 ml. of 0·95 per cent. aqueous solution of potassium ferricyanide and the whole well mixed. After the lapse of 1 to $1\frac{1}{2}$ minutes the contents of the cylinders are made up to 10 ml. with water and 13 ml. of dry *iso*-butyl alcohol added, the contents then centrifuged and 10 ml. of the *iso*-butyl alcohol layer from both the blank and the oxidised tests pipetted into fluorescence-free glass test tubes containing 1 ml. of ethyl alcohol. The tubes are compared in ultra-violet light (filtered through a nickel oxide glass screen) and additions of a standard thiochrome solution made to the blank until its fluorescence matches that of the oxidised mixture. Matching may be facilitated by viewing the tubes through a Wratten 18A filter. The standard thiochrome is prepared by mixing 1 ml. of a stock solution of aneurine hydrochloride (30 mg. in 1 litre of water acidulated with a few drops of hydrochloric acid) with 2 ml. of methyl alcohol and adding with constant agitation 2 ml. of a

30 per cent. w/v aqueous solution of sodium hydroxide : to this is added 2 drops of a 0·95 per cent. aqueous solution of potassium ferricyanide and the liquid shaken. After 1 to $1\frac{1}{2}$ minutes 10 ml. of *iso*-butyl alcohol is added, the mixture very well shaken, allowed to separate and the lower aqueous layer removed by means of a fine glass tube and discarded. To the *iso*-butyl alcohol is added a little ethyl alcohol as a clearing agent and the volume is made up to 30 ml. with more *iso*-butyl alcohol. The strength of this standard thiochrome solution is such that 1 ml. represents 1 μg. of aneurine.

In calculating results, if it be supposed that n grams of flour be represented by 1 ml. of the centrifuged extract and that s millilitres of standard thiochrome solution are required by the blank to match the fluorescence of the oxidised solution then, since 3 ml. of the centrifuged extract are used in the test and the volume of the final *iso*-butyl alcohol solution is 16·5 ml. of which 10 ml. is taken for matching, we have :

$$\text{International Units per g. of sample} = s \times \frac{16{\cdot}5}{10} \times \frac{1}{3n} \times \frac{1}{3} = \frac{0{\cdot}183s}{n}$$

In order to arrive at the amount of flour which is represented by 1 ml. of centrifuged extract it is necessary, when establishing the method for the first time, to perform a special experiment. Duplicate samples of a flour are weighed out and subjected to the adopted technique, but in the extraction acid of one sample is included a known amount of aneurine, say, 1 μg. per g. of flour used. From the difference found in the aneurine contents of the aliquot portions of the two extracts it is possible to calculate the amount of flour represented by 1 ml. of centrifuged extract. The figure thus obtained is independent of the sample of white flour used and can be regarded therefore as a constant in all subsequent work.

(*for National Wheatmeal*)[24]. The method described above for the determination of vitamin B_1 in white flour is applied using 42·5 ml. of a 2 per cent. solution of hydrochloric acid per 10 g. of sample. An equal quantity of each sample is made up in exactly the same way but with the addition of 10 μg. of aneurine hydrochloride (i.e. 1 μg. per g. of flour) contained in the 42·5 ml. of acid. Should there be any interfering fluorescence, as sometimes happens when the meal is made from grist containing a large proportion of Durum wheats, the acid digest should be submitted to a preliminary extraction with *iso*-butyl alcohol as

described in the method given above for the examination of yeast and related products.*

(*for Urine*)[22]. A 24-hour sample is collected with 10 ml. of glacial acetic acid as a preservative. In order to determine the content of vitamin B_1, 12 ml. of the acidified sample is pipetted into a centrifuge tube fitted with a ground-glass stopper and shaken with 10 ml. of *iso*-butyl alcohol. The mixture is centrifuged, the alcoholic layer discarded and a 9-ml. portion of the aqueous liquid is removed and diluted with 10 ml. of water. The reaction is adjusted to *p*H 4·2 to 4·5 with 0·2N acetic acid and the solution is poured into a narrow Gooch crucible containing 0·2 g. of Decalso brand of refined zeolite,† 60 to 90 mesh, which has previously been treated with 10 ml. of 1 per cent. acetic acid. The liquid is drawn through by gentle suction, the zeolite washed with 10 ml. of a 1 per cent. solution of acetic acid, then transferred to a centrifuge tube and the walls of the crucible are washed with 2 ml. of a 25 per cent. aqueous solution of potassium chloride, the washings being transferred to the same tube. The suspension of Decalso is shaken in order to complete the elution and from 0·2 to 0·5 ml. of 0·5 per cent. potassium ferricyanide solution is added, depending upon the amount of aneurine expected to be present, followed by 1 ml. of 20 per cent. w/v

* The procedure recommended for the determination of vitamin B_1 in National Flour and Bread by the Vitamin B_1 Sub-Committee appointed by the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute (*Biochem. J.*, 1943, **37**, 433), is essentially the same as that described above for Yeast and Related Products. The strength of the hydrochloric acid used for the preparation of the suspension is specified as 0·02N and when 2 g. of flour or bread is taken, the mixture is diluted to 25 ml. after enzymatic hydrolysis. An alternative procedure, for flour only, consists in creaming 2 g. of the sample with 25 ml. of 2 per cent. solution of hydrochloric acid, allowing to stand for 16 to 18 hours at room temperature and centrifuging until the supernatant liquid is practically clear. Before washing with *iso*-butyl alcohol, one drop of a 10 per cent. solution of hydrochloric acid is added, while the treatment with hydrogen peroxide is deferred until after the development of the thiochrome, when the mixture in each tube is treated with 0·25 ml. of a 6 per cent. solution of hydrogen peroxide and allowed to stand for 30 to 60 seconds, followed immediately by the addition of the 10 ml. of *iso*-butyl alcohol. In calculating the result, allowance is made for the change in volume during the preliminary washing with *iso*-butyl alcohol, the results being calculated in accordance with the following formula :—

$$\frac{4x}{10}\times\frac{25}{1{\cdot}5\times 2}\times\frac{r_2}{r_1}=\frac{10xr_2}{3r_1}\ \mu\text{g. aneurine per g.}$$

where x is the volume in ml. of the standard thiochrome extract required and r_1 and r_2 are the volumes in ml. of the extracts respectively before and after the preliminary washing. It is stated that "it is advisable to use a Wratten 18A or similar filter in conjunction with Wood's glass to reduce errors due to substances with fluorescences of tints other than the purplish-blue tint of thiochrome."

† This brand is manufactured by The Permutit Company Limited, Gunnersbury Avenue, Chiswick, London, W.4.

sodium hydroxide solution. If the yellow colour disappears rapidly, potassium ferricyanide solution should be added, drop by drop, until a slight yellow colour persists. *iso*-Butyl alcohol (4·9 ml.) is then added, the mixture vigorously shaken for 1 minute, the layers separated by centrifuging, 2 ml. of the separated *iso*-butyl alcohol transferred to a test tube and the bluish fluorescence produced on exposure to filtered ultra-violet light is compared visually with the fluorescence of standard solutions of thiochrome in the same solvent. In the case of urines from patients suffering from deficiency of vitamin B_1, a known amount of aneurine hydrochloride should be added to a duplicate sample and the figure obtained for the original amount present corrected in accordance with the percentage proportion of added material which is recovered. If such control experiments cannot be conducted a correction should be applied based upon the assumption that, owing to the presence of interfering substances only 75 per cent. of the aneurine is recovered. In the case of normal urines it is not necessary to apply any correction.

Discussion. Of biologically important compounds which react with diazotised *p*-amino-acetophenone, histamine, histidine and inositol give orange colours, while tyrosine and adrenaline form reddish compounds but none of these coloured derivatives is extracted by xylene. An alternative proposal for the determination of aneurine depending upon the diazo reaction and using the trichloracetate of ethyl *p*-aminobenzoate[25] was not found by the present author to be so effective as the method using *p*-amino-acetophenone.

In conducting the thiochrome test it is important to ensure that the *iso*-butyl alcohol employed is itself non-fluorescent in ultra-violet light which has been filtered through a Wood's glass window. Thiochrome is somewhat unstable in ordinary light and very unstable in ultra-violet light when in solution in *iso*-butyl alcohol. It is desirable, therefore, to minimise as far as possible the time taken in matching the fluorescence : if the preliminary approximation is completed rapidly, then the relatively rather long exposure to light required to complete the balancing will not appreciably disturb the result since destruction of thiochrome will be nearly equal in the tubes concerned. In the procedure evolved by Harris and Wang[14, 19] the preliminary extraction with *iso*-butyl alcohol of the test solution derived from pre-treatment of the sample helps to remove any interfering substances which might otherwise give a fluorescence. The final solutions of thio-

chrome in *iso*-butyl alcohol are washed with water in order to remove traces of thiochrome pyrophosphate which may otherwise be present. Some destruction of aneurine occurs if the addition of the ferricyanide solution is delayed for more than 2 minutes. While some investigators measure the intensity of the fluorescence by means of a Spekker fluorimeter employing a solution of quinine sulphate as standard, direct visual matching is preferred since errors due to instability of thiochrome in ultra-violet light are less pronounced and a distinction can be made between the specific blue fluorescence due to thiochrome and any non-specific yellow fluorescence. The effect of the latter is conveniently eliminated by viewing the tubes through a Wratten 18A filter while it may sometimes prove to be an additional assistance to interpose a Wratten 49A between the tubes and the source of light. According to Harris and Wang[14] this procedure gives results closely agreeing with those obtained biologically except in cases where the vitamin content is low, when variations of more than 15 per cent. may occur. Some results referable to the aneurine content of foodstuffs obtained by these investigators are presented in Table XXXIII.

The method given above for the extraction of the vitamin from bread is an elaboration of the procedure proposed by Pyke[13] and follows the recommendation of Johannson and Rich[26] by including an adjustment of the reaction after an initial period of incubation in acid-pepsin. In their work on the determination of vitamin B_1 in bread Dawson and Martin[20, 21], to whom the technique given above is due, adopted Pyke's[13] method for conducting the final thiochrome test, but since the latter differs only in minor details from the more recent and somewhat improved technique of Harris and Wang[14, 19] it has not been reproduced in the above account. Dawson and Martin[21] conducted a number of experiments with the object of determining the average loss of vitamin B_1 during the baking of bread and found for the white variety a loss of 22 per cent. of the total vitamin B_1 when aneurine was added to the flour, or 20 per cent. loss if added as a solution to the dough. The baking loss rose to 27 per cent. in the case of bread made with National wheatmeal but was only 19 per cent. when using germ meal (Hovis). In an experiment in which white bread was fortified by use of a B_1-rich yeast the loss of added vitamin was only 8 per cent. The addition to flour of 14 oz. of calcium carbonate per sack (280 lb.) did not exercise any influence upon the loss of vitamin during baking.

TABLE XXXIII. A SELECTION OF RESULTS ILLUSTRATIVE OF THE VITAMIN B_1 CONTENT OF CERTAIN FOOD PRODUCTS (After L. J. Harris and Y. L. Wang, *Biochem. J.*, 1941, **35**, 1050)

Product	Content of Vitamin B_1 I.U. per g.
Yeast, high potency: dried	200
,, ,, 74 per cent. water	57
,, brewers' (Guinness)	12
,, extracts (various samples)	4·8 to 13·4
Pork, raw dried powder	7·7 to 8·1
,, roast, dried powder	5·1
Bacon	4·0
Ham, sliced and canned	0·48
Flour, wholemeal	1·2
,, straight run, bleached	0·29
Bread, retail	0·22 to 0·24
,, fortified	0·4 to 0·7
,, yeast enriched	0·70 to 1·49
,, whole wheat, dried powder	0·87
Biscuits, wheatmeal	0·6 to 1·4
Barley, meal	0·43 to 0·67
,, pearl	0·44
Spinach, dried powder	1·53
Orange Juice, partly evaporated	0·66
,, dried	5·4
,, fresh	0·17
Potatoes	0·30 to 0·57
,, canned	0·12
Grass	0·52
Brussels Sprouts	0·28
Carrots, canned	0·17
Cauliflower	0·26
Beetroot, canned	0·12
Egg, yolk	0·50 to 0·54
,, whole	0·30
Milk, cows'	0·10 to 0·15
Liver, beef: roasted, dried powder	0·7
,, ,, raw, dried powder	0·93 to 1·10
,, sheep: raw, fresh	0·65 to 1·40
,, calf: raw, fresh	0·35 to 0·75
Herring, muscle, raw, fresh	0·03

The procedure outlined above for the determination of vitamin B_1 in flour was published at the request of the Ministry of Food and is the coordinated work of representative chemists of the Government Laboratory, the Research Association of British Flour Millers, the Dover Laboratories and the Aynsome Laboratories. The original Report includes details for matching the thiochrome fluorescence by the photo-electric method using a Spekker fluorimeter against quinine sulphate standards which are stronger than the unknown. According to Wokes the fading of

thiochrome solutions and standards can be retarded by the use of specially constructed cells whereby the liquids are maintained under an atmosphere of nitrogen[27]. In Britain, flour is fortified by the addition of 0·2 g. of aneurine hydrochloride per sack (280 lb.). It has been shown by J. J. C. Hinton[28], and independently by A. H. Ward[29], that the part of the wheat germ lying between the embryo and the endosperm and known as the scutellum contains a much higher concentration of vitamin B_1 than any other portion of the grain. Thus, in one particular experiment in which the whole wheat contained 1·21 I.U. per g., the scutellum, comprising 0·63 per cent. of the total weight of the grain, was found to contain 49·4 I.U. per g.[29].

The method of Harris and Wang for the examination of yeast and foodstuffs is directly applicable to the determination of vitamin B_1 in urine[30] but the alternative method reproduced here may prove to be more serviceable in the treatment of samples containing a high proportion of interfering substances. Whichever method be adopted it is generally advisable to run control determinations on material containing a known amount of added aneurine so that, if necessary, a correction for incomplete recovery can be applied.

References

1. H. W. Kinnersley and R. A. Peters, *Biochem. J.*, 1934, **28**, 667.
2. H. W. Kinnersley and R. A. Peters, *Biochem. J.*, 1938, **32**, 1516.
3. H. Willstaedt and F. Barany, *Enzymologia*, 1938, **2**, 316.
4. H. J. Prebluda and E. V. McCollum, *Science*, 1936, **84**, 488.
5. H. J. Prebluda and E. V. McCollum, *J. Biol. Chem.*, 1939, **127**, 495.
6. D. Melnick and H. Field, Jr., *J. Biol. Chem.*, 1939, **127**, 505, 515 and 531.
7. M. E. Auerbach, *J. Amer. Pharm. Assoc.*, 1940, **29**, 313.
8. G. Barger, F. Bergel and A. R. Todd, *Ber.*, 1935, **68**, 2257.
9. B. C. P. Jansen, *Rec. Trav. chim. Pays-Bas*, 1936, **55**, 1046.
10. L. R. Cerecedo and D. J. Hennessy, *J. Amer. Chem. Soc.*, 1937, **59**, 1617.
11. L. R. Cerecedo and F. J. Kaszuba, *J. Amer. Chem. Soc.*, 1937, **59**, 1619.
12. L. R. Cerecedo and J. J. Thornton, *J. Amer. Chem. Soc.*, 1937, **59**, 1621.
13. M. A. Pyke, *J. Soc. Chem. Ind.*, 1939, **58**, 338T.
14. L. J. Harris and Y. L. Wang, *Biochem. J.*, 1941, **35**, 1050.
15. K. Lohman and P. Schuster, *Naturwiss.*, 1937, **25**, 26.
16. K. Lohman and P. Schuster, *Biochem. Z.*, 1937, **294**, 188.
17. D. J. Hennessy and L. R. Cerecedo, *J. Amer. Chem. Soc.*, 1939, **61**, 179,
18. R. T. Conner and G. J. Straub, *Ind. Eng. Chem., Anal. Ed.*, 1941, **13**. 380.
19. Y. L. Wang and L. J. Harris, *Chem. & Ind.*, 1942, **61**, 27.
20. E. R. Dawson and G. W. Martin, *J. Soc. Chem. Ind.*, 1941, **60**, 241.
21. E. R. Dawson and G. W. Martin, *J. Soc. Chem. Ind.*, 1942, **61**, 13.
22. E. Egaña and A. P. Meiklejohn, *J. Biol. Chem.*, 1941, **141**, 859.
23. J. R. Nicholls, R. G. Booth, D. W. Kent-Jones, A. J. Amos and H. H. Ward, *Analyst*, 1942, **67**, 15.

24. R. G. Booth, *Analyst*, 1942, **67**, 162.
25. E. R. Kirch and O. Bergeim, *J. Biol. Chem.*, 1942, **143**, 575.
26. H. Johannson and C. E. Rich, *Cereal Chem.*, 1941, **18**, 473.
27. F. Wokes, *Analyst*, 1942, **67**, 135.
28. J. J. C. Hinton, *J. Soc. Chem. Ind.*, 1942, **61**, 143.
29. A. H. Ward, *Chem. & Ind.*, 1943, **62**, 11.
30. L. J. Harris and Y. L. Wang, *Biochem. J.*, 1941, **35**, 1068.

VITAMIN E (α-TOCOPHEROL)

In 1938 Karrer and Keller proposed a potentiometric method for determining this vitamin which depended upon its capacity to reduce a solution of gold chloride[1].

This procedure, although useful in some connections, was not found to be generally applicable and Emmerie and Engel[2,3] in searching for a more satisfactory method, discovered that tocopherol reduces ferric chloride rapidly provided αα′-dipyridyl is also present to remove the ferrous ions formed. Since, within a certain range, the latter compound yields a red colour proportional to the amount of ferrous salt produced, it was found possible to evolve an approximate colorimetric method for determining the vitamin. In Britain, the richest source of tocopherol is wheat germ oil and a procedure for the evaluation of this product is given below and, in addition, its adaptation to the examination of blood serum[4].

Method (*for Wheat Germ Oil*). To a weighed quantity (about 2 g.) of the sample is added 10 ml. of 2N potassium hydroxide in methyl alcohol, the mixture boiled under a reflux condenser for 10 minutes or until it becomes clear and then diluted with about 55 ml. of approximately 38 per cent. v/v methyl alcohol. The liquid is transferred to a separator and extracted by shaking, first with 60 ml. of ether (peroxide-free) and then with three separate portions each of 40 ml. The ether extracts are combined and washed with successive 15-ml. portions of water until the latter no longer reveal an alkaline reaction when tested with phenolphthalein indicator solution. The ether is dried by the addition of a little anhydrous sodium sulphate then filtered into a distilling flask and removed by distillation in an atmosphere of carbon dioxide or nitrogen. The residue is dissolved in about 5 ml. of benzene, the solution passed through a column (about 6 cm. long by 1 cm. diameter) of Floridin XS earth and the latter washed

with five portions each of about 5 ml. of benzene, the washings being added to the original eluate. The benzene solution is made up to a known volume, 5 ml. is evaporated to dryness in an inert atmosphere, the residue dissolved in 0·2 ml. of chloroform and the solution tested for vitamin A and carotene by the Carr-Price method (see p. 353) : if a reaction is obtained the remainder of the benzene solution is passed through a fresh column of Floridin XS earth and the latter washed with additional benzene as before. The whole of the benzene is then removed by distillation in an atmosphere of carbon dioxide or nitrogen, the residue dissolved in absolute ethyl alcohol and made up to a known volume. To a suitable aliquot part is added 1 ml. of a 0·2 per cent. solution of hydrated ferric chloride, $FeCl_3.6H_2O$, in absolute alcohol followed by 1 ml. of a 0·5 per cent. solution of $\alpha\alpha'$-dipyridyl in absolute alcohol and the mixture diluted to 20 ml. with the same solvent and set aside in a dark cupboard for 5 minutes. The intensity of any red colour which develops is measured as quickly as possible by means of a photometer or Lovibond tintometer and from the reading obtained is deducted the colour due to a control test made by diluting a mixture of 1 ml. each of the ferric chloride and the $\alpha\alpha'$-dipyridyl solutions to 20 ml. with absolute alcohol. If a Lovibond instrument is employed with a 1-cm. cell then the nett reading in red units multiplied by 0·0555 gives mg. α-tocopherol in the aliquot part of the solution taken for the colour test provided the value observed lies between 3·5 and 6·5 on the Lovibond red scale.

(*Concentrates*). The above technique is applied to 1 g. of the sample. If the latter does not give any reaction by the Carr-Price test indicating the absence of vitamin A the chromatographic treatment with Floridin earth may be omitted ; otherwise, the benzene solution is largely diluted and an aliquot part taken for treatment in the column.

(*Blood Serum*)[4]. To 10 ml. of the sample in a 250-ml. separator is added, with gentle shaking after each addition, 5 ml. of aqueous 0·2N potassium hydroxide, 15 ml. of 38 per cent. formaldehyde solution (previously neutralised to phenolphthalein) and 15 ml. of 95 per cent. ethyl alcohol. The mixture is extracted with 50 ml. of peroxide-free ether and then again with two further similar portions of the same solvent after adding 10 ml. of alcohol each time to prevent the formation of emulsions. The extracts are combined and washed, first with a 2 per cent. aqueous solution of potassium hydroxide, then with 25 ml. of a 1 per cent. solution

of sulphuric acid and finally several times with water. The ether is dried by the addition of a little anhydrous sodium sulphate then filtered into a distilling flask, 10 ml. of benzene added and the solvent removed by gentle distillation in an atmosphere of carbon dioxide. A further 10 ml. of benzene is added to the residue and at once removed by distillation. The residue (a very small drop) is taken up in 5 ml. of benzene and passed through a column (3 cm. long by 1 cm. in diameter) of Floridin XS earth which is then washed with five portions each of about 5 ml. of benzene, the latter being added to the original eluate. The whole of the benzene is removed by distillation in an atmosphere of carbon dioxide or nitrogen. The residue in the flask is dissolved in 5 ml. of a reagent mixture consisting of 1 ml. of a 0·2 per cent. solution of hydrated ferric chloride in absolute alcohol, 1 ml. of a 0·5 per cent. solution of αα′-dipyridyl in absolute alcohol and 5 ml. of benzene. The resulting solution is set aside in a dark cupboard for 10 minutes and the intensity of any red colour which develops is measured as quickly as possible by means of a photometer. From the reading obtained is deducted that due to the colour of the reagent mixture and the nett reading referred to a calibration curve prepared by conducting the test on a series of known quantities of α-tocopherol derived from a standard solution of the pure substance in benzene.

Discussion. In conducting any of the above procedures it is important to avoid working in bright daylight and since in the final reaction mixture the red colour rapidly intensifies on exposure to light it is essential to take readings quickly, preferably within 20 seconds. Vitamin A and carotenoids interfere with the colour reaction but, if originally present, they are removed by the chromatographic treatment. Wheat germ oil is generally found to contain between 0·1 and 0·3 per cent. of α-tocopherol. According to Karrer and Keller[1] maize germ oil is an extremely rich source of vitamin E and sometimes contains up to 10 per cent. but only traces occur in most other commercially available fixed oils. In their fresh condition, industrially prepared concentrates may generally be expected to contain from 2 to 35 per cent. of α-tocopherol. Nothing more than an approximate evaluation can be obtained by application of the colorimetric technique since, apart from the inherent inaccuracies of the method, the reaction is also given by the β and γ isomers of tocopherol which are generally found in association with the α compound but are biologically less active. Acetyl-tocopherol, which may occur in blood serum, does

not react in the colour test although it is extracted along with tocopherol itself. The acetyl compound can be hydrolysed by treating it with methyl alcoholic potassium hydroxide at 72° to 74° C. for 10 minutes and the liberated tocopherol isolated by diluting with water and extracting with ether. However, in estimating the acetyl-tocopherol content of an unknown sample, a compromise has to be sought between the quantity of alkali necessary for saponification and the maintenance of a sufficiently low concentration to reduce to a minimum destruction of the tocopherol.

REFERENCES

1. P. Karrer and H. Keller, *Helv. Chim. Acta*, 1938, **21**, 1161.
2. A. Emmerie and C. Engel, *Rec. Trav. chim. Pays-Bas*, 1938, **57**, 1351.
3. A. Emmerie and C. Engel, *Rec. Trav. chim. Pays-Bas*, 1939, **58**, 283.
4. A. Emmerie and C. Engel, *Rec. Trav. chim. Pays-Bas*, 1939, **58**, 895.

SECTION V

MISCELLANEOUS SUBSTANCES

INTRODUCTION

It would have been possible to increase considerably the number of monographs in this Section without, however, adding much to the utility of the book. Here, as elsewhere in this work, it has been necessary to make a selection based upon considerations of general interest and reliability but the following references to a few colorimetric procedures of more restricted scope may serve to rectify some omissions.

As indicated in Table I (p. 4) carbonyl chloride, or phosgene, present in low concentrations in air can be estimated by passing the sample through filter paper impregnated with *p*-dimethylaminobenzaldehyde and diphenylamine and comparing the yellow colour produced with a standard chart supplied with the appropriate leaflet of the Department of Scientific and Industrial Research. In cases where somewhat larger quantities of carbonyl chloride are required to be determined the sample may be bubbled through a saturated aqueous solution of aniline, the precipitated diphenylurea collected and the nitrogen content determined by treating with hot concentrated sulphuric acid followed by distillation of ammonia and its colorimetric estimation using Nessler's reagent[1]. The detection of phosgene present as impurity in chloroform has received some attention and a sensitive test has been devised[2] depending upon the fact that, if alcohol is used as preservative, any phosgene present will immediately react to form ethyl carbonate and hydrochloric acid whence the latter is detected in the presence of chloroform by means of a mixture of resorcinol and vanillin.

An interesting proposal for the determination of α-naphthol present as an impurity in β-naphthol is based upon the greater affinity of the former for diazo compounds[3]. A solution of diazotised *p*-nitraniline, containing 50 per cent. excess over the theoretical amount of hydrochloric acid but only a little free

nitrous acid, is added to an alcoholic solution of the sample followed by a specified quantity of sodium nitrite, when any α-compound present will be precipitated as *p*-nitrobenzene-azo-α-naphthol which can be separated and dissolved in aqueous sodium hydroxide to form a blue solution.

A colorimetric method for the determination of coumarin by coupling with *p*-nitroazobenzene after hydrolysing with alkali to sodium coumarinate has been developed by Clayton and Larmour[4] and employed in genetical studies directed towards the cultivation of sweet clover containing minimal concentrations of coumarin[4–6]. In a microchemical modification of the method, Roberts and Link[7] recommend the separate determination of melilotic acid and coumarin by heating an aliquot portion of the extract from the vegetable tissue with alkali, cooling, acidifying in order to close the coumarin ring, then adding the diazo solution and measuring the red colour due to melilotic acid while, in another portion of the same extract, acidification after alkaline hydrolysis is omitted, the colour then produced being due to melilotic acid and coumarin. The same colour test has also been applied to the examination of vanilla extract, the coumarin first being separated by steam distillation under reduced pressure in the presence of potassium sulphate[8].

A suggestion[9] for the colorimetric determination of capsaicin, the pungent principle of capsicum fruit, is worthy of mention since it would appear probable that it could be developed into a ready means for evaluating this commodity. The procedure is based upon a test originated by G. Denigès[10] which depends upon the formation of a blue colour when a chloroformic solution of vanadium oxychloride is added to a solution of capsaicin in acetone, but the utility of the existing technique is limited owing to the transient nature of the colour produced.

Lastly, it may be of interest to allude to the possibility of determining starch colorimetrically by the iodine reaction. A procedure embodying this principle was originally proposed by Dennstedt and Voigtländer[11] in 1895 but was not found to be reliable[12, 13]. More recently, Pucher and Vickery[14] have developed a promising technique for the examination of plant tissues in which the starch is extracted with hot concentrated aqueous solution of calcium chloride, then precipitated at an optimum acid reaction and salt concentration by means of iodine-potassium iodide reagent, the starch iodide separated by centrifuging and treated with alcoholic sodium hydroxide solution

whence the starch is regenerated. The determination is completed by dissolving the isolated starch in water acidified with acetic acid, adding a definite excess of iodine as potassium iodide-iodate mixture and measuring the intensity of the resulting blue colour. This process aims at attaining a high degree of specificity for the colour reaction : the precipitation of the starch with iodine eliminates a large group of polysaccharides which may be present in the starch extract while the interference of alkaloids and glucosides may be obviated by subjecting the sample of dried plant tissue to an initial extraction with alcohol. Substances of the amylohemicellulose type found to occur in certain starch-bearing seeds, fruits, leaves and stem tissues[15–17] give an intense blue-violet colour with iodine although, unlike starch, they are not digested by diastase. While this interference obviously imposes considerable limitation on the value of the colorimetric method it seems probable that the disturbing substances, even when present, occur only in small proportions relative to the quantity of starch.

REFERENCES

1. A. Kling and R. Schmutz, *Compt. rend.*, 1919, **168,** 773.
2. N. L. Allport, *Analyst*, 1931, **56,** 706.
3. J. Prochazka, *Ind. Eng. Chem.*, 1923, **15,** 944.
4. J. S. Clayton and R. K. Larmour, *Can. J. Research*, 1935, **C13,** 89.
5. T. M. Stevenson and J. S. Clayton, *Can. J. Research*, 1936, **C14,** 153.
6. W. L. Roberts and K. P. Link, *J. Biol. Chem.*, 1937, **119,** 269.
7. W. L. Roberts and K. P. Link, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9,** 438.
8. I. J. Duncan and R. B. Dustman, *Ind. Eng. Chem., Anal. Ed.*, 1937, **9,** 416.
9. L. F. Tice, *Amer. J. Pharm.*, 1933, **105,** 320.
10. G. Denigès, *Bull. Soc. pharm. Bordeaux*, 1932, **70,** 182.
11. M. Dennstedt and F. Voigtländer, *Forschungsber. Lebensm.*, 1895, **2,** 173.
12. F. T. Littleton, *Amer. Chem. J.*, 1897, **19,** 44.
13. H. Witte, *Z. Untersuch. Nahr. Genussm.*, 1903, **6,** 625.
14. G. W. Pucher and H. B. Vickery, *Ind. Eng. Chem., Anal. Ed.*, 1936, **8,** 92.
15. E. Schulze, *Z. physiol. Chem.*, 1892, **16,** 387.
16. S. B. Schryver and E. M. Thomas, *Biochem. J.*, 1923, **17,** 497.
17. A. R. Ling and D. R. Nanji, *J. Chem. Soc.*, 1925, **127,** 652.

ACETONE

When sodium hydroxide is added to a mixture of *o*-nitrobenzaldehyde and acetone, indigo is formed as a condensation product. This reaction was employed as a qualitative test for

acetone by Penzoldt[1] and has been modified for quantitative use by C. A. Adams and J. R. Nicholls[2].

Method[1]. To an aliquot portion of the distillate to be tested (containing not more than 0·02 g. of acetone and diluted with water to 10 ml.) is added 1 ml. of a recently prepared 1 per cent. solution of *o*-nitrobenzaldehyde in 50 per cent. ethyl alcohol (not methylated spirit, as this contains acetone). After mixing, 0·5 ml. of a 30 per cent. aqueous solution of sodium hydroxide is added, and the test solution allowed to stand for about 15 minutes, avoiding strong daylight. The resulting blue colour is compared with that developed in a set of standard solutions, containing from 0 to 20 mg. of acetone in 10 ml. which have been treated similarly at the same time.

Discussion. The gradation of colour produced is well marked and it is possible to have twenty readily differentiated standards within the suggested range. Adams and Nicholls[2] have based a useful qualitative test for *iso*-propyl alcohol on the same reaction by introducing a preliminary oxidation with bromine. Thus, to detect *iso*-propyl alcohol in the presence of ethyl alcohol, 10 ml. of the liquid, previously diluted so as to contain approximately 10 per cent. by volume of ethyl alcohol, is mixed with 5 ml. of saturated bromine water and allowed to stand in a dark place for 3 to 6 hours, and then examined for the presence of acetone as described above excepting that 2 ml. of 30 per cent. sodium hydroxide solution is used ; roughly quantitative results may be obtained by comparing with a series of standards similarly prepared.

REFERENCES

1. J. Penzoldt, *Deut. Arch. klin. Med.*, 1883, **34**, 127.
2. C. A. Adams and J. R. Nicholls, *Analyst*, 1929, **54**, 2.

ACETYLENE (IN AIR)

Methods so far proposed for the colorimetric determination of acetylene depend upon production of cuprous acetylide in presence of a protective colloid. Weaver[1] compared the red colour thus produced with an artificial standard consisting of a solution of mixed dyestuffs, while Schulze[2] passed the sample of gas through an ammoniacal cuprous solution until the depth of colour matched that of a previously prepared natural standard and Riese[3] added

a cuprous solution to dilute aqueous solutions of acetylene of known strength; all these investigators employed gelatin as protective colloid. With a view to avoiding the frothing which characterises the use of gelatin, Coulson-Smith and Seyfang recommend starch as the protective colloid and match the colours produced against standards prepared from ferric thiocyanate[4].

Method[4]. The following standard solution and special reagents are required :—

1. A Standard Solution of Iron made by dissolving 0·602 g. of ferric ammonium sulphate, $Fe_2(SO_4)_3(NH_4)_2SO_4.24H_2O$, in about 50 ml. of water, adding 10 ml. of 20 per cent. v/v sulphuric acid and diluting with water to 500 ml.

2. Starch Solution. This is prepared by mixing 2·5 g. of soluble starch with 2 to 3 ml. of cold water and pouring into 300 ml. of boiling water with stirring, boiling for 2 minutes, allowing to cool and diluting to 500 ml. This solution will remain in good condition for about 2 weeks.

3. The Ammoniacal Cuprous Chloride Solution is made by dissolving 0·80 g. of cuprous chloride in a mixture of 20 ml. of water and 9 ml. of strong ammonia (s.g. 0·880) contained in a 100-ml. stoppered flask : to this is added, first, 7 g. of hydroxylamine hydrochloride previously dissolved in 30 ml. of warm water, then, 20 ml. of the above starch solution, the mixture being finally diluted to 100 ml. with water. The reagent is preserved by adding 4 g. of copper wire cut into 1-cm. lengths and the surface is layered with light petroleum. Under these conditions the solution will remain colourless for a week in a stoppered flask.

The determination is conducted by the aid of an assembly similar to that depicted diagrammatically in Fig. 16. The whole apparatus is first swept out with air, or nitrogen, known to be free from acetylene. By opening the tap T3 and connecting the purifying tube A with the burette B by means of the tap T1, 100 ml. of the sample of air, or other gas, under test is drawn into the burette and then expelled *via* T1 and T2 through the Jena gas bubbler, which is not yet in the cylinder C. This ensures that the purifying tube A and all the tubing contains the sample gas. From 36 to 38 ml. of water is introduced into the cylinder C and then 10 ml. of the ammoniacal cuprous chloride solution is run in from a pipette the end of which dips under the water during

delivery. The bubbler is then placed in the cylinder and connected with the burette. A further quantity of the air, or other gas, under examination (100 ml.) is drawn into the burette and passed slowly (in 90 or 100 seconds) through the bubbler into the ammoniacal cuprous chloride solution. If a pronounced colour develops too quickly, only 50 ml., or even 25 ml., of the sample

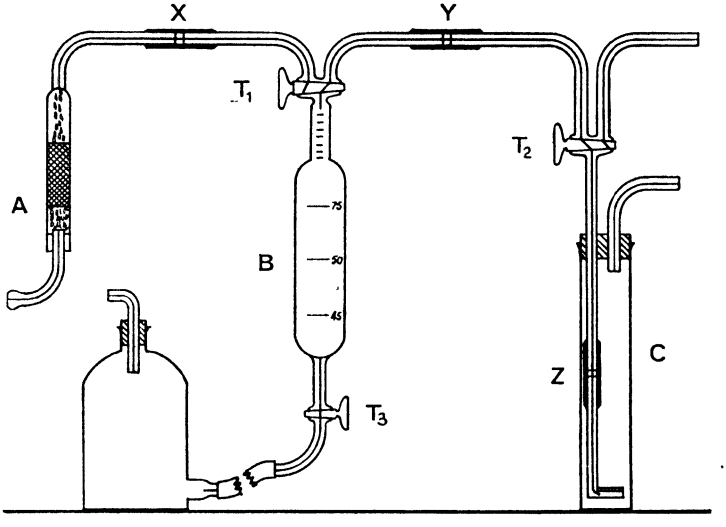


FIG. 16. APPARATUS FOR THE DETERMINATION OF ACETYLENE IN AIR

The purifying tube A, which should be about 8 cm. long by 1·8 cm. in bore is charged with six strips of filter paper moistened with solution of copper sulphate so folded as to ensure thorough contact with the gas and interposed between plugs of cotton-wool inserted at each end of the tube. The sampling burette B, connected with A by means of capillary tubing, has a capacity of 100 ml. from the tap to a mark on the bottom stem and is also graduated at 25-, 50- and 75-ml. levels. A small levelling bottle containing saturated salt solution is connected with the bottom tap. A Jena gas bubbler (33cG1) is attached to capillary tubing leading *via* two-way grooved taps to the sampling burette and is held in the glass cylinder C by means of a bung which also carries a gas exit tube. (After C. Coulson-Smith and A. P. Seyfang, *Analyst*, 1942, **67**, 39.)

should be passed ; on the other hand, if after the passing of 100 ml. the colour is faint, a further 100 ml. may be introduced.

After the reaction, the apparatus is disconnected at Y and the bubbler and cylinder are removed together. The small amount of liquid in the bubbler is blown out, and the bubbler is disconnected at Z. The contents of the cylinder are transferred to a Nessler glass, the bubbler is rinsed with 2 or 3 ml. of water into the cylinder, and the rinsings are added to the main bulk of the liquid. Into a second Nessler glass is added 40 ml. of water, 5 ml. of a 5 per cent. aqueous solution of potassium thiocyanate (delivered by

means of a safety pipette) and 3 ml. of the starch solution. After mixing, the liquid is diluted with water to the same volume as that in the first Nessler glass. The standard iron solution is then run from a burette into the second Nessler glass until the colour matches that of the cuprous acetylide when viewed vertically, preferably employing a B.D.H. Lovibond Nessleriser. The result is calculated on the basis that 1 ml. of the standard iron solution yields a colour with the thiocyanate-starch mixture equal in intensity to that due to the cuprous acetylide formed from 100 ml. of air containing 0·1 per cent. v/v of acetylene, the relation being linear within a range of concentrations from 0·02 to 0·25 per cent. v/v.

Discussion. Any appreciable concentration of hydrogen sulphide or phosphine is removed by passing the gas through the purifying tube A. The proportion found in acetylene as prepared from calcium carbide would not have any effect upon the cuprous chloride solution but greater quantities, particularly of hydrogen sulphide, derived from other sources may be present. Within the effective range of concentration of acetylene in air the intensity of the cuprous acetylide colour is linear ; hence, a deeply tinted solution can be halved or quartered and made up to the original volume, the iron titre being multiplied accordingly but it should be remembered that this cannot be done with the ferric thiocyanate standard, the colour in this case varying with the acid concentration.

It is sometimes necessary to ascertain whether a concentration of acetylene in air is sufficient to give a potentially explosive gas, the lower limit of such a mixture, as laid down by the Home Office, being 2 per cent. v/v. In order to analyse samples of air containing between 1 and 3 per cent. of acetylene the apparatus is swept out with pure air as before, the Jena gas bubbler is replaced in the cylinder C which has been charged with 115 ml. of water and 10 ml. of the ammoniacal cuprous chloride solution. Then 25 ml. of the sample of gas is slowly passed from the burette through the bubbler into this mixture and 25 ml. of the resulting cuprous acetylide solution diluted to 50 ml. in a Nessler glass and the colour evaluated as already described.

REFERENCES

1. E. R. Weaver, *J. Amer. Chem. Soc.*, 1916, **38**, 352.
2. A. Schulze, *Z. angew. Chem.*, 1916, **29**, 341.
3. W. Riese, *Z. angew. Chem.*, 1931, **44**, 701.
4. C. Coulson-Smith and A. P. Seyfang, *Analyst*, 1942, **67**, 39.

AMMONIA (IN WATER)

The colour test for the presence of traces of ammonia or ammonium salts using an alkaline solution of potassio-mercuric iodide, which was orignated in 1856 by J. Nessler[1], undoubtedly remains the best available for quantitative application. Alternative suggestions include the use of phenol and sodium hypochlorite to produce a blue coloration in the presence of ammonium salts[2], and the use of a fresh mixture of silver nitrate and tannic acid followed by measurement of the brownish tint due to the formation of free silver[3] ; however, no procedure superior to that of Nessler has so far been proposed. Because of the value attached to determinations of the ammonia content of water supplies for the purpose of assessing their suitability for domestic use it will be convenient to describe the method as it is applied to this type of sample, whence its appropriate adaptation to other purposes will be obvious. Numerous modifications of the potassio-mercuric iodide reagent have been proposed but it is doubtful if any of the suggestions offer any advantage over the one quoted here which is the formula adopted by Thresh and his collaborators[4]. The preparation of a more dilute reagent[5], favoured for biochemical work, is described on p. 279.

Method. Nessler's Reagent is prepared by dissolving 35 g. of potassium iodide and 12·5 g. of mercuric chloride in 800 ml. of water and adding a cold saturated aqueous solution of mercuric chloride until after repeated shaking a slight red precipitate remains ; to this is added 120 g. of sodium hydroxide, the mixture shaken until the alkali has dissolved and finally a little more saturated solution of mercuric chloride added and then sufficient water to produce 1 litre. The reagent is occasionally shaken during several days, then allowed to stand until the precipitate settles, the clear supernatant liquid being used for the tests.

(*Free Ammonia*). To 50 ml. of the sample of water to be examined, contained in a Nessler glass, is added 2 ml. of the above reagent, the liquid mixed and, after allowing 5 minutes for development, the yellow or brownish-yellow colour is matched against standards prepared at the same time by adding suitable quantities varying from 0·1 to 10 ml. of a 0·00315 per cent. aqueous solution of ammonium chloride to a series of Nessler glasses, diluting each to 50 ml. with ammonia-free distilled water and adding 2 ml. of Nessler's reagent. The standard solution is

made by diluting 10 ml. of a 0·315 per cent. aqueous solution of ammonium chloride to 1 litre with ammonia-free distilled water : 1 ml. of this diluted standard contains 0·01 mg. NH_3. The ammonia-free water is prepared by distilling a fairly large bulk of tap water acidified with phosphoric acid until 50 ml. of distillate separately collected gives no colour when mixed with 2 ml. of Nessler's reagent, then collecting the remainder which distils over. If the sample under examination contains an appreciable trace of magnesium or calcium a turbidity results on the addition of the alkaline Nessler's reagent, but this may be prevented by first adding 2 ml. of a 50 per cent. w/v aqueous solution of sodium potassium tartrate. In order to ensure that the latter solution is free from ammonia it may be preserved by adding to it about 5 per cent. of Nessler's reagent.

The amount of free ammonia in waters intended for drinking purposes may not be sufficient to give a measurable colour when 50 ml. is tested directly although the quantity present may still be significant for the purpose of judging its suitability for use. Thus, in the examination of potable waters it is customary to distil two separate 100-ml. portions from a 500-ml. sample contained in a copper flask then determine the proportion of ammonia in the distillates and calculate the results accordingly.

(*Albuminoid Ammonia or Protein Nitrogen*)[6]. To the 300 ml. of water remaining in the flask after distilling off the free ammonia is added 50 ml. of alkaline permanganate solution and the distillation resumed, 50-ml. portions being collected until only negligible quantities of ammonia can be detected. From the quantity of albuminoid ammonia thus found the proportion expressed as parts per 100,000 is readily calculated. The alkaline permanganate solution is prepared by mixing 1 litre of a 0·8 per cent. aqueous solution of potassium permanganate with 500 ml. of a 34 per cent. w/v aqueous solution of potassium hydroxide and boiling down to a volume of 1 litre.

Discussion. When the quantity of ammonia in the 50 ml. of sample taken for the colour test does not exceed 0·005 mg. the colour develops slowly and in such cases 15 minutes should be allowed to elapse before matching. If the Nessler's reagent be used in the proportion of 5 ml. for 50 ml. of water the colour develops more decisively and quickly attains its maximum value even in the presence of minute quantities of ammonia, but, in laboratories where many determinations are made daily, the extra amount of reagent used is a significant consideration. When

distilling in the presence of alkaline permanganate, trouble due to bumping is frequently encountered and it is a good plan to minimise this tendency by introducing a roll of copper gauze. While it is generally conceded that potable waters intended for drinking purposes should not normally yield more than 0·01 part per 100,000 each of free and albuminoid ammonia the source of supplies and the proportion of other constituents needs careful consideration in the interpretation of analyses. It is worthy of note that certain very pure artesian well waters derived from below submerged chalk strata contain relatively high proportions of free ammonia.

Method Using Permanent External Standards. Four discs each containing nine glass colour standards are available for use with the B.D.H. Lovibond Nessleriser. They are applicable to the method described above and in the aggregate cover a range from 0·001 to 0·1 mg. NH_3. When using these external standards it is important to use Nessler's reagent that has been made precisely in accordance with the instructions given above as otherwise considerable variations in the sensitivity may be encountered and thus lead to serious error.

REFERENCES

1. J. Nessler, *Chem. Gaz.*, 1856, **14**, 445 and 460.
2. P. Thomas, *Bull. Soc. chim.*, 1912, **11**, 796.
3. K. C. Makris, *Z. anal. Chem.*, 1930, **81**, 212.
4. J. C. Thresh, J. F. Beale and E. V. Suckling, "The Examination of Waters and Water Supplies," 5th Ed., by E. V. Suckling, London: J. & A. Churchill, 1943.
5. O. Folin and H. Wu, *J. Biol. Chem.*, 1919, **38**, 89.
6. J. A. Wanklyn, E. T. Chapman and M. H. Smith, *J. Chem. Soc.*, 1867, **20**, 445.

BENZOYL PEROXIDE (IN FLOUR)

Benzoyl peroxide is sometimes used for bleaching flour and the usual methods for its detection depend upon the oxidising action of the unchanged peroxide. In effecting its bleaching action the peroxide is reduced to benzoic acid and, if the whole of the added peroxide is so changed, none remains to give an indication that the flour has been treated. J. R. Nicholls[1] has suggested a method involving isolation of benzoic acid and unchanged benzoyl peroxide by steam distillation, reduction of the latter to benzoate

by treatment with acetone and sodium hydroxide followed by permanganate oxidation of extraneous organic material, isolation of the total benzoic acid and its colorimetric determination by controlled oxidation to salicylic acid and application of the ferric-salicylate test. The colorimetric determination may conveniently be conducted by applying the modification of Nicholls's original technique[2] advocated by Edwards, Nanji and Hassan[3] as described on p. 129.

Method[1]. About 40 g. of calcium chloride and 100 ml. of water are placed in a litre flask ; 50 g. of the flour to be tested and 10 ml. of concentrated hydrochloric acid are added and the flask is connected with a steam-distillation apparatus. Steam is passed, very cautiously at first, until the mass ceases to froth and becomes fluid, and then rapidly, the flask being heated to prevent increase in the bulk of the liquid. About 300 ml. of distillate is collected, saturated with common salt and extracted twice with 50 ml. of ether. The ether is evaporated at a temperature of about 30° C., a rapid current of air being drawn over the surface. When the residue is almost dry, 5 ml. of acetone is used to wash down the sides of the vessel and to dissolve the residue and 5 ml. of 2N sodium hydroxide is added : the mixture is diluted with an equal volume of water and the acetone is boiled off. After the liquid has been cooled to 50° to 60° C. it is treated with an aqueous solution of potassium permanganate until an excess is evident by the colour, then 10 ml. of 2N sulphuric acid is added and the solution is just decolorised by the addition of oxalic acid. Any insoluble fatty acids are filtered off and the solution is extracted twice with about 20 ml. of a mixture of equal parts of ethyl ether and light petroleum. The extracts are evaporated to dryness, conveniently in a boiling tube, at about 30° C., a current of air being drawn over the surface. When the last trace of solvent has disappeared, any benzoic acid will be visible as a crystalline deposit. The residue is dissolved in 10 ml. of warm water, 5 ml. of 0·1N sulphuric acid added and the remainder of the test conducted precisely as described for the colorimetric determination of benzoic acid commencing with the addition of 1 ml. of the iron reagent for oxidation (see p. 130).

Discussion. Considering its composition, benzoyl peroxide is a remarkably stable substance. It can be heated with water and acids without decomposition and it can be distilled unchanged in steam. It is insoluble in water and, while insoluble, is resistant to oxidising and reducing agents but when dissolved in organic

solvents its oxidising character is revealed. When 1 vol. of 0·1N sodium hydroxide is added to 1 vol. of an acetone solution of benzoyl peroxide and the mixture immediately diluted with about 5 vols. of water the reaction proceeds quantitatively according to the equation :—

$$\begin{matrix} C_6H_5CO{-}O \\ \quad\quad\quad | \\ C_6H_5CO{-}O \end{matrix} + 2NaOH + CH_3COCH_3 \rightarrow 2C_6H_5COONa + CH_3CHO + HCHO + H_2O$$

and, if the excess alkali is determined by titration with 0·1N acid using phenolphthalein as indicator, the reaction affords a rapid method of determining the appropriate strength of commercial preparations containing the bleaching agent[1]. Under the conditions outlined 1 ml. of 0·1N sodium hydroxide is equivalent to 0·0121 g. $C_6H_5CO.O.O.COC_6H_5$. It is with the object of facilitating the complete reduction of benzoyl peroxide to benzoic acid that acetone and sodium hydroxide are added to the material isolated by steam-distillation in the colorimetric procedure described above.

REFERENCES

1. J. R. Nicholls, *Analyst*, 1933, **58**, 4.
2. J. R. Nicholls, *Analyst*, 1928, **53**, 19.
3. F. W. Edwards, H. R. Nanji and M. K. Hassan, *Analyst*, 1937, **62**, 172.

CARBON (COMBINED, IN STEEL)

When steel is dissolved in nitric acid a brown colour is produced by the carbide in the metal and this phenomenon forms the basis of the well-known Eggertz[1, 2] test. Any carbon present as graphite is not acted upon by the nitric acid and hence is not included in the colorimetric determination. The standard colours must be prepared from steels of known carbon content and in other respects of similar composition to the samples under test.

Method. A suitable quantity of the sample and of a standard steel of approximately the same composition, both of which should be in the form of drillings, are weighed out into test tubes (about 15×1·5 cm.) and nitric acid (s.g. 1·2, made by adding 100 ml. of acid s.g. 1·42 to 155 ml. of water) slowly added. After the initial

violent reaction has subsided, the tubes are immersed in boiling water so that their lower parts only are below the surface. In no case must the top of the liquid in the tubes be below the level of the boiling water, or iron oxide will deposit on the sides of the tubes and will form a brown basic nitrate when dissolved, thus introducing an error into the colour measurement. The time taken for solution of the samples varies from about 20 minutes for a low carbon steel to 45 minutes for a steel with a high carbon content. The weight of steel to be taken and the quantity of acid employed should be chosen according to the probable carbon content as shown in Table XXXIV.*

TABLE XXXIV. WEIGHT OF SAMPLE AND VOLUME OF ACID TO BE TAKEN FOR THE COLORIMETRIC DETERMINATION OF CARBON

Expected Carbon Content per cent.	Weight of Sample to be taken g.	Volume of Nitric Acid (s.g. 1·2) to be used ml.
1·2 to 1·7	0·05	3
1·1 to 1·3	0·05	3
0·8 to 1·1	0·1	4
0·4 to 0·8	0·1	3
0·2 to 0·4	0·1	2
0·1 to 0·3	0·3	6

When the samples have completely dissolved the tubes are cooled at once by immersion in cold running water, the solutions transferred to the long narrow stoppered Eggertz tubes which are usually graduated in increments of 0·1 ml. and the latter set up in the special matching stand which is provided with an opal glass back. The comparison is made by diluting the standard to a convenient known volume and then adding water to the sample until the same intensity of colour is obtained. The volumes are then taken as being proportional to the carbon content. In all operations prior to the actual matching the solutions should be kept away from bright light.

For low carbon steel the procedure should be modified by dissolving 1 g. of the sample in 12 ml. of nitric acid (s.g. 1·2), diluting to 60 ml. with water, filtering through a dry paper and comparing the colour with a similarly prepared standard by the dilution method as before. In order to determine combined

* Table XXXIV is reproduced from "Select Methods of Metallurgical Analysis," by W. A. Naish and L. E. Clennell (Chapman & Hall, Ltd., London, 1929), with the kind permission of Dr. Naish.

carbon in white cast iron, 0·05 g. should be dissolved in 7 ml. of nitric acid (s.g. 1·2), the solution diluted to, say, 20 ml. and the colours quickly compared. In the case of grey cast iron, 0·05 g. should be dissolved in 5 ml. of nitric acid (s.g. 1·2), the solution cooled, 2 or 3 ml. of water added, the graphite filtered off, the filter washed and the combined filtrate and washings compared with standards similarly prepared from grey cast iron of similar carbide content.

Discussion. Notwithstanding its limitations, this method, which was originally proposed 80 years ago, is still widely used since it is so much more convenient for routine control purposes than the combustion method. Colorimetric determinations should always be conducted in duplicate and the standard steels employed should have had approximately the same heat treatment as the samples. If much graphitic carbon is present the values obtained by the colorimetric method for the combined carbon are inclined to be low. The presence of manganese will result in lighter colours being produced and it is therefore advisible that the standards should contain about the same proportion of this metal. Similarly, if appreciable concentrations of nickel, cobalt, copper, chromium, or over 1 per cent. of silica are present the colour is liable to be altered and in these instances it will be necessary to employ standards of appropriate composition. In any case, frequent checks should be made by applying the combustion procedure. For a critical study of the methods employed for the determination of carbon in steel a paper by Congdon, Brown and Friedel may usefully be consulted[3].

REFERENCES

1. V. Eggertz, *Jernkontorets Ann.*, 1862, 54.
2. V. Eggertz, *Jernkontorets Ann.*, 1874, 176.
3. L. A. Congdon, F. J. Brown and R. K. Friedel, *Chem. News*, 1924, **129**, 253.

CARBON DISULPHIDE (AS IMPURITY IN BENZENE)

Diethylamine in the presence of copper serves as a useful reagent for the colorimetric determination of traces of carbon disulphide[1] since the latter combines with the amine to produce the equivalent of sodium diethyl-dithio-carbamate which serves so well as a colorimetric test for copper.

Method[2]. To a measured quantity of the sample to be examined, conveniently 1 ml., is added 1 ml. of a 1 per cent. solution of diethylamine in benzene (AnalaR grade) or other miscible solvent free from carbon disulphide, followed by 1 ml. of a 0·03 per cent. solution of copper acetate in absolute alcohol and the mixture finally diluted to 10 ml. with absolute alcohol. Any yellow or brown colour produced is compared with standards similarly prepared containing from 0·025 to 0·1 mg. of carbon disulphide. A standard solution may be prepared by appropriately diluting with AnalaR benzene a 1 per cent. w/v solution of carbon disulphide in the same solvent. The colours should be compared after the tests have stood for 20 minutes.

Discussion. This method will detect 1 part per million of carbon disulphide in benzene and the procedure is equally applicable to toluene, carbon tetrachloride, acetone, ether and alcohol. Thiophen, dimethyl sulphide and ethyl mercaptan do not interfere but thioacetic acid gives the reaction.

REFERENCES

1. N. Tischler, *Ind. Eng. Chem., Anal. Ed.*, 1932, **4**, 146.
2. T. Callan, J. A. R. Henderson and N. Strafford, *J. Soc. Chem. Ind.*, 1932, **51**, 193T.

CARBON TETRACHLORIDE

Fujiwara observed that carbon tetrachloride gives a red colour with pyridine in the presence of strong alkali hydroxide[1]. A similar colour reaction is given by chloroform and W. H. Cole suggested a procedure for applying the test to the quantitative determination of the latter compound[2]. However, the method as applied by these investigators yields colours which are unstable but, as the result of an exhaustive examination of the reaction R. P. Daroga and A. G. Pollard[3] have succeeded in producing a colour which is stable for many hours, thus establishing a sensitive colorimetric method which is capable of yielding reliable results.

Method[3]. Into a 50-ml. narrow-necked flask is transferred 10 ml. of a 20 per cent. aqueous solution of sodium hydroxide followed by exactly 20 ml. of pyridine. The solution under test (the isolated carbon tetrachloride dissolved in acetone) is added, the flask is loosely corked to prevent evaporation of pyridine, and, with

constant shaking, is immersed for 5 minutes in water at 100° C. The flask and its contents are cooled under running water for 2 minutes, the upper red-coloured layer is separated and matched against standards similarly prepared. Colours of suitable intensity for measuring in a 1-cm. cell using the Lovibond tintometer are produced by operating with quantities of carbon tetrachloride lying between 0·03 and 0·5 mg.

Discussion. Daroga and Pollard developed the above method

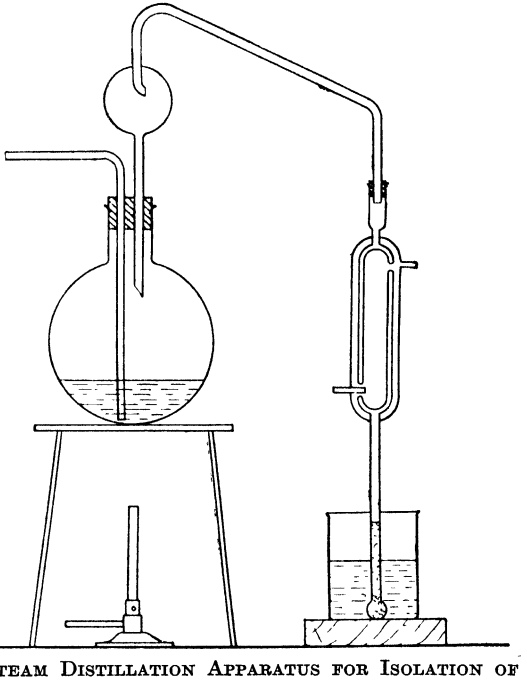


FIG. 17.—STEAM DISTILLATION APPARATUS FOR ISOLATION OF CARBON TETRACHLORIDE OR CHLOROFORM

The essential feature is the delivery tube fused on to the end of the condenser and terminated by a bulb about 12·5 mm. in diameter provided with four holes. This bulb and about 15 cm. of the tube above are filled with solid glass beads wetted with the absorbing liquid. (Simplified after the apparatus of J. C. Higgins, T. G. Morris and A. G. Pollard, *J. Soc. Chem. Ind.*, 1939, **58**, 272.)

for the purpose of studying the diffusion of carbon tetrachloride vapours through soil and they report[3] that attempts to effect a preliminary isolation by means of organic solvents proved to be impracticable but satisfactory results were obtained by resorting to steam-distillation. To a suspension of the soil sample in about 100 ml. of water they added 1 ml. of pyridine and submitted the mixture to steam-distillation using the apparatus recommended by Higgins, Morris and Pollard[4] (see Fig. 17). The distillate

(50 ml.) was collected in a receiver containing 10 ml. of acetone, the mixture diluted to 100 ml. with more acetone and the carbon tetrachloride determined by applying the colorimetric method to a suitable aliquot part.

For the determination of carbon tetrachloride in a current of air Daroga and Pollard found Schott's gas-washing bottle with sintered glass disc to be the only satisfactory form of apparatus. They recommend that the air current containing carbon tetrachloride be drawn through a Schott's apparatus containing 50 ml. of acetone at the rate of about 1 litre per minute and that a suitable proportion of the resulting acetone solution be submitted to the colorimetric test.

As already indicated, the test is not specific for carbon tetrachloride since pink or red colours are produced by any compound conforming to a structure represented by R-C-halogen$_3$ including bromoform, chlorbutol, chloral and iodoform while the method has been developed for the colorimetric determination of chloroform (see p. 394).

Method Using Permanent External Standards. A Lovibond Comparator disc containing nine glass standards based upon the above technique and covering a range from 0·03 to 0·51 mg. CCl_4 has been issued.

REFERENCES

1. K. Fujiwara, *Sitzungsb. Abhandl. Naturforsch. Ges., Rostock*, 1914, **6**, 1.
2. W. H. Cole, *J. Biol. Chem.*, 1926–7, **71**, 173.
3. R. P. Daroga and A. G. Pollard, *J. Soc. Chem. Ind.*, 1941, **60**, 218.
4. J. C. Higgins, T. G. Morris and A. G. Pollard, *J. Soc. Chem. Ind.*, 1939, **58**, 272.

CHLORINE (FREE, IN WATER)

The utility of the well-known *o*-tolidine test[1, 2] is generally acknowledged. The yellow colour produced is not specific for chlorine, being formed also by nitrites which are present in many contaminated waters; but the colour due to chlorine is readily destroyed by sodium thiosulphate whereas that due to nitrite is not affected, hence it is a simple matter to determine which substance is responsible for the reaction. Owing to the difficulty of preparing standard solutions of known free chlorine content it is usual to employ artificial standards made by mixing solutions of potassium dichromate and copper sulphate in specified proportions

or to use permanent glass standards such as those offered in the Lovibond series.

A method depending upon the green colour produced by the action of chlorine on benzidine[3] is less satisfactory. Another procedure deserving of mention depends upon the formation of a coloured meriquinone known as Wurster's red when free chlorine in dilute solution reacts with *p*-aminodimethylaniline (*asym.* dimethyl-*p*-phenylenediamine). This reaction, first characterised by Willstätter and Piccard[4], was originally suggested for analytical use by Kolthoff[5] while others have effected improvements in the colorimetric technique[6, 7]. However, it is necessary to include buffers in the test mixtures since the development of the colour is greatly influenced by the reaction of the sample, while solutions of the organic dyestuff, methyl red, must be employed for the artificial standards since no inorganic substances of precise composition have been found to yield the correct tints. The instability of the colour produced is a still greater disadvantage and Byers and Mellon[8], after making a careful study, conclude that it offers no advantage over the *o*-tolidine method described below.

Method. A reagent solution is made by dissolving 1 g. of pure *o*-tolidine in 100 ml. of concentrated hydrochloric acid (AnalaR grade) and diluting to 1 litre with distilled water. The test is applied by adding 0·5 ml. of the reagent solution to 50 ml. of the sample contained in a Nessler glass and, after allowing to stand for 5 minutes, matching any yellow colour produced against artificial standards made in accordance with the data presented in Table XXXV.

Method Using Permanent External Standards. Owing to the inconvenience of making natural or artificial solution standards the Lovibond range of discs serves a particularly useful purpose in this instance. Two discs are available for use with the B.D.H. Lovibond Nessleriser which together cover the range from 0·01 to 0·5 part per million free Cl, the technique of the test being identical with that given here. The same procedure is used when the colours are observed by means of the Lovibond Comparator except that, if desired, the tests may be conducted on a smaller scale: five discs are available which are variously designed for use in conjunction with cells of selected dimensions and which together cover the range from 0·02 to 5·0 parts per million free Cl.

Discussion. Many other oxidising agents besides chlorine and nitrites produce a colour with *o*-tolidine among which may be mentioned ferric salts, persulphates, permanganates, dichromates,

TABLE XXXV. DATA FOR THE PREPARATION OF ARTIFICIAL STANDARDS FOR THE DETERMINATION OF FREE CHLORINE IN WATER BY THE *o*-TOLIDINE METHOD

Chlorine p.p.m.	Quantity of Solution to be Diluted to 50 ml.		Chlorine p.p.m.	Quantity of Solution to be Diluted to 50 ml.	
	Potassium Dichromate: 0·05 per cent. w/v in 0·2 per cent. v/v Sulphuric Acid ml.	Copper Sulphate: 0·3 per cent. w/v in 0·2 per cent. v/v Sulphuric Acid ml.		Potassium Dichromate: 0·05 per cent. w/v in 0·2 per cent. v/v Sulphuric Acid m.l	Copper Sulphate: 0·3 per cent. w/v in 0·2 per cent. v/v Sulphuric Acid ml.
0·01	0·25	0·50	0·10	2·50	5·00
0·02	0·45	0·75	0·15	3·25	7·50
0·03	0·75	1·00	0·20	5·25	8·00
0·04	1·00	1·50	0·25	6·50	8·50
0·05	1·35	2·00	0·30	7·50	8·50
0·06	1·65	2·50	0·35	9·00	9·00
0·07	1·90	3·00	0·40	9·50	9·50
0·08	2·15	3·50	0·45	10·00	10·50
0·09	2·25	4·00	0·50	10·50	10·50

peroxides, bromine, iodine, nitric acid, nascent oxygen and ozone. Apart from nitrites and ferric salts there is not much likelihood that any of the other substances mentioned will be present in natural waters or even in sewage. A colour equivalent to 0·1 part per million of chlorine is given by 0·9 part per million of nitrogen present as nitrite while about the same tint is produced by the presence of 1 part per million of ferric iron. It should be noted that if ammonia is present in the sample under examination (i.e. if the chloramine process is being used) 20 minutes should be allowed for the colour development.

REFERENCES

1. E. B. Phelps, *Bull. Ohio State Brd. Health*, 1913, No. 1.
2. J. W. Ellms and S. J. Hauser, *J. Ind. Eng. Chem.*, 1913, **5**, 914.
3. R. C. Stratten, J. B. Ficklen and W. A. Hough, *Ind. Eng. Chem., Anal. Ed.*, 1932, **4**, 2.
4. R. Willstätter and J. Piccard, *Ber.*, 1908, **41**, 1458.
5. I. M. Kolthoff, *Chem. Weekblad*, 1926, **23**, 203.
6. K. Alfthan, *J. Amer. Water Works Assoc.*, 1928, **20**, 407.
7. L. W. Hoase and G. Gad, *Z. anal. Chem.*, 1936, **107**, 1.
8. D. H. Byers and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 202.

CHLOROFORM

The colorimetric method for the determination of carbon tetrachloride based upon the Fujiwara reaction[1] using pyridine and sodium hydroxide is also applicable to chloroform provided the latter is dissolved in 0·05N hydrochloric acid. The details given below have been worked out by R. P. Daroga and A. G. Pollard[2]. Another useful, but less sensitive and somewhat less accurate method depends upon the violet colour produced by chloroform in the presence of β-naphthol and sodium hydroxide : this test was originated by Lustgarten[3] and has been studied and improved by Moffitt[4].

Method Using Pyridine[2]. The determination is conducted by the procedure given for carbon tetrachloride (p. 389) excepting that the chloroform is collected and dissolved in 0·05N hydrochloric acid instead of in acetone. Red colours of suitable intensity for measuring in a 1-cm. cell using the Lovibond tintometer are produced by operating with quantities of chloroform lying between 0·03 and 0·4 mg.

Method Using Pyridine and Permanent External Standards. A Lovibond Comparator disc containing nine glass standards based upon the above technique and covering a range from 0·03 to 0·45 mg. $CHCl_3$ has been issued.

Method Using β-Naphthol[4]. The following two solutions are required :—

1. A 2 per cent. Solution of β-Naphthol in 40 per cent. w/v aqueous solution of potassium hydroxide. The potash solution used for dissolving the β-naphthol should not be warmer than room temperature.
2. A Standard Solution (0·5 per cent. by vol.) of Chloroform dissolved in industrial methylated spirit or ethyl alcohol (95 per cent.).

In applying this test it is a good plan to make a series of standards before operating on the sample. This is done by transferring 10 ml. of the β-naphthol reagent to each of several Nessler glasses, adding from a pipette a quantity of the standard solution of chloroform varying from 0·2 to 0·6 ml. followed by sufficient industrial methylated spirit to make the total volume of the mixture 11 ml. In practice it is better to add the methylated spirit before the chloroform solution and to deliver both from a pipette the tip of which dips slightly below the

surface of the liquid. The mixtures are shaken and allowed to stand for 5 to 10 minutes so that the blue colour may attain its maximum intensity. In order to apply the test, about 5 ml. of the sample under examination should be distilled with 75 ml. of methylated spirit, at least 50 ml. of distillate being collected. The distillate should then be treated in the manner described above and the colour compared with those obtained by similarly treating varying quantities of standard chloroform solution as nearly as possible at the same time. Since the colour fades rapidly it is essential that the comparison be made within a few minutes.

Discussion. The method using pyridine is discussed on p. 390 in connection with the use of the same reaction for the determination of carbon tetrachloride. The β-naphthol reaction is not capable of yielding results of more than approximate accuracy but is sometimes advantageous where methods based upon the action of alcoholic potash on chloroform break down owing to the presence of other readily decomposed chloro-compounds. Moffitt ascertained that none of the following chloro-compounds interfered with the method: *sym.*-dichlorethane, trichlorethylene, *sym.* dichlorethyl ether. Moreover, the presence of acetone does not disturb the reaction. The method was found to be satisfactory for making approximate determinations of chloroform present in compound liniment of aconite, B.P.C., 1923. Apparently the method was not applied to A.B.C. liniments containing oil. A similar colour reaction is also given by α-naphthol but, although the sensitivity of the test using this reagent is greater, it is more subject to interference by other substances than the procedure described.

REFERENCES

1. K. Fujiwara, *Sitzungsb. Abhandl. Naturforsch. Ges., Rostock*, 1914, **6**, 1.
2. R. P. Daroga and A. G. Pollard, *J. Soc. Chem. Ind.*, 1941, **60**, 218.
3. S. Lustgarten, *Monatsh.*, 1882, **3**, 715.
4. W. G. Moffitt, *Analyst*, 1933, **58**, 2.

DULCIN (*p*-PHENETYL CARBAMIDE)

A colorimetric method of determination depending upon quantitative hydrolysis to *p*-phenetidine followed by diazotisation and coupling with phenol has been proposed by Longwell and Bass[1].

Method[1]. A quantity of the solution to be examined, expected to contain about 0·01 g. of dulcin, is brought to a volume of 10 ml. by dilution or concentration as necessary, 50 ml. of 3N sulphuric acid added and the mixture boiled under a reflux during 4 hours. After allowing to cool, the liquid is rendered alkaline by the addition of a 40 per cent. w/v aqueous solution of sodium hydroxide and steam-distilled into 15 ml. of 0·1N hydrochloric acid, at least 500 ml. of distillate being collected. A suitable aliquot part of the distillate, say 20 ml., is diluted to 50 ml. with water, transferred to a 100-ml. Nessler glass containing 4 ml. of 0·1N hydrochloric acid, the liquid cooled to just under 10° C. and 1 ml. of a 1 per cent. aqueous solution of sodium nitrite added. After standing for 10 minutes, 0·5 ml. of a 1 per cent. aqueous solution of phenol is added followed by 3 ml. of an 8 per cent. aqueous solution of sodium hydroxide. The reddish-yellow colour produced is matched against a series of standards prepared by similarly acidifying, diazotising and coupling measured quantities, ranging by increments of 0·2 ml. from 2·5 to 4·5 ml., of a 0·01 per cent. aqueous solution of *p*-phenetidine hydrochloride. The result is multiplied by 1·037 in order to convert into terms of dulcin.

Application to Food and Beverages[1]. Samples containing either alcohol, essential oils, or both, should be saturated with salt and extracted with light petroleum. The latter is washed with water and the washings added to the main aqueous portion which is then extracted four times with ethyl acetate using 30 ml. on each occasion. These extracts, containing the dulcin, are transferred to the flask which is to be used for the hydrolysis and the solvent removed by distillation and drying in a current of air. The residue is taken up with 10 ml. of water and 50 ml. of 3N sulphuric acid and the hydrolysis and determination carried out as described above.

Discussion. Saccharin is not hydrolysed to any great extent under the conditions of the determination and, if present, would be mainly retained during the steam distillation : if, however, some does find its way into the final distillate it will not exercise any influence on the azo colour due to dulcin, nor will the determination be disturbed by ammonia. If sugar is present in the sample to be examined it is essential to apply the preliminary separation of dulcin by extraction with ethyl acetate. Although the colorimetric method is primarily of value for the estimation of small concentrations (0·1 to 0·2 per cent.) of dulcin added to food or

beverages it can be applied to the examination of the compound itself provided that any impurity present does not contain a primary amino group ; samples of dulcin are better examined by hydrolysing with 18N sulphuric acid and volumetrically determining the ammonia formed[2].

REFERENCES

1. J. Longwell and C. S. Bass, *Analyst*, 1942, **67**, 14.
2. J. F. Hirst, F. Holmes and G. W. G. Maclennan, *Analyst*, 1941, **66**, 450.

ETHYL ALCOHOL

See Section III : Substances of Clinical and Biochemical Significance, p. 214.

FORMALDEHYDE

A high degree of accuracy cannot be expected from any existing proposals for the colorimetric determination of this substance but three procedures capable of yielding approximate results warrant description. As a general method, the reaction of formaldehyde with phenylhydrazine hydrochloride in the presence of potassium ferricyanide to give a carmine-red coloured compound is the best[1]. For the special purpose of determining formaldehyde in pharmaceutical tablets Evers and Caines[2] recommend an application of Schiff's reaction for aldehydes using reduced rosaniline[3] while in the examination of milk for the presence of preservatives the addition of formalin is conveniently detected by the use of hydrochloric acid containing a small proportion of nitric acid as originally suggested by Shrewsbury and Knapp[4]. Eegriwe[5] has shown that when a trace of formaldehyde is treated with a mixture of 1 : 8-dihydroxynaphthalene-3 : 6-disulphonic acid (chromotropic acid) and moderately strong sulphuric acid and the mixture heated a violet colour is produced. This test has been applied quantitatively[6] but its utility is limited by the instability of the chromotropic acid reagent and the involved procedure necessary for its preparation.

Method Using Phenylhydrazine Hydrochloride (General)[1]. To

10 ml. of the solution to be examined, expected to contain between 0·005 and 0·15 mg. of formaldehyde is added 2 ml. of a freshly prepared 1 per cent. aqueous solution of phenylhydrazine hydrochloride, 2 ml. of a freshly prepared 2·5 per cent. aqueous solution of potassium ferricyanide followed by 3 ml. of concentrated hydrochloric acid. After allowing to stand for 5 minutes the intensity of the red colour produced is matched against standards similarly prepared from appropriate dilutions of formaldehyde solution of reagent quality (36 to 38 per cent. w/v).

Method Using Schiff's Reagent (Pharmaceutical Tablets)[2]. The reagent is prepared by triturating 0·2 g. of rosaniline hydrochloride (basic fuchsin or basic magenta) in a mortar with 10 ml. of water, pouring off the liquor, passing into the latter sulphur dioxide gas until it is completely saturated, allowing to stand for 24 hours and finally diluting with water to 200 ml. A tablet is weighed, transferred to a flask containing 200 ml. of water and the mixture boiled under a refluxing condenser for 30 minutes in order to convert any paraformaldehyde which may be present into formaldehyde. After allowing to cool, the liquid is diluted to 500 ml. with water and a portion of this solution filtered. A standard solution (containing 0·038 mg. formaldehyde per ml.) is made by diluting 1 ml. of 38 per cent. w/v reagent quality material to 1 litre with water. From this a set of standards is prepared by taking quantities of 0·1 to 1 ml. in ten test tubes and diluting each to 10 ml. with water while into another test tube is transferred 10 ml. of the filtered tablet solution. In a second rack, eleven test tubes of uniform bore each containing 2 ml. of the Schiff's reagent are set up, the contents of the tubes in the first rack then poured as quickly as possible into the second series of tubes and the latter well agitated. After standing for 3 minutes the purple colour of the test mixture is matched against that of the standards. The amount of formaldehyde in the tablet taken for assay is equivalent to $(a \times 0{\cdot}019)$ g. where a=ml. of standard solution required to match the colour produced by the sample.

Method Using Hydrochloric Acid with Nitric Acid (Milk)[4]. The reagent should be freshly made by mixing 0·1 ml. of concentrated nitric acid (or 1·6 ml. of N nitric acid) with 100 ml. of concentrated hydrochloric acid. To 5 ml. of the milk to be examined contained in a test tube is added 10 ml. of the reagent, the mixture vigorously shaken and the tube immersed for 10 minutes in a water-bath maintained at a temperature of 50° C. The tube and its contents are then cooled rapidly to about 15° C. A violet colour indicates

the presence of formaldehyde and, within a range of concentration between 0·2 and 6 p.p.m., its intensity is roughly proportional to the amount. If the colour obtained is deeper than that shown by a standard containing 6 p.p.m. of formaldehyde then the sample should be diluted with pure milk prior to applying a quantitative test.

Discussion. For most purposes Schryver's method using phenylhydrazine hydrochloride and potassium ferricyanide is the method of choice. The red colour produced is easy to match and is stable for some hours while the reaction is not given by acetaldehyde. It may be applied to the determination of formaldehyde added as a preservative to meat by heating in a boiling water-bath 10 g. of the minced sample with water to every 10 ml. of which has been added 2 ml. of a 1 per cent. solution of phenylhydrazine hydrochloride. After about 5 minutes the mixture is filtered through a plug of cotton-wool and the test as already described applied to an appropriate portion of the filtrate[7]. The procedure given for the examination of pharmaceutical tablets is not disturbed by the presence of lactose, sucrose or menthol.

For the determination of formaldehyde in milk the method of Shrewsbury and Knapp is undoubtedly superior to all others and, besides being capable of an approximately quantitative application, it possesses the particular advantage that the blank is quite white. Any colour developed as a result of dyes added to the milk is distinguishable by the fact of its appearance prior to the mixture being heated to 50° C. It should be noted that when formaldehyde is added to milk it gradually decomposes hence the amount found in any particular case may well be much below that actually added.

REFERENCES

1. S. B. Schryver, *Proc. Royal Soc.*, 1910, **82(B)**, 226.
2. N. Evers and C. M. Caines, *Yearb. Pharm.*, 1921, 321.
3. H. Schiff, *Compt. rend.*, 1865, **61**, 45.
4. H. S. Shrewsbury and A. W. Knapp, *Analyst*, 1909, **34**, 12.
5. E. Eegriwe, *Z. anal. Chem.*, 1937, **110**, 22.
6. M. J. Boyd and M. A. Logan, *J. Biol. Chem.*, 1942, **146**, 279.
7. G. S. Buchanan and S. B. Schryver, Local Government Board, Food Report No. 9, 1909.

FURFURALDEHYDE

The red colour produced by the reaction of aniline salts with furfuraldehyde was originally proposed by Tolman and Trescott as a test indicative of the pentosan content of vegetable material and applicable to distillates from plant tissues.[1] Besides being expeditious, the method, unlike that depending upon precipitation with phloroglucinol, does not respond to methyl-furfuraldehyde, hydroxymethyl-furfuraldehyde or formaldehyde. The general procedure given below is that recommended by Stillings and Browning[2] who have studied the effect of all the variable factors involved. In addition, applications to the direct determination of furfuraldehyde in vinegar[3] and to the detection and approximate estimation of Japanese mint oil in peppermint oils[4] are described.

Method[2]. An amount of the hydrochloric acid distillate, or other solution, expected to contain between 0·05 and 0·45 mg. of furfuraldehyde is transferred to a 100-ml. graduated flask, a drop of phenolphthalein indicator added and the liquid exactly neutralised with a 10 per cent. aqueous solution of sodium hydroxide. The mixture is diluted with water to approximately 40 ml. and, if necessary, sufficient sodium chloride added so that the total weight of salt in the flask is not less than 2 gm. To 50 ml. of glacial acetic acid, 5 gm. of freshly redistilled aniline is added from a specially calibrated pipette and this mixture is added to the neutralised solution under examination after the temperature of both has been adjusted to 20° C. The final mixture is diluted to 100 ml. with water, stored in the dark at 20° C. for 55 minutes and the intensity of the colour compared with that produced by standards similarly prepared. In this test comparisons are best made by using an instrument capable of providing an external standard such as a recording spectrophotometer or a Lovibond tintometer.

Application to Vinegar[3]. A reagent is made by dissolving 6 ml. of freshly redistilled aniline in 24 ml. of glacial acetic acid and diluting to 60 ml. with amyl alcohol. To 20 ml. of vinegar is added 10 ml. of the reagent and, after shaking, the mixture is allowed to stand in the dark for 15 minutes. Under these conditions the amyl alcohol separates as a distinct layer which will be coloured red if furfuraldehyde is present in the sample. The intensity of the colour produced is compared with that of standards similarly prepared.

Application to Peppermint Oil[4]. Exactly 0·1 ml. of the sample is mixed in a test tube with 5·0 ml. of a 2 per cent. solution of freshly redistilled aniline in glacial acetic acid, added from a burette. The reaction mixture is set aside in the dark for 10 minutes then the intensity of the red component of the orange colour is measured by means of a Lovibond tintometer using a 1-cm. cell. Genuine peppermint oil gives only a pale colour, the intensity varying slightly among individual samples and being least with oils of European origin, while Japanese mint oil (from *Mentha arvensis*) yields a colour about seven times as strong. The approximate proportion of the latter added as an adulterant to peppermint oil may be assessed from the intensity of the red component of the colour by reference to Table XXXVI.

TABLE XXXVI. RELATION BETWEEN THE PROPORTION OF JAPANESE MINT OIL PRESENT AND THE RED COMPONENT OF THE COLOUR PRODUCED (After D. C. Garratt, *Analyst*, 1935, **60**, 369)

Japanese Mint Oil per cent.	Colour observed in 1-cm. cell after 10 minutes Lovibond Red Units	
	American and English Peppermint Oils	French and Italian Peppermint Oils
nil	0·4 to 0·9	0·1 to 0·3
10	1·1 to 1·4	0·6 to 0·9
20	1·4 to 1·8	0·9 to 1·3
30	1·8 to 2·4	1·3 to 1·9
40	2·4 to 2·8	1·9 to 2·3
50	above 2·8	above 2·3

Discussion. The general method given above is stated by Stillings and Browning[2] to be sensitive to 0·1 part per million of furfuraldehyde. Neither malt nor wine vinegar contain furfuraldehyde but a slight reaction is given by cider vinegar while commercial distilled vinegars generally contain from 10 to 60 parts per million. It has been shown by Lampitt, Hughes and Trace[3] that furfuraldehyde only develops in vinegar when the latter is heated above 80° C., the production at 100° C. being appreciable and increasing with time, whilst acetic acid coloured with caramel shows no increase in content of furfuraldehyde under the same conditions.

In utilising the test for furfuraldehyde as it is applied to oil of peppermint caution should be exercised in the interpretation of results given by English oils in view of the wider range of colour

produced when the reaction is applied to genuine specimens, the value having been observed to vary from 0·4 to 1·2 red units compared with 0·4 to 0·6 for American and 0·1 to 0·3 for European oils. If Japanese mint oil be steam-distilled and the first 5 per cent. rejected the residual oil has a low content of furfuraldehyde, hence, by altering the method of fractionating Japanese oils, it may be possible to produce a mint oil adulterant which could not be detected by this method. In a further communication[5] D. C. Garratt shows that the same test may be applied to the detection of light camphor oil (9·2 red) in rosemary oil (0·8 red) or clove oil (23·0 red) in bay (1·4 red) and in pimento berry oil (1·1 red).

REFERENCES

1. L. M. Tolman and T. C. Trescott, *J. Amer. Chem. Soc.*, 1906, **28**, 1619.
2. R. S. Stillings and B. L. Browning, *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 499.
3. L. H. Lampitt, E. B. Hughes and L. H. Trace, *Analyst*, 1927, **52**, 260.
4. D. C. Garratt, *Analyst*, 1935, **60**, 369.
5. D. C. Garratt, *Analyst*, 1935, **60**, 595.

GLYCERYL TRINITRATE (NITROGLYCERIN)

The colorimetric determination of this substance is of value for the assay of medicinal tablets which, for official purposes, are required by the British Pharmacopœia, 1932, to contain 0·5 mg. in a chocolate basis. Wilfred Smith[1] has proposed a procedure which is an elaboration of that of Anderson[2] and is based upon the volatility of glyceryl trinitrate in steam, hydrolysis with sodium hydroxide, reduction of the nitrate, distillation of the ammonia produced, followed by its colorimetric determination using Nessler's reagent. An alternative method which was originally suggested by Scoville[3] and has been developed by Meek[4], utilises the phenol-disulphonic acid reaction for nitrates. The methods, both of which have given good results in the present author's hands, are described here, and either may well be adapted to the determination of small concentrations of glyceryl trinitrate present in other commodities.

Method by Determination as Ammonia (*for Tablets*)[1]. To five tablets contained in a 500-ml. Kjeldahl flask is added 25 ml. of a saturated aqueous solution of sodium sulphate, 75 ml. of water and sufficient N sulphuric acid to make the liquid just acid to

litmus paper. A still-head is attached and the mixture distilled just to dryness into a flask containing 10 ml. of 0·1N sodium hydroxide, the outlet tube of the condenser being kept below the surface of the alkali. The condenser and outlet tube are washed down, the sodium hydroxide solution is evaporated to dryness, 2 ml. of water added followed by 0·3 g. (±0·01 g.) of reduced iron and 2 ml. of 50 per cent. v/v sulphuric acid. The mixture is allowed to stand for 10 minutes, then boiled for 2 minutes and quantitatively transferred to a steam distillation apparatus by washing in with water. After the liquid has been rendered alkaline by the addition of 4 ml. of saturated aqueous solution of sodium hydroxide the liberated ammonia is steam-distilled into a flask containing 10 ml. of 0·1N sulphuric acid, the process being continued until the volume of distillate equals 500 ml. The ammonia in a suitable aliquot part of this distillate is then determined colorimetrically using Nessler's reagent as described on p. 382. A control experiment on the reagents is conducted in the same way excepting that in order to obtain a final colour which is deep enough to measure it will generally be necessary to concentrate the 500 ml. of distillate to 100 ml. The relative intensities of the colours may be determined by direct comparison in Nessler glasses but, more conveniently, by means of a colorimeter or tintometer. From the difference between the nitrogen content of the experimental distillate and the control the glyceryl trinitrate present can be calculated by applying the factor 5·40.

Method of Determination as Nitrate (*for Tablets*)[4]. A weighed portion of the powdered tablets, expected to contain about 1 mg. of glyceryl trinitrate, is transferred to a stoppered cylinder containing exactly 5 ml. of glacial acetic acid and shaken continuously for an hour. The mixture is filtered, 1 ml. of the filtrate transferred to a porcelain dish, 2 ml. of phenoldisulphonic acid added (see p. 145), the liquid stirred, allowed to stand for 15 minutes, diluted with 8 ml. of water and cautiously rendered alkaline with ammonia (10 per cent. aqueous solution). The resulting yellow liquid is transferred to a 25-ml. stoppered measuring cylinder and, when cool, the volume adjusted to 20 ml. and the temperature to 20° C. and the intensity of the colour matched against standards. The colour due to 1 mg. of glyceryl trinitrate may be reproduced by diluting 1 ml. of a 1 per cent. alcoholic solution to 50 ml. with glacial acetic acid and applying the test as described to 1 ml. of the resulting liquid. Alternatively, a standard colour of the same intensity may be prepared by transferring 1 ml. of a 0·045 per

cent. aqueous solution of silver nitrate to a porcelain dish, evaporating to dryness, adding phenoldisulphonic acid and proceeding as above.

Discussion. Of the two methods given, the one depending upon isolation as ammonia occupies much more time but, on the other hand, is likely to be amenable to more general application. The use of Devarda's alloy for reducing the nitrate should be avoided as it frequently contains traces of ammonia.

Potassium nitrate should not be employed for preparing standards for the phenoldisulphonic acid procedure described above as there is a tendency for the colours to be slightly too intense. It should be noted that the colours are sensitive to change of temperature, the values being about 10 per cent. higher at 25° C. than at 15° C. The 0·045 per cent. solution of silver nitrate may be prepared by diluting 26·4 ml. of 0·1N to 1 litre.

REFERENCES

1. W. Smith, *Quart. J. Pharm.*, 1935, **8**, 370.
2. E. L. Anderson, *J. Assoc. Off. Agric. Chem.*, 1932, **15**, 140.
3. W. L. Scoville, *Amer. J. Pharm.*, 1911, **83**, 359.
4. H. O. Meek, *Quart. J. Pharm.*, 1935, **8**, 375.

METHYL ALCOHOL

An approximate colorimetric determination of this substance depending upon its controlled oxidation to formaldehyde followed by addition of Schiff's decolorised rosaniline reagent[1] was originally suggested by Denigès[2] and has since been the subject of study by several investigators[3–7].

Method. The following special reagents are required :—

1. An Oxidising Solution made by dissolving 3 g. of potassium permanganate in a mixture of 15 ml. of phosphoric acid (89 per cent. w/w) and 70 ml. of water and diluting the solution to 100 ml. with more water.

2. A 5 per cent. w/v Solution of Oxalic Acid in a cooled mixture of equal volumes of sulphuric acid and water.

3. Decolorised Rosaniline Reagent made by dissolving 1 g. of powdered rosaniline hydrochloride (basic fuchsin or basic magenta) in 600 ml. of warm (not hot) water, allowing to cool, adding 10 g. of anhydrous sodium sulphite previously dissolved

in 100 ml. of water followed by 10 ml. of concentrated hydrochloric acid and finally diluting to 1000 ml. with water and allowing to stand, with occasional shaking, for 24 hours before use[7].

To 5 ml. of the liquid to be tested is added 2 ml. of the oxidising solution, the mixture allowed to stand for 10 minutes and then 2 ml. of the oxalic-sulphuric acid mixture added. The colourless liquid is now poured into 5 ml. of the decolorised rosaniline reagent contained in another tube. After allowing to stand for 10 minutes the colour is matched against standards prepared at the same time and in the same manner from solutions containing known concentrations of methyl alcohol, the quantity of the latter present in the final test mixtures being arranged to vary by convenient increments from 0·05 mg. upwards.

Discussion. At best this method can only be expected to yield approximate results. In a study of the test as it is applied to the detection of methyl alcohol present as impurity in ethyl alcohol Ballard and Hersant[7] have shown that the temperature at which the reaction is conducted has an important bearing upon the character and intensity of the colour produced. Thus at temperatures of 10° C. and less, an intense violet colour develops even in the absence of methyl alcohol while a pale green results at temperatures of 15° C. and upwards. In the presence of 0·2 mg. of methyl alcohol a pale blue-green is formed at ordinary room temperatures which, with varying quantities up to 4 mg., changes through blue tints to a deep magenta, while at 5° to 10° C. increasing intensities of violet are produced. Successive batches of Schiff's reagent, which should be stored away from light and not be more than a month old, are liable to vary in sensitivity according to the character of the dyestuff used and the concentration of sulphur dioxide. Dimethyl ether is stated[8] to give a response equivalent to 93 per cent. of that yielded by methyl alcohol while, according to Espinosa[9], normal amounts of aldehydes, *iso*-butyl, amyl and propyl alcohols, acetic and tartaric acids present as impurities in beverages do not interfere. On the other hand it is pointed out by Ballard and Hersant[7] that in testing pure ethyl alcohol a slight reaction is always obtained due partly to the incomplete inhibition of the reaction between acetaldehyde and the reagent, and also to the production of traces of formaldehyde on oxidation of ethyl alcohol. Glycerol must be absent as it gives a response equal to about one-tenth that due

to methyl alcohol. Schryver and Wood[10] recommend converting methyl alcohol into formaldehyde by controlled oxidation with ammonium persulphate followed by application of the phenylhydrazine-ferricyanide colour reaction described on p., 397 but as it is necessary to determine by trial the correct amount of persulphate needed for each individual case it has not been selected for description here. Unfortunately, other oxidation methods interfere with the subsequent application of the phenylhydrazine-ferricyanide colour test for formaldehyde.

REFERENCES

1. H. Schiff, *Compt. rend.*, 1865, **61**, 45.
2. G. Denigès, *Compt. rend.*, 1910, **150**, 529 and 832.
3. C. Simmonds, *Analyst*, 1912, **37**, 16.
4. T. von Fellenberg, *Biochem. Z.*, 1918, **85**, 45.
5. R. M. Chapin, *J. Ind. Eng. Chem.*, 1921, **13**, 543.
6. L. O. Wright, *Ind. Eng. Chem.*, 1927, **19**, 750.
7. C. W. Ballard and E. F. Hersant, *Quart. J. Pharm.*, 1942, **15**, 97.
8. B. F. Dodge, *Ind. Eng. Chem., Anal. Ed.*, 1932, **4**, 23.
9. N. S. Espinosa, *Anal. Asoc. Quím. Argentina*, 1930, **18**, 57.
10. S. B. Schryver and C. C. Wood, *Analyst*, 1920, **45**, 164.

OXYGEN (DISSOLVED, IN WATER)

The method, originated by Winkler[1], depending upon the oxidation of manganous hydroxide to the manganic state and the subsequent liberation of iodine from potassium iodide is the most satisfactory that has been proposed. While it is generally recommended that the quantity of iodine liberated be determined by titration with standard sodium thiosulphate, Rideal and Burgess[2] suggested the following simple colorimetric procedure.

Method[2]. For this determination about twenty uniform, white glass, narrow-mouthed, stoppered bottles having a known total capacity of approximately 130 ml. are required. One of these bottles is filled to within about 3·5 ml. of its total capacity with the water to be examined, 0·5 ml. of a nearly saturated aqueous solution of manganese chloride added, and then 1·5 ml. of a solution containing 30 per cent. of sodium hydroxide and 10 per cent. of potassium iodide. The stopper is inserted, the contents mixed and, after the oxidation of the manganous hydroxide has taken place and the precipitate has mostly settled down, the stopper is withdrawn and 1·5 ml. of concentrated hydrochloric acid added.

The stopper is again inserted, the contents of the bottle mixed and, if possible, the whole cooled slightly by immersion in a basin of cold water in order to obviate the tendency for the stopper to become loose due to the elevation of temperature occasioned by the addition of the acid. The intensity of any yellow colour, due to liberated iodine is matched in diffused daylight against standards prepared in similar bottles. In order to make the standards, 90 ml. of distilled water, 1·5 ml. of a 10 per cent. aqueous solution of potassium iodide and 0·15 ml. of concentrated hydrochloric acid are introduced in each of fifteen of the uniform white glass bottles. From these mixtures the correct amount of iodine is liberated by adding appropriate quantities of 0·0125N potassium permanganate (0·395 g. per litre, whence 10 ml. is equivalent to 1 mg. oxygen). For example, if the full capacity of the bottle is 128 ml. then 12·8 ml. of the permanganate solution is added in order to produce a tint which will correspond to 1 part by weight per 100,000 of dissolved oxygen. In this way, by adding proportionate amounts of permanganate, standards ranging from 0·1 to 1·5 parts per 100,000 can be prepared. When the proper amounts of permanganate have been run in, the bottles are filled up with distilled water, except for a small bubble of air, the stoppers tightly inserted and the contents mixed by agitation. Provided these standards are stored in the dark they will remain stable for at least a week or two and any liability to change may conveniently be checked by noting when sensible colour begins to develop in a " blank " containing only water, potassium iodide and hydrochloric acid.

Method Using Permanent External Standards. Two standard discs are available for use with the B.D.H. Lovibond Nessleriser the one marked " A " being designed for the examination of boiler feed water and covering the range from 0·05 to 1·0 ml. of dissolved oxygen per litre (0·007 to 0·14 part per 100,000) and the disc " B " containing standards from 0·04 to 0·16 part per 100,000 which is suitable for use in testing natural waters. The technique adopted is similar to, but not identical with, that described above and when the Nessleriser is used the test should be conducted with the aid of a solution of manganous chloride and a reagent containing potassium iodide and alkali which are supplied in ampoules especially for use with the instrument.

Discussion. Nitrite interferes with this colorimetric modification of the Winkler method but, since the reaction takes place in a solution from which free oxygen has been already removed by

manganous hydroxide, no trouble is experienced consequent upon nitric oxide acting as a carrier of oxygen; hence the reaction with nitrite will proceed stoichiometrically and its presence can be allowed for on the basis that 1·4 parts of nitrous nitrogen correspond to 0·8 part of oxygen. In cases where the sample contains appreciable quantities of trivalent iron it may perhaps be best to compensate for its influence by preparing standards with the water under test after the free oxygen has been removed by boiling.

REFERENCES

1. L. W. Winkler, *Ber.*, 1888, **21**, 2843.
2. S. Rideal and W. T. Burgess, *Analyst*, 1909, **34**, 193.

PHENOLS (IN WATER, URINE AND BLOOD)

The determination of traces of phenols is of importance in the control of road drainage into rivers, while the concentration of phenols in blood and urine is of some interest owing to their possible significance as an index of intestinal putrefaction. With regard to the first problem, several methods have been proposed of which that due to J. J. Fox and A. J. H. Guage[1, 2], which depends upon the formation of a yellow or orange dye when the mixed phenols are coupled with diazotised sulphanilic acid, is probably the best known. Other investigators have suggested the use of *p*-nitraniline[3, 4] in place of sulphanilic acid while Baylis[5] applied the Gibbs reaction[6] in which the phenols are converted into blue indophenols by reaction with 2 : 6-dibromoquinone chloroimide. This latter method, being subject to disadvantages as, for example, slow production of colour and excessive sensitivity to the reaction of the medium, led Houghton and Pelly[7] to develop an improved procedure in which the phenols are converted into indophenols by oxidation with hypochlorite in the presence of dimethyl-*p*-phenylenediamine. The coloured compounds thus produced, unlike those characterising the Gibbs method, are soluble in organic solvents whence a high degree of sensitivity is attainable by extracting the colour with carbon tetrachloride. Again, the colour develops satisfactorily in 3 minutes provided the reaction of the sample lies within the relatively wide range from *p*H 7·0 to 8·5. A description of this method, together with that of Fox and Guage, is included in this monograph.

With regard to the examination of urine, Folin and Denis[8] proposed a procedure based on the ability of phenols to reduce hexavalent tungsten and molybdenum to coloured products of lower valence but since uric acid also reduces in the same way it is necessary to provide for its separation by means of silver lactate. The original reagent was improved and rendered more specific by Folin and Ciocalteu[9] and this modification is given below. The most promising method for blood appears to be that due to Theis and Benedict[10] using diazotised *p*-nitraniline ; this has the advantage that it is not affected by uric acid, which therefore need not be removed.

Method for Water (Diazo Reaction)[1, 2]. The following special reagents are required :—

1. Sulphuric Acid (1 in 4) prepared by mixing 1 vol. of concentrated acid with 3 vols. of water.
2. Sulphanilic Acid Solution containing 1·91 g. in 250 ml. of water.
3. Sodium Nitrite Solution containing 0·85 g. in 250 ml. of water : this should be recently prepared.
4. Diazotised Sulphanilic Acid prepared immediately before use by mixing 5 vols. of the sulphanilic acid solution with 1 vol. of sulphuric acid (1 in 4), cooling to 4° C. and adding slowly 5 vols. of the sodium nitrite solution.
5. Strong Standard Solution of Cresols containing 1 g. of mixed cresols per litre, the mixture consisting of 35 per cent. *o*-cresol, 40 per cent. *m*-cresol and 25 per cent. *p*-cresol. This solution is stable if stored in a cool dark place.
6. Diluted Standard Solution of Cresols containing 0·05 mg. mixed cresols per ml. freshly prepared by diluting 5 ml. of the strong standard solution with 95 ml. of water. This standard gives an orange colour in the test.
7. Additional Standard Solution of Xylenols made from the fraction of the tar acids from coal tar boiling from 205° to 230°C. and adjusted to contain 0·05 mg. mixed xylenols per ml. This standard gives a deep orange colour in the test.
8. Additional Standard Solution of Naphthol containing 0·05 mg. per ml. This standard gives a red colour in the test.

A measured volume of the sample under examination (in the case of aqueous extracts of road dressings, 250 ml. is a suitable quantity) is acidified by the addition of 10 ml. of sulphuric acid (1 in 4) and extracted three times with a total of 100 ml. of chloro-

form. The combined chloroformic liquid is extracted by shaking with two portions each of 20 ml. of a 10 per cent. aqueous solution of sodium hydroxide and the mixed alkaline solutions are diluted with water to 100 ml., or other suitable volume. An appropriate aliquot portion of this solution is transferred to a Nessler glass, neutralised to litmus paper with sulphuric acid (1 in 4), diluted to 40 ml. with water, 5 ml. of the diazotised sulphanilic acid solution added followed by 5 ml. of an 8 per cent. aqueous solution of sodium hydroxide and the liquid mixed. The colour produced is matched against standards prepared by adding suitable quantities, not exceeding 2 ml., to 40 ml. of distilled water followed by 5 ml. of diazotised sulphanilic acid solution and 5 ml. of an 8 per cent. aqueous solution of sodium hydroxide. According to the hue of the colour produced by the sample so it may be necessary to choose one or the other of the three standard solutions listed above or, alternatively, a mixture of two or more may give a colour more closely resembling that yielded by the sample under examination.

Discussion. It should be noted that very dilute solutions of various tar products, *e.g.* phenol, the three cresols, higher boiling tar acids, as well as quinoline, *iso*-quinoline and naphthalene, in non-sterile water rapidly undergo biological change. For this reason the examination of samples of water for tar acids must be carried out at the earliest possible moment after collection. A delay of 48 hours may result in the oxidation of a considerable proportion of the tar products. The above test is capable of detecting less than 1 part per million of tar acids. The initial extraction serves to separate the tar phenols from vegetable tannins which, being composed of phenolic condensation products of various acids, possess hydroxyl groups in suitable positions for reacting with diazonium solutions. The shades of colour produced by vegetable extracts with diazotised sulphanilic acid are similar to those given by coal tar phenols, and it is therefore impossible to distinguish between the two classes by the application of the test above. Fox and Guage[2] conducted experiments in which aqueous extracts of various vegetable substances prepared by allowing 12 parts of London tap water and 1 part of vegetable débris to stand for 24 hours were found to contain phenolic substances to the following extent when determined by a direct application of the colour test (omitting the extraction with chloroform) using xylenols as standard : Bracken, 11 ; pear-tree leaves, 7 ; straw, 3 ; sawdust, 2 ; sphagnum moss, 0·4 ; turf,

0·15 ; all calculated in parts per 100,000 of extract. Weaker extracts prepared from 1 part of vegetable débris and 1000 parts of tap water showed proportions of phenolic substances varying from 0·02 to 0·07 part per 100,000. Peaty waters, flax-retting waters, and silts from pure streams were also found to contain small proportions of phenolic substances. River water which has been polluted by tar and in which the concentration of tar acids amounts to 1 part in 100,000 may well be fatal to fish life.

Method for Water (Diazo Reaction) Using Permanent External Standards. A disc is issued containing nine coloured glasses for use with the B.D.H. Lovibond Nessleriser. The series covers the range from 0·01 to 0·09 part per 100,000 equivalent to 0·005 to 0·045 mg. of tar acids in the 50-ml. volume employed in the final colour matching. An arbitrary mixture of phenols has been used for making the solutions on which the glass colour standards are based and generally these closely match the colours produced in testing aqueous extracts of road dressings and road drainage. The technique of the test adopted for use with the Nessleriser is precisely the same as described above.

Method for Water (Indophenol Reaction)[7]. The following special reagents are required :—

1. Dimethyl-*p*-phenylenediamine Reagent. A 0·1 per cent. solution of *p*-nitrosodimethylaniline is prepared by adding the requisite amount to water previously heated to just below boiling point ; after cooling, the solution is filtered and will then keep for at least a week. The diamine reagent is prepared immediately before use by shaking the above solution with a large excess of zinc dust until decolorisation is complete, the reduction being hastened by the addition of 1 drop of a 10 per cent. solution of copper sulphate for every 10 ml. of liquid. The excess of zinc is filtered off and the completed reagent then usually exhibits a pale pink tint which, however, does not appear to be detrimental.
2. Sodium Hypochlorite Solution prepared by mixing together about 60 g. of crystalline sodium carbonate, 40 g. of bleaching powder and 400 ml. of water, filtering and diluting the latter so that it contains 0·05 per cent. of available chlorine.
3. Strong Standard Solution of Phenol containing 1 mg. per ml. This should be standardised by titration with 0·1N bromate-bromide solution.

4. Diluted Standard Solution of Phenol containing 0·01 mg. per ml. freshly prepared by diluting 10 ml. of the strong standard solution to 1 litre with water.

It is always preferable to make a preliminary test in order to ascertain the approximate concentration of phenol present in the sample and the volume of hypochlorite solution required. When the phenol content is expected to be greater than 0·15 part per million 2 ml. of a 5 per cent. aqueous solution of sodium bicarbonate is added to 100 ml. of the sample followed by 2 ml. of the diamine reagent and the hypochlorite run in gradually from a burette, with gentle agitation. The first addition of hypochlorite results in the formation of a pronounced pink colour, but this is completely discharged on adding more hypochlorite and, in the presence of over 0·1 part per million of phenol, the colour of the solution changes to pure blue, owing to the formation of indophenol. No further addition of hypochlorite should be made once the solution is free from any red tinge since an excess of chlorine tends to bleach the blue colour; however, the titration may be overdone by 0·5 ml. without appreciably affecting the results. The approximate concentration of phenol in the sample is then determined by matching the blue colour obtained against the colour produced in a similar manner by a known volume of the diluted standard solution of phenol, the matching being made in Nessler glasses by the balancing-column method. To determine the phenol content accurately, the sample is diluted so as to contain 0·15 to 0·3 part per million of phenol and treated with sodium bicarbonate, diamine reagent and hypochlorite in the manner described above and the blue colour produced matched exactly against one of a set of appropriate standards. The comparisons should be made after allowing the mixtures to stand for about 3 minutes.

When the phenol content is less than 0·15 part per million, 200 ml. of the sample is transferred to a separator followed by 4 ml. of a 5 per cent. aqueous solution of sodium bicarbonate and 4 ml. of the diamine reagent. The correct quantity of hypochlorite solution (already ascertained from a preliminary test) is then gradually run in so that the pink colour which first appears is just discharged. The indophenol is now extracted with two 10-ml. portions followed by one 5-ml. portion of carbon tetrachloride and the extracts are dried by the addition of a little anhydrous sodium sulphate. The aqueous solution is finally

extracted with a further 5 ml. of carbon tetrachloride which is also used to wash out any indophenol remaining on the sodium sulphate used for drying the previous extracts. The dry extracts are combined, diluted to a known convenient volume and some of the liquid transferred to a Nessler glass or a tube of suitable bore and matched against standards prepared in precisely the same manner.

Discussion. The tint of the indophenols produced from waters polluted with tar acids is generally sufficiently near to that of a phenol standard for satisfactory comparison but cases may arise where it is preferable to employ the xylenol standard already mentioned in the description of the diazo method. The colour of the indophenol solutions obtained from a few of the higher phenols is given in Table XXXVII from which it will be seen that no

TABLE XXXVII. COLOUR OF INDOPHENOLS IN WATER AND IN CARBON TETRACHLORIDE (After G. U. Houghton and R. G. Pelly, *Analyst*, 1937, **62**, 117)

Phenol	Colour of Solution in Water	Colour of Solution in Carbon Tetrachloride
Phenol	Blue	Purple
o-Cresol	Deep blue	Purple
m-Cresol	Deep blue	Purple
p-Cresol	No reaction	—
m-Xylenol	Pale blue	Blue
α-Naphthol	Purple	Purple
β-Naphthol	Very pale green	Green
o-Chlorophenol	Turquoise blue	Blue
p-Chlorophenol	Pale blue	Pale purple
Trichlorophenol	No reaction	—

reaction is obtained with *p*-cresol. This must be regarded as a severe limitation on the utility of the method since it is obvious that in certain circumstances low results will be obtained. This defect also characterises methods based upon the Gibbs reaction[6]. Again, the sensitivity of the indophenol method varies considerably for different phenolic substances and, of those examined by Houghton and Pelly[7], is highest with *o*-cresol and lowest with β-naphthol. Primary aromatic amines interfere since they give coloured indamines ; aniline, *o*-toluidine, *m*-toluidine, *m*-xylidine and the naphthylamines all react, but, with the exception of α-naphthylamine, the sensitivity is equivalent to only about 1 part per million compared with 0·01 part per million for phenols when working on a 200-ml. sample. α-Naphthylamine may be recognised by the magenta-coloured solution which its indamine forms in carbon tetrachloride solution. Except in special cases, these

amines are unlikely to be present in water unless accompanied by a large excess of phenol.

Method for Urine[8, 9]. Reagents are required as follows :—

1. A 3 per cent. Solution of Silver Lactate in a 3 per cent. aqueous solution of lactic acid ; the latter may be prepared by diluting 3·5 ml. of ordinary concentrated acid (s.g. 1·21) to 100 ml. with water.

2. Acid Sodium Chloride : a saturated solution of sodium chloride each litre of which contains 10 ml. of concentrated hydrochloric acid.

3. Folin and Ciocalteu's Reagent[9]. This is made by dissolving 100 g. of sodium tungstate, $Na_2WO_4.2H_2O$, and 25 g. of sodium molybdate, $Na_2MoO_4.2H_2O$, in 700 ml. of water, contained in a 1500-ml. flask adding 50 ml. of phosphoric acid (s.g. 1·75) and 100 ml. of concentrated hydrochloric acid. The flask is connected to a refluxing condenser by means of a ground-glass joint or by using a rubber stopper wrapped in tin foil*, the mixture boiled for 10 hours then 150 g. of lithium sulphate, 50 ml. of water and a few drops of bromine added. The boiling is continued without a condenser for 15 minutes, the golden-yellow solution allowed to cool and diluted to 1 litre.

4. Strong Standard Solution of Phenol containing 1 mg. per ml. This should be standardised by titration with 0·1N bromate-bromide solution.

5. Diluted Standard Solution of Phenol containing 0·1 mg. per ml. freshly prepared by diluting 10 ml. of the strong standard solution to 100 ml. with water.

(*Free Phenols*). To 10 ml. of the sample contained in a 50-ml. graduated flask is added the acid solution of silver lactate until no further precipitation occurs (say from 2 to 20 ml.) then about 0·5 ml. of dialysed iron solution is introduced and the mixture diluted to the mark with water. The contents of the flask are passed through a dry filter, 25 ml. of the filtrate is transferred to another 50-ml. graduated flask, sufficient acid sodium chloride solution added to precipitate all the silver, the mixture diluted to the mark with water and the contents of the flask filtered through a dry paper. To apply the colour test, 20 ml. of the filtrate is transferred to a third 50-ml. graduated flask, 5 ml. of Folin and Ciocalteu's phenol reagent added followed by 15 ml. of a saturated aqueous

* If tin foil is employed care must be taken to ensure that the solution does not come into contact with the metal.

solution of sodium carbonate and the mixture finally diluted to the mark with water previously warmed to 35° C. After 20 minutes the intensity of the blue colour produced is compared by means of a colorimeter with that given by treating 5 ml. of the diluted standard solution of phenol with 10 ml. of Folin and Ciocalteu's reagent, 25 ml. of a saturated aqueous solution of sodium carbonate and diluting to 100 ml. with warm water.

(*Total Phenols*). To 20 ml. of the filtrate obtained after precipitating the excess silver with acid sodium chloride solution is added 0·5 ml. of concentrated hydrochloric acid, the tube containing the mixture covered with a watch glass, the liquid heated rapidly to boiling over a free flame and the tube then immersed in a boiling water-bath for 10 minutes. At the end of this time the contents of the tube are cooled, transferred to a 100-ml. graduated flask, 10 ml. of Folin and Ciocalteu's reagent added followed by 25 ml. of a saturated aqueous solution of sodium carbonate and the mixture finally diluted to the mark with water previously warmed to 35° C. After 20 minutes the colour produced is compared with that of a standard prepared as already described under the determination of the free phenol.

It will be seen from the above description that in the determination of free phenols the final colour solution contains the equivalent of 2 ml. of the original sample in 50 ml. of liquid while, in the case of the total phenol determination the equivalent of 2 ml. of the sample is contained in 100 ml. of the final colour solution. In each case the standard colour solution contains 0·5 mg. of phenol in 100 ml. The concentration of combined phenol is found by difference.

Method for Blood[10]. Special reagents are required as follows :—

1. *p*-Nitraniline Hydrochloride Solution made by dissolving 1·5 g. of the base in a mixture of 460 ml. of water and 40 ml. of concentrated hydrochloric acid.

2. Diazotised *p*-Nitraniline Solution freshly prepared by adding 0·75 ml. of a 10 per cent. aqueous solution of sodium nitrite to 25 ml. of the *p*-nitraniline hydrochloride solution.

3. Strong Standard Solution of Phenol containing 1 mg. per ml. This should be standardised by titration with 0·1N bromate-bromide solution.

4. Diluted Standard Solution of Phenol containing 0·0025 mg. per ml. freshly prepared by diluting 2·5 ml. of the strong standard solution to 1 litre with water.

(*Free Phenols*). A protein-free filtrate is prepared by mixing 5 ml. of the sample with 10 ml. of water, slowly adding 5 ml. of 2/3N sulphuric acid and, after shaking, adding 5 ml. of a 10 per cent. aqueous solution of sodium tungstate, mixing and passing through a dry filter. To 10 ml. of the filtrate is added 1 ml. of a 1 per cent. solution of gum acacia, 1 ml. of a 50 per cent. aqueous solution of sodium acetate, $NaC_2H_3O_2.3H_2O$, and 1 ml. of the diazotised *p*-nitraniline solution. After standing for 1 minute, 2 ml. of a 20 per cent. aqueous solution of sodium carbonate, Na_2CO_3, is added and the reddish colour produced is compared by means of a colorimeter with the colour due to similarly applying the test to 10 ml. of the diluted standard solution of phenol.

(*Total Phenols*). Another 10 ml. of the protein-free blood filtrate prepared as above is transferred to a large test tube, 0·25 ml. of concentrated hydrochloric acid is added, the tube covered with a watch glass, the liquid heated rapidly to boiling over a free flame and the tube then immersed in a boiling water-bath for 10 minutes. The solution is cooled, neutralised with solution of sodium hydroxide and the colour test applied as already described for the determination of free phenols starting with the addition of 1 ml. of gum acacia solution. The same quantities of acid and alkali should be added to the standard solution before applying the colour test.

Discussion. Phenols occurring in the blood and bodily excretions constitute part of the oxidation products of amino acids of the aromatic series such as tyrosine, tryptophane and phenylalanine. The blood of normal individuals appears to contain from about 1·7 to 3·2 mg. per ml. of total phenols. At the present time little clinical significance can be attached to the phenol concentration of either blood or urine since the cause of variations has not been elucidated.

REFERENCES

1. J. J. Fox and A. J. H. Guage, *J. Soc. Chem. Ind.*, 1920, **39**, 260T.
2. J. J. Fox and A. J. H. Guage, *J. Soc. Chem. Ind.*, 1922, **41**, 173T.
3. S. Hilpert and R. Gille, *Angew. Chem.*, 1933, **46**, 326.
4. T. Folpmers, *Chem. Weekblad*, 1934, **31**, 330.
5. J. R. Baylis, *J. Amer. Water Works Assoc.*, 1928, **19**, 597.
6. H. D. Gibbs, *J. Biol. Chem.*, 1927, **72**, 649.
7. G. U. Houghton and R. G. Pelly, *Analyst*, 1937, **62**, 117.
8. O. Folin and W. Denis, *J. Biol. Chem.*, 1915, **22**, 305.
9. O. Folin and V. Ciocalteu, *J. Biol. Chem.*, 1927, **73**, 627.
10. R. C. Theis and S. R. Benedict, *J. Biol. Chem.*, 1924, **61**, 67.

RANCIDITY OF FIXED OILS AND FATS

Since Kreis described his test for rancidity in oils and fats[1] many investigators have attempted to give it a quantitative significance indicating the degree of deterioration. The original test depends upon the production of a red colour when an ethereal solution of phloroglucinol is shaken with an oxidised fat treated with concentrated hydrochloric acid. According to Powick[2] the substance present in rancid fats which gives rise to the red colour is epihydrinaldehyde. This compound can be produced by the oxidation of an aqueous solution of acrolein with hydrogen peroxide[3] and the pure substance has been described as a volatile, unstable compound melting considerably below 0° C. but it is thought to be present in autoxidised fat in a bound and non-volatile form[4]. Suggestions for rendering the test quantitative have included the substitution of an alcoholic solution of phloroglucinol for the original reagent using ether as solvent[5], while Pyke recommends acetone and quotes a relation between the intensity of the colour obtained as expressed in Lovibond red units and the percentage concentration of epihydrinaldehyde[6]. Lampitt and Sylvester[7] use a modification of Kerr's technique[8] in which the oil, dissolved in light petroleum, is shaken with concentrated hydrochloric acid and an ethereal solution of phloroglucinol and the intensity of colour of the separated acid layer measured in a 2-cm. cell by means of a Zeiss photometer, a combination of light filters transmitting sharply at 5400Å being employed. By substituting trichloracetic acid for hydrochloric acid and amyl acetate as a common solvent for the oil and the phloroglucinol W. P. Walters, M. M. Muers and E. B. Anderson[9] conduct the reaction in one phase and thus effect an increase in the sensitivity of the test. This method and the procedure originated by Pyke have been selected for description here but it may be appropriate to observe that many analysts remain sceptical of the validity of colorimetric expressions of the degree of rancidity and still favour Kerr's practice[8] of stating the limiting dilution capable of giving a perceptible tint.

Method of Pyke[6]. The oil (0·5 to 1 g.) is dissolved in sufficient of a 1 per cent. solution of phloroglucinol in acetone to give a 10 per cent. solution. A number of drops of concentrated sulphuric acid equal to the number of ml. of solution are added and the mixture is placed in a water-bath at 10° C. for 15 minutes. The intensity

of the resulting red colour is measured by means of a Lovibond tintometer using a 1-cm. cell the percentage of epihydrinaldehyde present in the sample being given by the expression $\frac{R-0{\cdot}625}{97}$, where R represents Lovibond red units.

Discussion. Traces of water inhibit the reaction while increase of temperature considerably intensifies the colour produced. At room temperature the colour was observed to develop linearly with time of standing up to a period of at least 1 hour. If a precipitate is produced in the reaction mixture, as may happen when the oil or fat has been freshly extracted from a cereal product by means of ether, the solution should be filtered through a dry paper into the tintometer cell.

Method of Walters, Muers and Anderson[9]. Kreis values are expressed in terms of E for Zeiss-Pulfrich photometer observations or T if the Lovibond tintometer is used. $E=Z/lc$ and $T=R/lc$ where Z is the photometer reading, $\log_{10}(I_0/I)$, R is red units on the tintometer, l is cell length in cm. and c is concentration of oil in gm. per ml. of final solution. A convenient filter for use with the photometer consists of a combination of Wratten No. 62 Mercury Green and Wratten No. 45H: this transmits a fairly narrow symmetrical band around 5400Å. About 3 ml. of the melted fat or oil is weighed into a test tube, 1 ml. of a 0·5 per cent. w/v solution of phloroglucinol in amyl acetate is added, the mixture vigorously stirred (or air-bubbled through for 1 minute), then 2 ml. of trichloracetic acid solution (made by dissolving x gm. of the acid in $0{\cdot}382x$ ml. of amyl acetate) added and the tube immersed in a water-bath at 45° C. ($\pm 0{\cdot}1$° C.) for 15 minutes the contents being continuously stirred or agitated by a stream of air. The tube is removed and the contents immediately diluted with 10 ml., or more, of an ice-cold solution made by diluting 1 vol. of the above trichloracetic acid solution with 2 vols. of amyl acetate. The intensity of the colour is measured at once and expressed in the units E or T: if the tintometer is used the dilution of the test and the length of cell are so chosen that the intensity of the colour measured does not exceed 5 Lovibond red units. Along with the actual test a blank is conducted using amyl acetate in place of the phloroglucinol solution and the tintometer reading due to the blank subtracted from the value found for the sample; except for highly coloured oils the correction is usually quite small or even negligible. If the photometer is used the blank solution is introduced into the compensating cell; a further

correction may be made for any colour produced by interaction of acid and phloroglucinol and in this connection a maximum photometer reading (compared with distilled water) of 0·06 to 0·07 unit per cm. has been observed.

Discussion. It is claimed by the originators that the above test will detect oxidation at a very early stage and fresh fats and oils that have not been stored under oxygen-free conditions give a positive reaction. Pure methyl oleate, freshly distilled *in vacuo* in absence of oxygen, gave a negative result, as did oleic acid prepared from it, while beef kidney fat removed from a freshly killed animal and kept in an oxygen-free atmosphere before clarifying at as low a temperature as possible, behaved similarly. After a few hours' exposure to the air all gave weak positive reactions. Applying the method to a sample of fresh butter fat Walters, Muers and Anderson observed a value for T of 20·2 (E 1·38) while for a slightly tallowy specimen T was 28·8 (E 2·02) and for a butter fat which tasted very tallowy T was found to be 60·7 and E 4·31. It was also noted that the Kreis value does not increase indefinitely with oxidation. An experiment is quoted in which a fresh butter fat was aërated at a temperature of 65° C. during a period of about 200 hours, portions being removed at intervals for the determination of T, which quickly rose after 50 hours from about 20 to 215, then to 530 when aëration had proceeded for 70 hours; thereafter the value of T rapidly diminished to about 25 after 130 hours and was still slowly falling when the last determination was made 200 hours after the commencement of the trials.

REFERENCES

1. H. Kreis, *Chem.-Ztg.*, 1902, **26,** 897 and 1014.
2. W. C. Powick, *J. Agric. Res.*, 1923, **26,** 323.
3. J. Pritzker and R. Jungkunz, *Z. Unters. Lebensm.*, 1929, **57,** 419.
4. K. Täufel and F. K. Russow, *Z. Unters. Lebensm.*, 1933, **65,** 540.
5. A. Taffel and C. Revis, *J. Soc. Chem. Ind.*, 1931, **50,** 87T.
6. M. A. Pyke, *Analyst*, 1935, **60,** 515.
7. L. H. Lampitt and N. D. Sylvester, *Biochem. J.*, 1936, **30,** 2237.
8. R. H. Kerr, *J. Ind. Eng. Chem.*, 1918, **10,** 471.
9. W. P. Walters, M. M. Muers and E. B. Anderson, *J. Soc. Chem. Ind.*, 1938, **57,** 53.

SACCHARIN (*o*-BENZOIC-SULPHONE-IMIDE)

On treating saccharin with hot dilute mineral acids the ammonium salt of sulpho-benzoic acid is formed, whence small quantities of the sweetening agent may be determined by appli-

cation of Nessler's reaction. After studying all the known colour reactions, A. F. Lerrigo and A. L. Williams concluded that hydrolysis followed by determination of the ammonia produced constituted the most satisfactory basis for evaluating products with respect to their content of saccharin and they developed the best conditions for carrying out the procedure[1].

Method[1]. The solution to be examined, preferably containing between 0·5 and 1 mg. of saccharin, is acidified with dilute hydrochloric acid and shaken with ether. After separation, the aqueous layer is drawn off and again extracted with another portion of ether, the two ethereal solutions transferred to a tared 100-ml. flask and the solvent removed by distillation. After drying at 100° C. for an hour, the weight of residue is determined and a known quantity of pure saccharin rather less than the weight of the ethereal extract is introduced into another 100-ml. flask and 25 ml. of 3N hydrochloric acid is added to each. The flasks are covered by watch glasses and placed on a boiling water-bath for 2 hours. After cooling, the solutions are made alkaline to litmus paper by the addition of 3N sodium hydroxide, diluted to 100 ml. with water and the ammonia content of the two solutions compared by treating suitable aliquot parts with Nessler's reagent in the usual manner as described on p. 382.

Application to Food and Beverages[1]. For liquid samples 50 ml. is transferred to a 100-ml. flask, 25 ml. of boiling water added followed by 3 ml. of glacial acetic acid. After mixing, a slight excess of a 20 per cent. aqueous solution of lead acetate is added, the whole diluted to 100 ml. and filtered. A suitable portion of the filtrate is used as the solution to be examined, the procedure already described being applied. Solid foods are similarly treated with boiling water, the resulting mixture being acidified with acetic acid and clarified with lead acetate prior to extraction of the saccharin with ether. Alcoholic beverages should be reduced to half their original volume by evaporation before the clarification process is applied.

Discussion. The colour produced when a solution of hydrolysed saccharin is treated with Nessler's reagent is slightly different from that given by a solution of ammonium chloride, hence it is always advisable to employ saccharin itself as the standard of reference. Since, at best, this method can only be expected to attain an accuracy of ± 10 per cent. there is nothing to be gained by distillation of the ammonia prior to its colorimetric determination. The amount of saccharin added to foodstuffs will, of course, vary

considerably according to the character of the product and the degree of sweetness which it is desired to attain, but as a guide to the amount of material required to be taken for effecting satisfactory determinations it may be observed that the addition of 0·02 per cent. will cause most foods to taste quite sweet. In commercial practice it is customary to describe pure *o*-benzoic-sulphone-imide as "Saccharin 550" and the water-soluble sodium salt, $CO.C_6H_4.SO_2NNa.2H_2O$, as "Saccharin 500," the presumption being that the numerals indicate the sweetening power on the basis that the sweetness of sucrose is represented by unity. Actually, the relative sweetness varies quite considerably according to the food or beverage that is flavoured but, apart from the uncertainty thus introduced, if *o*-benzoic-sulphone-imide be taken as 550 then soluble saccharin, meeting all the requirements of the British Pharmacopœia and containing not less than 98 per cent. of the sodium salt, would be more correctly described as "Saccharin 409."

REFERENCE

1. A. F. Lorrigo and A. L. Williams, *Analyst*, 1927, **52**, 377.

TANNIN AND GALLIC ACID

A useful colorimetric method, devised by C. A. Mitchell[1], is based upon the fact that the presence of a tartrate causes ferrous sulphate to react immediately with gallotannin to form a soluble compound which, unlike the ink produced by ferrous sulphate alone, is fairly stable. The coloration ranges from reddish-violet in a very dilute solution to bluish-violet in one which is relatively more concentrated and its intensity is approximately proportional to the amount of tannin substance present. The results may be expressed either in terms of pyrogallol, crystalline gallic acid, $C_6H_2(OH)_3COOH.H_2O$ or anhydrous gallic acid, for in each instance the pyrogallic group is the tinctogenic agent, the water of crystallisation and the carboxyl group or (in the case of gallotannin) any glucose present, merely serving to dilute that group. Mitchell has also shown that osmium tetroxide gives a violet colour with compounds containing three adjacent hydroxyl groups such as gallotannin, gallic acid and pyrogallol, and also with catechol, and a procedure based upon this reaction serves as an alternative method for the colorimetric determination of tannin.[2]

Method Using Ferrous Tartrate[1]. A standard 0·1 per cent.

aqueous solution of gallic acid is prepared from material previously freed from water of crystallisation by drying at 100° C. A standard coloration is made by adding 1 ml. of this solution to 95 ml. of water, conveniently contained in a Nessler glass provided with a Hehner side tubulure and tap, diluting to 100 ml. and adding 2 ml. of ferrous tartrate reagent made by dissolving 0·1 g. of crystalline ferrous sulphate and 0·5 g. of sodium potassium tartrate in 100 ml. of water. A suitable quantity of the solution under examination is similarly treated and the colours so produced matched by appropriately diminishing the height of the more strongly coloured liquid.

Application to Galls[1]. An average sample is crushed and 5 g. of the resulting powder extracted by treating with several successive portions of about 150 ml. of water, boiling for 1 hour each time, filtering, and diluting the mixed extracts to 500 ml. A ten times dilution of this solution is made and 1 ml. of the resulting 0·1 per cent. extract taken for the colorimetric determination of the total tinctogenic substances in terms of gallic acid using the method described above. The gallic acid present as such in the 0·1 per cent. aqueous extract is then determined by precipitating the gallotannin in 10 ml. with a slight excess of an aqueous solution of quinine hydrochloride, adding a little Spanish clay to assist coagulation of the quinine tannate, allowing to stand for 5 minutes, filtering, washing the precipitate with several small portions of water, diluting the combined filtrate and washings to 100 ml. and applying the colorimetric determination to a suitable aliquot part of this solution. The value thus found is subtracted from the figure for total gallic acid and the result converted to tannin by applying the factor 1·85 for Aleppo galls or 2·10 for Chinese or British galls.

Application to Myrobalans and Roasted Galls[1]. The yellow colour from such products interferes, to some extent, with the colorimetric method. As it is difficult to eliminate the colouring matter without also removing some of the tannin it is necessary to make a correction by adding a trace of caramel or dyestuff to the standards prior to the addition of the ferrous tartrate reagent. The factor for converting gallic acid into chebulinic acid, the tannin of myrobalans, is 2·14.

Discussion. The ferrous tartrate reagent does not give any coloration with phenol or salicylic acid or other compounds containing either one or two hydroxyl groups. In a summary of his work Mitchell[3] writes : " . . . in commerce it is generally accepted

that ' white ' galls (*i.e.* those from which the larvæ have escaped) are less valuable than blue or green galls, which still contain the larvæ. From the point of view of the ink-maker, however, much will depend upon the degree of oxidation which has taken place within the galls. For example, the green galls in a commercial sample contained 61·8 per cent. of tannin and 2·5 per cent. of gallic acid, whilst the white galls in the same sample contained 52·9 per cent. of tannin and 7·4 per cent. of gallic acid. The total tinctogenic values, however, were 23·9 and 24·0 respectively, so that whilst the tannin had decreased, the gallic acid had correspondingly increased, and the value of either to the ink manufacturer (though not to the tanner) was the same.

" Roasted galls are a commercial product and are valued because they yield a darker ink. The effect of this process of heating to about 220° C. is to convert some of the gallotannin first into gallic acid and then into pyrogallol. Thus a sample of the green galls, mentioned above, was found, after being roasted, to contain only 37·0 per cent. of tannin, whereas the gallic acid and pyrogallol (in terms of gallic acid) amounted to 20·0 per cent., and the total tinctogenic value (in terms of pyrogallol) was 26·6. Hence, roasting the galls reduces the amounts of soluble extract and gallotannin, but greatly increases the proportions of non-tannin tinctogenic substances."

Nicholson and Rhind recommend that the matching of the colours be conducted by means of artificial light filtered through dark blue crinkled paper[4]. P. H. Price[5] attempted to extend the method to the determination of the catechol tannins but could not obtain satisfactory results ; however, it was later shown by Glasstone[6] that maximum colour production is subject to the reaction of the medium, that due to catechin being limited to *p*H 7·0 to 7·5 while full colours are given by catechol and protocatechuic acid at *p*H 7·0 to 10·3 and *p*H 6·3 to 10·4 respectively.

Method Using Osmium Tetroxide[2]. The reagent consists of the ordinary 1 per cent. solution of " osmic acid " (as used for microscopical work) diluted ten times with water and a suitable standard for comparison is made by adding 1 ml. of the resulting reagent to 100 ml. of a solution containing 1 mg. of pyrogallol, catechol, or gallic acid. The violet colour is allowed to develop for 5 minutes and then compared with that produced by an appropriate portion of the sample solution which has been similarly treated.

Application to Tea[2]. A weighed quantity, i.e. 1 g., is extracted by treating with several successive portions of boiling water under

a reflux condenser during 90 minutes, the united extracts diluted to a known volume and the tannin determined by the above method using anhydrous gallic acid as the standard. Another portion of the extract is precipitated with quinine hydrochloride as described under the application of the ferrous tartrate method to galls and the proportion of gallic acid in the filtrate determined with the osmium tetroxide reagent; the difference in the two values so obtained is converted to tannin by multiplying by the factor 1·10.

Application to Coffee and Hops[2]. The method as given for tea is applicable to these commodities, the factor for the conversion of gallic acid into the tannin of coffee being the same as that employed for tea while, for conversion to lupulo-tannin, the content in terms of gallic acid is multiplied by 2·65.

Discussion. Osmium tetroxide gives no coloration with phenol, salicylic acid, phloroglucinol or resorcinol. Unlike ferrous tartrate it does not give different colorations with pyrogallol and catechol, but produces a similar violet tint with each thus enabling simultaneous determinations to be made of the two compounds in the presence of each other, whence the results can be expressed in terms of either. Pyrogallol reacts much more rapidly than catechol, but in both cases the full colour develops in 5 minutes and begins to darken in 15 minutes, presumably due to the absorption of oxygen. The factors employed in this method for the conversion of gallic acid into the various tannins are empirical. A sample of China tea examined by Mitchell[2] was found to contain 5·3 per cent. of tannin and the corresponding value for a specimen of Ceylon tea was 10·0 per cent. The same investigator reported the tannin content of a sample of raw Costa Rica coffee as 2·3 per cent. which fell to 1·7 per cent. on roasting while a consignment of raw Nairobi coffee contained 1·9 per cent. which was reduced to 1·2 per cent. by the same treatment. The method is not applicable to the determination of tannin in cutch as the extracts of this substance give a colour with osmium tetroxide which differs from that given by catechol, pyrogallol or gallic acid and no exact comparison is possible.

REFERENCES

1. C. A. Mitchell, *Analyst*, 1923, **48**, 2.
2. C. A. Mitchell, *Analyst*, 1924, **49**, 162.
3. C. A. Mitchell, "Recent Advances in Analytical Chemistry," Vol. I, London: J. & A. Churchill, 1930, p. 185.
4. W. N. Nicholson and D. Rhind, *Analyst*, 1924, **49**, 505.
5. P. H. Price, *Analyst*, 1924, **49**, 361.
6. S. Glasstone, *Analyst*, 1925, **50**, 49.

INDEX TO AUTHORS

ABDERHALDEN, E., 213
Adair, F. L., 310
Adams, C. A., 378
Agnew, W. J., 40, 59
Albanese, A. A., 277
Albright, F., 335
Alfthan, K., 393
Allen, H. O., 88
Allen, J., 319, 326
Allport, N. L., 50, 70, 126, 298, 299, 301, 310, 315, 316, 318, 377
Almy, L. H., 174, 175, 176
Alt, A., 278
Alten, F., 12, 38, 72
Ammundsen, E., 232
Amor, A. J., 166
Amos, A. J., 370
Andersen, A., 350, 358
Anderson, A. B., 70
Anderson, A. K., 179
Anderson, E. B., 417, 419
Anderson, E. L., 402
Andreasch, R., 59
Andrews, J. C., 214
Andrews, K. C., 214
Andrews, L., 59
Anwyl-Davies, T., 203
Archer, H. E., 279
Armstrong, A. R., 250, 252
Armstrong, W. D., 127
Arnold, V., 211
Aschaffenburg, R., 250, 256, 257, 258, 259
Ashley, S. E. Q., 95
Askew, H. O., 46
Atack, F. W., 12, 46
Atkins, W. R. G., 151
Aubrey, P., 36
Auerbach, M. E., 360
Aumonier, F. S., 27
Autenrieth, W., 260, 266

BABBITT, D., 154, 273
Bach, A., 81
Bach, J. M., 118
Bachman, C., 289, 291
Bainbridge, H. W., 38
Baker, L. C., 214
Ball, W. C., 137
Ballard, C. W., 405
Bamford, F., 287, 289, 299
Bambach, K., 70
Bandier, E., 346
Barany, F., 370
Barbour, R. F., 199
Barger, G., 302, 370
Barkan, G., 59
Barker, J. H., 326
Barker, P., 165
Barker, S. B., 245
Barrenscheen, H. K., 102
Barron, E. S. G., 214
Bass, C. S., 395
Baubigny, H., 196
Baxter, J. G., 351
Baylis, J. R., 408
Beal, D., 247
Beale, J. F., 384
Beam, W., 12
Beaumont, G. E., 196
Behre, J. A., 209, 210
Bell, R. D., 156
Benedetti-Pichler, A. A., 216
Benedict, S. R., 209, 210, 219, 222, 223, 283, 409
Bennekou, I., 310
Bennett, R. R., 182
Benoit, C., 213
Berenblum, I., 161
Bergel, F., 370
Berger, K. C., 137
Bergeret, —, 16
Bergh, A. A. Hijmans van den, 193, 195
Bergheim, O., 371
Bergheimer, E., 199
Berglund, H., 216
Berl, E., 140
Berman, L., 238
Bernhard, A., 59
Bernheim, A. R., 241
Besthorn, E., 126
Bettel, W., 90
Bier, A., 199
Bird, O. D., 290
Blair, A. A., 74
Blankenstein, A., 214
Blau, F., 53
Blom, J., 151

Bloom, A., 335
Bloor, W. R., 199, 332, 333
Blotner, H., 215
Bloxham, C. L., 25
Blum, W., 74
Booth, R. G., 370, 371
Borchers, W., 112
Bordeianu, C. F., 174
Bourne, M. C., 222
Boursnell, J. C., 276
Boyd, M. J., 183, 399
Boyd, W. J., 278
Brand, E., 291
Bratton, A. C., 154, 270
Braun, A. D., 87
Breh, F., 97
Briggs, A. P., 72, 156
Bright, H. A., 81
Brinkmann, R., 238
Brodigan, C. B., 53
Brown, A. L., 238
Brown, F. J., 388
Brown, R. A., 291
Browning, B. L., 400, 401
Buchan, J. L., 65
Buchanan, G. S., 399
Bueding, E., 267, 268
Bukatsch, F., 291
Bullen, S. S., 332, 333
Burgess, L. L., 97
Burgess, W. T., 406
Burke, A. D., 126
Burmester, B. R., 55
Bushill, J. H., 214
Butler, A. M., 291
Byers, D. H., 392

CAINES, C. M., 397
Caley, E. R., 104
Callan, T., 27, 28, 50, 389
Callow, N. H., 335
Callow, R. K., 335
Calvery, H. O., 31
Capen, R. G., 75
Carmichael, E. A., 203
Carr, F. H., 290, 302, 350, 353, 356, 357, 358, 372
Carroll, M. F., 23
Carruthers, C., 291
Carter, G., 216
Cartland, G. F., 291
Cary, C. A., 278
Case, E. M., 267
Caspari, W. A., 25
Cassel, H. R., 53
Cazeneuve, P., 42
Cerecedo, L. R., 360
Chain, E., 161
Chakravarti, S. N., 318
Chamot, E. M., 151
Chaney, A. L., 31
Chanutin, A., 206
Chapin, R. M., 406
Chapman, A. Chaston, 140, 204
Chapman, E. T., 384
Charnass, D., 244
Chatterji, S. K., 289
Chello, L., 245
Chen, K. K., 301
Childs, A. E., 137
Cholak, J., 70
Chon, C. Y., 335
Ciocalteu, V., 87, 250, 251, 252, 253, 278, 335, 336, 337, 409, 414, 415
Ciogolea, G., 36
Claesson, P., 59
Clark, E. P., 38
Clark, R. E. D., 109, 110
Clarke, S. G., 12, 14
Clayton, J. S., 376, 377
Clegg, J. W., 231, 232
Clennell, L. E., 387
Clifford, P. A., 70
Close, H. G., 281
Cockburn, T., 49
Cocking, T. T., 63, 310, 337, 359
Cohen, B., 226
Cohen, H. R., 30
Cohen, S. L., 291
Cole, W. H., 389
Collins, H. S., 16, 17
Collip, J. B., 38
Congdon, L. A., 388
Conn, L. W., 50
Connell, W. B., 140
Conner, C. L., 339
Conner, R. T., 360
Conway, E. J., 128
Cooper, L. H., 199
Corbet, R. E., 351
Corfield, C. E., 304
Corley, R. C., 218
Coulson-Smith, C., 379
Cox, S. J., 357
Crawford, W. G., 117
Creveld, S. van, 218
Crews, S. K., 310, 357
Criston, P., 213
Cruickshank, J., 203
Crum, W., 72

Cullen, G. E., 283
Cunningham, T. R., 89
Cuny, L., 36
Cuthbertson, D. P., 274
Cutting, W. C., 270

DAGLISH, C., 308
Dangerfield, W. G., 275, 276
Dann, W. J., 347
Dare, A., 234
Daroga, R. P., 298, 345, 389, 390, 391, 394
Daubney, C. G., 289
Davidson, A. M. M., 42, 45
Davidson, J., 75
Davies, D. R., 174
Davies, W. C., 174
Davis, M. E., 310
Davis, W. A., 22
Dawson, E. R., 360, 368
Dechene, E. B., 335
Deckert, W., 126
Deemer, R. B., 31
Delépine, M., 50
Delory, G. E., 195, 208, 232, 247, 260, 283
Delpy, M., 140
Demuth, F., 250
Denigès, G., 30, 81, 160, 298, 321, 376, 404
Denis, W., 72, 165, 286, 409
Dennstedt, M., 376
DeTurk, E. E., 137
Dibdin, W. J., 199
Dickens, F., 128
Dickinson, D., 135
Dille, J. M., 189
Dische, Z., 245
Dixon, J. K., 46
Dodds, E. C., 196
Dodge, B. F., 406
Doisy, E. A., 156, 210
Dornheggen, H., 333
Dracass, W. K., 335
Drekter, I. J., 59
Drummond, J. C., 349, 350
Dubský, J. V., 72
Dudley, H. W., 301
Duffield, W. D., 16, 29, 30
Dufty, L., 72
Duncan, I. J., 377
Dunstan, W. R., 325
Durien, E., 140
Dustman, R. B., 377

EAGLES, B. A., 213
Eastland, C. J., 326
Eden, A., 96, 98
Edisbury, J. R., 350, 351, 355, 358
Edwards, F. W., 129, 130, 140, 142, 143, 162, 163, 385
Eegriwe, E., 183, 245, 397
Egaña, E., 360
Eggertz, V., 386, 387
Elgart, S., 244
Ellms, J. W., 393
Elvehjem, C. A., 44, 126
Emerson, K., Jr., 270
Emmens, C. W., 335
Emmerie, A., 371
Emmett, A. D., 290
Engel, C., 371
Espinosa, N. S., 405
Evans, B. S., 12, 15, 38, 40
Evans, J., 216
Evelyn, K. A., 215
Evers, N., 25, 26, 46, 47, 48, 302, 326, 397
Evlanova, A. V., 70
Ewins, A. J., 326

FAIRHALL, L. T., 96, 126
Fasal, H., 278
Feigl, F., 36, 91, 102
Fellenberg, T. von, 406
Fenton, H. J. H., 178
Ferguson, W. S., 340, 341
Ferris, C. A., 127
Ficklen, J. B., 393
Field, H., Jr., 349, 359
Findley, T., 128
Fine, J., 266
Fischer, Emil, 174
Fischer, H., 32, 70, 126
Fiske, C. H., 156
Fitter, H. R., 36
Fleming, R., 211
Fletcher, A. E., 151
Fletcher, W., 245
Flinn, F. B., 30
Foix, A., 53
Folin, O., 184, 204, 207, 213, 216, 219, 220, 221, 222, 223, 250, 251, 252, 253, 277, 282, 283, 285, 335, 336, 337, 384, 409, 414, 415
Folley, S. J., 250
Fölling, A., 248
Folmers, T., 416
Forbes, A. P., 335
Foss, M. R., 282

Foster, G. E., 335
Foulger, J. H., 282
Foulk, C. W., 104
Fox, J. J., 408, 410
Francis, C. K., 140
Francis, A. G., 65
François, A., 325
Frankston, J. E., 277
Franseen, C. C., 259
Fraser, R. W., 335
Freak, G. A., 12
Frederick, R. C., 148
Freeman, H. F., 342
Frenkel, L., 282
Frezouls, J., 199
Friedel, R. K., 388
Friedemann, T. E., 244
Friedgood, H. B., 334
Fuchs, H. J., 245
Fujiwara, K., 389, 394
Fuller, A. T., 269
Fürth, Otto, 278
Fürth, O. von, 244

GAD, G., 393
Gaebler, O. H., 97
Gallego, M., 121
Gamble, F. W., 182
Ganassini, D., 199
Gardner, W. M., 87
Garratt, D. C., 66, 310, 314, 401, 402
Gaunt, W. E., 275
Gebhardt, T. H., 50
Gentzkow, C. J., 282
Georges, L., 314, 315
Germuth, F. G., 154
Gettler, A. O., 216
Gibbs, H. D., 250, 256, 257, 290, 408, 413
Gibson, J. G., 2nd, 215
Gies, W. J., 260
Gille, R., 416
Gladstone, J. H., 151
Glasstone, S., 423
Goldman, D., 333
Goldscheider, I., 243
Gonder, K. L., 36
Görtz, S., 291
Gowers, W. R., 226
Graham, W. R., Jr., 250, 252, 254, 259
Grant, J., 14
Grau, E., 199
Greathouse, L. H., 73
Greenfield, J. G., 203
Greenwald, I., 210
Grendel, F., 50
Griess, J. P., 152, 154
Gross, J., 210
Grossfeld, J., 129
Grove, D. C., 309, 310
Gauge, A. J. H., 408, 410
Guilbert, H. R., 342
Gutzeit, H., 16

HABERSTOCK, J., 126
Hackford, J. E., 25
Haddock, L. A., 35, 46, 47, 48, 106, 107, 108
Haden, R. L., 231, 232
Hadley, W. H., 168, 169, 170, 171
Haessler, H., 238
Hagedorn, H. C., 219
Hahn, F., 199
Hald, J., 346
Haldane, J. S., 226, 228, 229, 230, 231, 234, 236
Hamano, S., 359
Hamence, J. H., 24, 151
Hammett, F. S., 247
Hammett, L. P., 7
Hamner, H. L., 89
Hampshire, C. H., 303, 308
Handler, P., 347
Hanriot, M., 129
Hansen, A., 245
Hardwick, P. J., 198
Harper, D. A., 4
Harris, J. E., 97, 100
Harris, J. S., 244
Harris, L. J., 347, 360, 367, 368, 369, 370
Harris, R. I., 259
Harrison, G. A., 182, 226, 233, 234
Harrison, J. B. P., 23
Harrop, G. A., Jr., 245
Hart, E. B., 250
Harvey, C. O., 65
Haskins, H. D., 238
Haslewood, G. A. D., 195, 196, 208, 247, 260, 283
Hassan, M. K., 129, 130, 140, 142, 143, 162, 163, 385
Hatfield, W. D., 12
Hauptmann, A., 199
Hauser, S. J., 393
Hazeloop, E., 214
Heard, C. R. C., 298
Heide, C. von der, 134
Heinzelmann, A., 87

Heise, K., 59
Heitzmann, L., 210
Helch, H., 315
Heller, K., 102
Heller, V. G., 126
Helmer, O. M., 206
Henderson, J. A. R., 50, 389
Hendrickson, A. R., 154, 273
Hennessy, D. J., 360, 370
Herbert, F. K., 222, 259
Herd, C. W., 70
Herd, M., 49
Hersant, E. F., 405
Hess, W. C., 214
Hibbard, P. L., 126, 325
Higgins, J. C., 390
High, J. H., 6
Hill, C. A., 16, 17, 60
Hill, R., 53
Hillebrand, W. F., 38, 115
Hiller, A., 230, 264
Hilpert, S., 416
Hine, C. H., 215
Hinks, E., 132
Hinton, J. J. C., 370
Hirst, J. F., 397
Hoar, T. P., 49
Hoase, L. W., 393
Hoffman, J. I., 81, 111
Hoffman, W. S., 96
Hofmann, K. A., 36
Hogden, C. G., 264, 265, 267
Holman, W. M., 160
Holmes, F., 397
Holmes, H. B., 351
Holtorff, A. F., 334
Hopkins, F. Gowland, 245
Hoppe-Seyler, F., 238
Hoskins, W. M., 127
Hough, G. J., 77
Hough, W. A., 393
Houghton, G. U., 408, 413
Howe, P. E., 267
Hubbard, D. M., 70, 87
Hubbard, R. S., 275
Hudson, J. H., 178
Hughes, E. B., 125, 401
Hughes, J. S., 342
Huldschinsky, E., 43
Hunter, G., 213, 238
Hurd, L. C., 88

IBELE, S., 126
Ihl, A., 218
Illing, E. T., 151
Illosvay de Nagy Ilosva, L., 152
Ilndovina, R., 199
Isaacs, L., 165
Isaacs, M. L., 128, 291

JACKLIN, J., 208
Jacob, F., 134
Jacobs, H. R. D., 96
Jacobs, M. B., 31
Jaffe, M., 121, 204
James, G. V., 273
James, L. H., 89
James, W. O., 298
Jansen, B. C. P., 360
Jenner, H. D., 252
Jensen, B. N., 219
Jesperson, J. C., 188, 189, 190, 192
Jesserer, H., 266
Johannson, H., 368
Johnson, A. H., 50
Johnson, B. C., 81
Johnson, C. R., 56
Johnson, M. O., 140
Johnson, W. A., 128
Jolles, A., 165, 277, 278
Jones, A. J., 129, 134, 163
Jones, A. O., 216
Jones, B., 95
Jones, Clemens, 88, 160
Jones, E. G., 46
Jones, H. O., 96
Jones, N. R., 298, 299, 301, 310, 315, 316, 318
Jones, R. T. Parry, 27, 28
Jonescu, A., 129, 130, 143
Jonxis, J. H. P., 238
Jordan, R. C., 216
Jorissen, A., 143, 162, 164
Josland, S. W., 46
Jungkunz, R., 419
Jungman, H., 245

KAHANE, E., 81
Kahn, B. S., 37, 275
Kamm, O., 97
Kantor, J. L., 260
Kao, C. H., 299
Kapeller-Adler, R., 239
Karpenko, V., 50
Karrer, P., 351, 371, 373
Karush, F., 29
Kassell, B., 29, 370
Kaszuba, F. J., 370

Kay, H. D., 250, 252, 254, 259
Keefer, C. S., 195
Keller, C. C., 308
Keller, H., 371, 373
Kendall, A. I., 244
Kent-Jones, D. W., 70, 370
Keresztesy, J. C., 291
Kerr, R. H., 417
Kersting, R., 151
Kharasch, M. S., 310
Kidson, E. B., 46
Kinnersley, H. W., 359
King, E. J., 156, 165, 166, 172, 195, 208, 231, 232, 246, 249, 260, 283
King, W. A., 90
Kirch, E. R., 371
Kirkpatrick, H. F. W., 291
Klein, D., 267
Kling, A., 377
Klooster, H. S. van, 46
Knapp, A. W., 397, 399
Knippenberg, E., 12, 38
Knoop, F., 238
Knowles, H. B., 9
Knudsen, L. F., 310
Kober, S., 289
Koch, F. C., 334
Kodicek, E., 347, 348
Koenemann, R. H., 245
Koenig, R. A., 56
Koenig, V. L., 290
Kohn-Abrest, C., 87
Kolthoff, I. M., 12, 72, 102, 151, 326, 392
Komm, E., 210
König, W., 349
Koones, H. F., 291
Kopp, E., 151
Koppanyi, W. S., 186
Kozelka, F. L., 215
Kramer, B., 37
Kraus, I., 278
Krause, A. G., 180
Kreis, H., 417, 418, 419
Kronenberger, F., 218
Krop, S., 189
Krumholz, P., 102
Kryĭgsman, B. J., 245
Kurchin, E., 278
Kurmies, B., 72
Kuttner, T., 30

LABAT, A., 199
Lamb, F. W., 70
Lampitt, L. H., 11, 45, 214, 401, 417
Lang, A., 245
Lang, K., 72
Lang, R., 125
Lange, C., 201, 203
Langley, W. D., 239, 240, 241
Laporte, C., 36
Larmour, R. K., 377
Larsen, K. T., 188, 189, 190, 192
Laszlo, D., 245
Law, N. H., 111, 112
Leavenworth, C. S.. 127
Legal, E., 248
Legault, R. R., 310
Lehner, V., 117
Leiboff, S. L., 156, 275
Leopoldi, G., 70, 126
Lerrigo, A. F., 151, 420
Letonoff, T. V., 180
Letts, E. A., 151
Levvy, G. A., 186, 188
Levy, E. D., 346, 349
Lida, E., 235
Lieben, F., 266
Liebhafsky, H. A., 70
Lincoln, A. T., 165
Lindsay, W. G., 174
Lindt, V., 96
Ling, A. R., 377
Link, K. P., 376, 377
Littleton, F. T., 377
Lloyd, L. L., 87
Lockemann, G., 140
Logan, M. A., 183, 399
Lohman, K., 360
Long, C. N. H., 245
Longwell, J., 395
Looney, J. M., 213, 278
Lopez, R. C., 199
Lorenz, R., 199
Lott, W. L., 126
Lu, G. D., 269
Lucas, M., 96
Lugg, J. W. H., 213, 214
Lundell, G. E. F., 9, 81, 111
Lüscher, E., 278
Lustgarten, S., 394
Lutz, R. E., 126
Lynch, G. Roche, 65, 108, 289
Lyons, E., 53

MACALLUM, A. B., Jr., 286
Macdonald, C. A., 344, 345
MacLachlan, E. A., 291
MacLean, H., 219
Maclennan, G. W. G., 397

Maechling, E. H., 30
Magnuson, H. J., 31
Mahr, C., 36
Makris, K. C., 384
Malaquin, P., 321
Malatesta, G., 53
Maltby, J. G., 22
Mann, T. Barton, 342
Marenzi, A., 235
Marenzi, A. D., 213
Marrian, G. F., 291
Marshall, E. K., Jr., 154, 269, 270, 273
Marshall, H., 72
Martin, G. W., 360, 368
Masen, J. M., 282
Mathews, A. P., 349
Maunsell, P. W., 137
Maxson, R. N., 53
May, C. E., 278
Mayençon, —, 16
McCabe, C. R., 119
McCance, R. A., 102, 104, 105
McCollum, E. V., 359
McIntosh, J. F., 267
McLean, R., 259
McLellan, B. G., 351, 358
McNary, R. R., 70
McNaught, K. J., 45, 46
McNee, J. W., 195
Mead, T. H., 351
Mecklenburg, W., 174
Meek, H. O., 402
Meigs, E. B., 278
Meiklejohn, A. P., 360
Melaven, A. D., 10
Mellanby, J., 203
Mellon, M. G., 55, 392
Melnick, D., 347, 349, 359
Melzer, F., 291
Mendel, B., 243
Menière, P., 87
Merejkovsky, B. K., 53
Merwe, A. J., van der, 42
Messiner, L., 102
Meulengracht, E., 241
Meyer, A. H., 30, 160
Meyer, J., 118
Meyer, R. K., 291
Meysenbug, L. von, 165
Michaelis, L., 214
Middleton, A. R., 91
Middleton, G., 356, 357
Miller, B. F., 245
Miller, C. W., 156
Miller, E. J., 70
Miller, H. L., 91
Miller, L. C., 291
Miller, R., 298
Milton, R. F., 16, 29, 30, 219, 224, 243, 244, 279
Mink, F., 260
Mitchell, C. A., 155, 178, 421, 422, 424
Mitchell, R. L., 42, 45
Moffitt, W. G., 394, 395
Mogerman, W. D., 8
Mohler, E., 129, 133, 143
Moir, J. Chassar, 301
Monier-Williams, G. W., 11, 25, 64, 132, 151
Monk, H. E., 359
Montequi, R., 121
Moon, C. D. B., 126
Morf, R., 351
Morin, C., 298
Morris, C. J., 273
Morris, H. J., 31
Morris, J. L., 210
Morris, T. G., 390
Morton, F., 70
Morton, R. A., 338, 359
Muers, M. M., 417, 419
Müller, J. A., 53
Müller, P., 196
Muntz, J. A., 245
Murphy, W. S., 189
Murray, W. M., Jr., 95
Myers, V. C., 199

NAGAYA, T., 245
Nagel, G., 112
Nagler, J., 31
Naish, W. A., 387
Nanji, D. R., 377
Nanji, H. R., 129, 130, 140, 142 143, 162, 163, 385
Neave, F. K., 250, 256, 257, 258, 259
Nessler, J., 382
Neuber, F., 36
Neurath, F., 165
Nicholls, J. R., 63, 129, 143, 162, 163, 370, 378, 384, 385
Nicholson, W. N., 423
Nickolls, L. C., 289
Nicloux, M., 214
Nicolet, B. H., 183
Niederl, J. B., 216
Nightingale, E., 350, 358
Nola, E. di, 53

Noponen, G. E., 151
Nordbö, R., 245

OBERMER, E., 279
Okáč, A., 72
Okuma, K., 72
Olson, L. C., 137
Oppel, W. W., 218
Oppenheimer, E., 199
Osgood, E. E., 238
Osler, T. G., 65
Osman, A. A., 281
Osmond, F., 155
Ossian, H., 59
Otto, J., 287, 289
Overhoff, J., 57
Overholser, L. G., 6

PAGE, G. R., 303, 308
Palmer, A. E., 154
Palmer, W. W., 226
Parri, W., 165
Partridge, M. W., 310
Patterson, J., 218
Pawletta, A., 118
Peacock, G., 291
Pearson, E. A., 326
Pechmann, A., 218
Pelly, R. G., 408, 413
Penrose, L., 247, 248
Penzolt, J., 378
Perlich, R. W., 96
Perlzweig, W. A., 346, 347, 349
Pesez, M., 151
Peterman, F. I., 180
Peters, J. P., 238
Peters, R. A., 359
Peterson, W. J., 342
Pettit, D. S., 289
Phelps, E. B., 393
Piccard, J., 392
Pichard, P., 72
Pierson, G. G., 53
Pilkington, F., 199
Pincus, G., 291
Pinder, H., 210
Platt, B. S., 269
Pohland, E., 87
Poirot, G., 36
Pollard, A. G., 160, 389, 390, 391, 394
Pollard, W. B., 53
Powell, A. D., 46
Powell, A. R., 6
Powick, W. C., 417
Pozzi-Escot, E., 53
Pratt, D. S., 151
Prebluda, H. J., 359
Price, E. A., 290, 350, 353, 356, 357, 358, 359, 372
Price, J. W., 267
Price, P. H., 423
Prister, A., 53
Pritzker, J., 419
Prochazka, J., 377
Prudhomme, R. O., 319, 320
Prunty, F. T. G., 214
Pryde, J., 216
Pucher, G. W., 127, 376
Putnam, G. L., 97
Pyke, M. A., 368, 417

QUASTEL, J. H., 247, 248

RADT, P., 218
Radulescu, D., 310, 313, 314
Ragins, I. K., 278
Ramage, H., 72
Rappaport, F., 245
Rây, P., 50
Rây, R. M., 50
Ray, T. W., 76, 77
Raymond, W. D., 347
Raynes, E. G., 351, 358
Rea, F. W., 151
Reddrop, J., 72
Redemann, C. E., 283
Redfield, H. W., 151
Reifenstein, E. C., Jr., 335
Reifer, I., 245
Reimann, F., 178
Reinecke, R. M., 217
Reinhold, J. G., 199
Resuggan, J. C. L., 143
Revis, C., 419
Rhind, D., 423
Rich, C. E., 368
Richards, A. N., 128
Richards, M. B., 80
Rideal, S., 406
Riehm, H., 174
Rienäcker, G., 126
Riese, W., 378
Riesen, I. von, 218
Riesser, O., 245
Riffart, H., 126
Rimington, C., 213

Robb, G. D., 279
Roberts, W. L., 376, 377
Roberts, W. M., 259
Robeson, C. D., 351
Robinson, F. C., 5
Robinson, H. M., 70
Robinson, H. W., 264, 265, 267
Robinson, K. L., 135, 136
Robinson, R. J., 97
Robinson, W. D., 349
Robison, R., 249
Rodillon, G., 165
Roe, J. H., 37, 216, 217, 290
Rogers, G., 310
Rojahn, C. A., 320
Romijn, G., 46
Rollet, A. P., 91
Rose, E. R., 278
Rose, W. C., 206
Rosenheim, A., 43
Rosenheim, O., 350
Rosenkränzer, F., 174
Rossouw, S., 214
Rothera, A. C. H., 248
Rouse, A. H., 180
Roy, M. B., 318
Ruemele, T., 278
Russow, F. K., 419
Rutz, M. H., 291

SACKETT, G. E., 200
Sage, C. E., 50
Samuels, L. T., 291
Sanchez, J. A., 320
Sand, H. J. S., 25
Sandell, E. B., 96, 126
Sarett, H. P., 346, 349
Sargant, W., 199
Sayers, R. R., 227
Scharer, H., 257, 258
Scherrer, J. A., 8, 9
Schiff, H., 397, 398, 404, 405
Schiff, W., 126
Schluiter, J. J. de la Fontaine, 196
Schmutz, R., 377
Schneider, L., 72
Schoeller, W. R., 6
Schoonover, I. C., 102
Schoop, K., 351
Schou, S. A., 308
Schreiner, O., 165
Schricker, J. A., 31
Schroenheimer, R., 201
Schryver, S. B., 377, 399, 406
Schulze, A., 378
Schulze, E., 377
Schuster, P., 360
Schwaibold, J., 112
Scott, B. A., 172
Scott, L. D., 216
Scott, R. O., 53, 59
Scotti, G., 165
Scoville, W. L., 402
Scudi, J. V., 290
Seaber, W. M., 338, 339, 341, 342
Seifert, R., 320
Self, P. A. W., 304
Seliwanoff, T., 216
Sendroy, J., Jr., 128
Servantie, G., 245
Seyfang, A. P., 379
Sharlit, H., 277, 278
Shaw, F. H., 327, 330, 331, 332
Shaw, G. E., 344
Shaw, P. A., 106
Sheftel, A. G., 273
Sheppard, S. E., 178
Sherman, C. C., 128
Shiels, D. O., 70
Shinn, L. A., 183
Shinn, M. B., 152, 153
Shinohara, K., 213
Shipp, H. L., 102, 104, 105
Short, F. W., 325
Shrewsbury, H. S., 397, 399
Shupe, I. S., 315
Sideris, C. P., 81, 96, 97
Siemssen, J. A., 53
Simmonds, C., 406
Simmons, C. C., 259
Sizer, I. W., 266
Skrimshire, G. H., 50, 70
Slater, R. H., 65
Smith, A. H., 226
Smith, A. Nutter, 164
Smith, E. R., 214
Smith, G. S., 135
Smith, J. L., 226
Smith, L., 151
Smith, Maurice I., 302
Smith, M. H., 384
Smith, S., 289
Smith, Sydney, 310
Smith, W., 402
Smith, W. H., 9
Sommer, H. H., 50
Somogyi, M., 208, 225, 244
Sonstadt, E., 53
Sottery, C. T., 7
Southgate, H. W., 216

Sperry, W. P., 201
Spies, T. D., 349
Splittgerber, A., 151
Sprengel, H., 148
Spurge, G., 90
Stadie, W. C., 238
Staples, A., 171
Stare, F. J., 44
Stas, J. S., 287, 289
Steiger, G., 127
Steinitz, H., 218
Stevens, S. G. E., 50
Stevenson, S. G., 143
Stevenson, T. M., 377
Stewart, C. P., 289
Stieglitz, E. J., 154
Stillings, R. S., 400, 401
St. Lorant, I., 178
Stock, A., 87
Stoll, K., 162
Story, R. V., 70
Stout, P. R., 325
Stover, N. M., 42
Strafford, N., 4, 8, 9, 81, 82, 86, 389
Stratten, R. C., 393
Straub, G. J., 360
Strohecker, R., 126
Subbarow, Y., 156
Suckling, E. V., 384
Suitsu, K., 72
Sulkowitch, H., 335
Sullivan, M. X., 211
Summerson, W. H., 245
Sutthoff, W., 151
Svedberg, A., 283
Szego, C. M., 291
Swaminathan, M., 291, 345
Swank, H. W., 55
Sweeney, O. R., 199
Sylvester, N. D., 11, 45, 125, 417

TAFFEL, A., 419
Talbot, N. B., 291
Tanret, C., 302
Tasker, H. S., 96
Täufel, K., 419
Taylor, A. E., 156
Taylor, G., 24
Taylor, W. A., 199
Taylor-Austin, E., 6
Teed, F. L., 70
Thayer, L. A., 165, 166
Theis, R. C., 409
Thomas, E. M., 377
Thomas, P., 384
Thornton, J. J., 370
Thresh, J. C., 382
Tice, L. F., 377
Tietz, E. B., 333
Tillmans, J., 151, 278
Timmis, G. M., 310
Tischler, N., 389
Tisdall, F. F., 37
Titel, C., 87
Todd, A. R., 370
Todd, F. J., 315
Todd, W. R., 126
Tolman, L. M., 400
Tolstoi, E., 223
Tompsett, S. L., 36, 55, 70, 213, 274
Tønnesen, M., 310
Toyoda, H., 214
Trace, L. H., 401
Trebler, H. A., 50
Trelles, R. A., 118
Treschow, C., 151
Trescott, T. C., 400
Trevan, J. W., 38
Tribe, A., 151
Truog, E., 30, 137, 160
Tschopp, Emilio, 156, 174
Tschopp, Ernest, 156, 174
Tubis, M., 335
Twort, F. W., 279

UCKO, H., 199
Underhill, F. P., 180
Urk, H. W. van, 302
Uzel, R., 154

VANDENBELT, J. M., 290
Van Slyke, D. D., 228, 230, 238, 245, 267, 283
Vas, M., 245
Vassel, B., 211, 213
Vaughan, E. J., 6
Vaughan, J., 195
Vickery, H. B., 127, 376
Vilter, S. P., 343
Vitali, D., 292
Vogel, H. W., 46
Voigtländer, F., 376
Voisenet, E., 278
Voss, K., 291
Vougerichten, E., 349

WAGENAAR, G. H., 81
Wakefield, E. G., 275
Walpole, G. S., 93
Walt, C. F. J. van der, 42
Walter, F. K., 199
Walters, H. E., 73
Walters, W. P., 417, 419
Wang, Y. L., 347, 348, 360, 367, 368, 369, 370
Wangerin, A., 316
Wanklyn, J. A., 384
Ward, A. H., 370
Ward, H. H., 370
Wardell, E. L., 199
Warington, R., 60
Wasicky, R., 298
Watson, A. F., 349
Watson, G. M., 298
Weaver, E. R., 378
Weber, H., 42
Weehuizen, F., 137, 138, 139
Weiland, H., 12, 38, 72
Weiss, F., 144
Weller, A., 112
Werner, A. E. A., 273
Wertheimer, E., 213
Westfall, B. B., 128
Wheeler, G., 291
Whidden, H. L., 334
Whitehorn, J. C., 327
Wibaut, J. P., 57
Wichman, W. J., 70
Wiese, A. C., 81
Wilken-Jorden, T. J., 214
Willard, H. H., 73, 127
Williams, A. L., 420
Williams, H. Amphlett, 24
Williams, M. Whitley, 151
Williamson, C. S., 228
Willstaedt, H., 316, 370
Willstätter, R., 392
Wilson, E. S., 298
Wilson, L. T., 250
Winkler, L. W., 406, 407
Winkler, S., 278
Winslow, E. H., 70
Winsor, H. W., 59
Winter, O. B., 70, 128
Withrow, J. R., 199
Witte, H., 377
Wokes, F., 308, 369
Wolfe, J. K., 291
Wolter, O., 238
Wong, S. Y., 226, 235
Wood, C. C., 406
Wood, D. R., 151
Wormall, A., 275, 276
Wortis, H., 267, 268
Wright, L. O., 406
Wu, H., 219, 221, 222, 223, 226 231, 282, 335, 384
Wuth, O., 199
Wyatt, P. F., 8, 9, 81, 82, 86

YANTS, W. P., 227
Yoe, J. H., 6, 53
Young, G., 291

ZAHL, P., 291
Zimmermann, W., 334, 335
Zinzadze, C., 30, 31
Zondek, H., 199
Zwikker, J. J. L., 186

INDEX TO SUBJECTS

ACETONE, 377
Acetylene, 378
for gold, 50
in air, apparatus for, 380
Acetylsalicylic acid, salicylic acid in, 163
Adrenaline, 326
by arsenomolybdic acid, 328
persulphate, 327
in blood, 329, 332
gland extracts, 326
Albumin, 260
by biuret method, 262
wet oxidation, 261
Albuminoid ammonia, 383
Alfalfa, β-carotene in, 342
Alizarin S,
for aluminium, 7
borates, 135
Alkaline hæmatin, 226, 230
Alkaline hæmatin technique, for hæmoglobin, 230
Allobarbitone, 192
Allobarbitonum, 192
Allocaine, 318
5-Allyl-5-*iso*butyl-barbituric acid, physical data for, 191
5-Allyl-5-(1-methylbutyl)-barbituric acid, 191
5-Allyl-5-*cyclo*pentenyl-barbituric acid, 190
5-Allyl-5-phenyl-barbituric acid, physical data for, 192
5-Allyl-5-*iso*propyl-barbituric acid, physical data for, 190
5-Allyl-5-*iso*propyl-1-methyl-barbituric acid, 191
Aluminium, 7
in alloys, 9
food, 11
mercury cathode cell for, 10
silicates in, 166, 170
Aluminium alloys, silicates in, 166, 170
Aluminon, 7, 9
Alurate, physical data for, 190
American peppermint oil, distinction from European and Japanese oils, 401, 402
p-Amino-acetophenone, for vitamin B_1, 359
Amino-acid nitrogen, 184
p-Aminobenzenesulphonamide, 269
for nitrites, 152
p-Aminobenzoyldiethylaminoethanol hydrochloride, 318
p-Aminodimethylaniline,
for chlorine, 392
cystine and cysteine, 211
sulphides, 174
1-Amino-8-naphthol-3:6-disulphonic acid, for procaine, 316
1-Amino-2-naphthol-4-sulphonic acid, for phosphates, 158
Ammonia, 382
Ammonium aurine tricarboxylate,
for aluminium, 7
preparation of, 9
Ammonium cobaltothiocyanate, 43
Ammonium molybdate,
for arsenic, 16
phosphates, 155
silicates, 165
Ammonium persulphate, for manganese, 73, 75
Ammonium polysulphide, for cyanides, 138
Ammonium thiocyanate,
for cobalt, 43
iron, 53
Amytal, physical data for, 190
5-Amyl-5-(2-bromallyl)-barbituric acid, 191
Androsterone, 290, 334
Aneurine, 359
Aniline,
for furfuraldehyde, 400
phosgene, 375
industrial toxic gas, 4
Animal tissue,
creatine and creatinine in, 205
manganese in, 78
silicates in, 172
Antimony, 12
in copper and its alloys, 14
lead, 15
tin, 14
Antimony potassium tartrate,
arsenic in, 22
lead in, 62
Antimony trichloride, for vitamin A, 290, 350

Antrypol, 275
Apomorphine, 299
Aromatic powder of chalk with opium, morphine in, 312
Arsenic, 16
  arsenomolybdate method, apparatus for, 30
  by ammonium molybdate, 16
    electrolytic method, 25
    Gutzeit method, 16
    sodium molybdate, 29
  contamination of food, as war gas, 24
  in antimony potassium tartrate, 22
    bismuth salts, 21
    carbonates, 20
    complex organic materials, 22
    dyestuffs, 22, 23
    food, 22, 24
    inorganic salts, 20
    insoluble organic compounds, 20
    iron compounds, 21
    nitrates, 21
    tartar emetic, 22
  lead electrode cell for, 26
  Gutzeit apparatus for, 17
Arsenic trichloride, for vitamin A, 350
Arsenomolybdic acid, for adrenaline, 326
Arsenomolybdic acid test, table showing response of adrenaline and allied substances, 331
Arsenophosphotungstic acid, for uric acid, 283
Arsine, 4
Aschaffenburg and Neave's method for phosphatase, 256
Atropa Belladonna, 298
Atropa acuminata, 297
Atropine, 292
  in belladonna leaf and root, 294
    galenicals, 295
    hypodermic tablets, 294
    stramonium leaf, 295
Aurine - tricarboxylic acid, ammonium salt,
  for aluminium, 7
  preparation, 9
Auxiliary solution, 60

BACON, vitamin B_1 in, 369
Barbital, physical data for, 190
Barbitone, 192
Barbitonum, 192
Barbiturates, 186
  in blood, 186
    tissues, 187
    urine, 187
Barbituric acid, preparation of derivatives, 188
Barbituric acids, trade names of, 192
Barley,
  β-carotene in, 342
  vitamin B_1 in, 369
Bay oil, clove oil in, 402
Bayer 205, 275
Bearing metals, aluminium in, 9
Bedside method, for sulphanilamide, 273
Beetroot, vitamin B_1 in, 369
Belladonna leaf and root, solanaceous alkaloids in, 295
Benedict's method for glucose, 222
Benzaldehyde, for tryptophan, 277
Benzene,
  carbon disulphide in, 388
  industrial toxic gas, 4
Benzenediazonium chloride *p*-sulphonic acid, for vitamin B_1, 359
Benzidine,
  for chlorine, 392
    gold, 50
    sulphates, 273
Benzoic acid, 129
  by Jonescu's method, 129
    Mohler's method, 129, 133
  distinction from salicylic and *p*-hydroxybenzoic acids, 143
  in butter, 131
    cream, 131
    fruit, 131
    fruit cordials, 133
    milk, 131
    vegetable foods, 131
o-Benzoic-sulphone-imide, 419
o-Benzoquinone, for cystine and cysteine, 211
Benzoyl chloride, for œstrogens, 289
Benzoyl peroxide, 384
Beryllium, 31
Beverages,
  dulcin in, 396
  saccharin in, 420
Bibliographies of metals in foods, references to, 6
Bilirubin, 193

Biological material,
citrates in, 127
iron in, 55
lead in, 69
metals in, references to bibliographies, 6
zinc in, 122
2 : 2′-Bipyridine, for iron, 53
Biscuits, vitamin B_1 in, 369
Bismuth, 32
by rapid method, 35
in brass, 35
bronze, 35
in copper, 34
gunmetal, 35
organic material, 35
Bismuth salts, arsenic in, 21
Bismuth tetroxide, for manganese, 72
Biuret test, 260, 262
Blood,
adrenaline in, 329, 332
amino-acid nitrogen in, 185
Antrypol in, 275
barbiturates in, 186
bilirubin in, 193
bromides in, 196
cholesterol in, 199
carboxyhæmoglobin in, 226
creatine and creatinine in, 208
dextrose in, 218
ethyl alcohol in, 214
fructose in, 217
glucose in, 218
hæmoglobin in, 226
icterus index of, 241
iron in, 55
lactic acid in, 243
magnesium in, 70
non-protein nitrogen in, 245
phenols in, 408, 415
phosphatase in, 250
phosphates in, 158
potassium in, 100
protein in, 260, 262, 264
pyruvic acid in, 267
sulphanilamide in, 269
sulphates in, 273
sulphonamides in, 269
suramin in, 275
table showing normal hæmoglobin content of, 229
urea in, 278
uric acid in, 283
vitamin E in, 372
Blood analyses, 181
Blood serum,
potassium in, 98, 99
sodium in, 102, 105
Blue galls, 423
Blue values, 350
Borates, 135
Boric acid, 135
Borocaine, 317
Bracken, in tap water, 410
Brain tissue, barbiturates in, 188
Brass,
aluminium in, 9
antimony in, 12
bismuth in, 35
Bread,
loss of vitamin B_1 on baking, 368
vitamin B_1 in, 363, 369
5-(2 - Bromallyl) - 5 - (1 - methylbutyl)-barbituric acid, 191
5 - (2 - Bromallyl) - 5 - *iso*propyl - 1 - methyl-barbituric acid, 190
Bromide intoxication, 199
Bromides,
by fluorescein, 197
gold chloride, 197, 198
in cerebrospinal fluid and in blood, 196
Bromine, for histidine, 238
Bronze,
aluminium in, 9
bismuth in, 35
Brucine for nitrates, 145
Brussels Sprouts, vitamin B_1 in, 369
Burnt sugar, 60
Butisol, 190
Butobarbitol, 190
Butter,
benzoic acid in, 131
p-hydroxybenzoic acid in, 142
phosphatase in, 259
vitamin A in, 354
5 - Butyl - 5 - allyl - barbituric acid, physical data for, 191
5 - *sec*Butyl - 5 - (2 - bromallyl) - barbituric acid, 191

CACOTHELINE test for tin, 109
Calcium, 36
Calcium compounds, manganese in, 76, 79
Calcium phosphate, lead in, 63, 67

Camphor oil, in rosemary oil, 402
Camphorated tincture of opium, morphine in, 311
Canned fish, *p*-hydroxybenzoic acid in, 142
Capsicum fruit, capsaicin in, 376
Carbon, 386
Carbon disulphide,
  in benzene, 388
  industrial toxic gas, 4
Carbon monoxide, industrial toxic gas, 4
Carbon monoxide poisoning, detection of, 238
Carbon tetrachloride, 389
Carbonates, arsenic in, 20
Carboxyhæmoglobin, 226
  by preliminary determination of hæmaglobin, 235
    tannic acid, 237
α-Carotene, 338
β-Carotene, 338
  table showing content in forage and other foods, 342
Carr-Price reagent,
  preparation of, 352
  storage and delivery apparatus for, 357
Carr-Price test, for vitamin A, 290, 352
Carrots, β-carotene in, 341
Cast iron, titanium in, 115
Catechol, 421
Cauliflower, vitamin B_1 in, 369
Cereals, β-carotene in, 341
Cerebrospinal fluid,
  bromides in, 196
  colloidal gold reaction, 201
  protein in, 260, 263
Ceylon tea, 424
Chebulinic acid, 422
Cheese, phosphatase in, 259
China tea, 424
Chlorides, 128
Chlorine,
  in water, 391
  industrial toxic gas, 4
Chloroform, 394
  by pyridine, 389
  phosgene in, 375
Chloroform of belladonna, solanaceous alkaloids in, 295
Chocolate, vitamin A in, 355
Cholesterol, in blood 199
Choline hydrochloride, for potassium, 96
Chromium,
  by diphenyl-carbazide, 40
    permanganate, 39
  in plant ash, 41
    rocks, 41
    soil, 41
    steel, 39, 40
Chromotropic acid, for formaldehyde, 397
Cider vinegar, 401
Cinchonine, for bismuth, 33
Citrates, 127
Clay, titanium in, 116
Clove oil, in bay or pimento berry oil, 402
Cobalt, 43
  by ammonium thiocyanate, 43
    nitroso-R salt, 44
  in food, 45
    nickel salt, 43, 44
    organic materials, 45
    soil, 45
Cobalt salts,
  for barbiturates, 186
  nickel in, 96
Cobalt steels, nickel in, 92
Cocarboxylase, 268, 360
Cocksfoot grass, 340
Cocoa, vitamin A in, 355
Codeine,
  for lactic acid, 243
  morphine in, 310
Codeine phosphate, morphine in, 310
Cod-liver oil, vitamin A in, 349
Cœruleomolybdate test, 155
Coffee, tannin and gallic acid in, 424
Colloidal gold reaction, 201
Colorimetric standards, 2
Compound liniment of aconite, chloroform in, 395
Continuous extraction apparatus, 304
Copper, 46
  antimony in, 12, 14
  beryllium in, 32
  bismuth in, 34
  by dithio-oxamide, 49
    sodium diethyl-dithio-carbamate, 46
  in organic material, 49
    tomato purée, 49
Copper alloys, beryllium in, 32
Copper sulphate,
  for diamorphine, 298
    ephedrine, 299

Cordials, *p*-hydroxybenzoic acid in, 141
Costa Rica coffee, 424
Coumarin, 376
Cream,
  benzoic acid in, 131
  phosphatase in, 258
Creatine, 204
  in animal tissue, 205
    blood, 208
    liver tissue, 206
    meat extract, 209
    rabbit tissue, 206
    urine, 206
Creatinine, 204, 257
  in animal tissue, 205
    blood, 208
    liver tissue, 206
    meat extract, 209
    rabbit tissue, 206
    urine, 206
Curcuma longa, 135
Curcumin, for borates, 135
Cysteine, 211
Cystine, 211
Cyanhæmoglobin, 226
Cyanides, 137
  by prussian blue method, 138
    Weehuizen's method, 138
  in water, 138
Cyanide solutions, gold in, 51
Cyanogen bromide, for nicotinic acid and its derivatives, 343
Cyclonal, 190
Cyclopal, 190
Cyclopen, 190

DACTYLIS glomerata, 340
Decalso, 366
Decolorised rosaniline,
  for formaldehyde, 397
    methyl alcohol, 404
Delvinal, 190
Dextrose, 218
  by Benedict's method, 222
    Folin and Wu's method, 219
    Milton's method, 224
Diabetes mellitus, 218
Dial, physical data for, 190
5 : 5-Diallyl-barbituric acid, physical data for, 190
Diamorphine, 298
Diamorphine hydrochloride, 310
Dianisidine, for œstrogens, 289
Diazobenzene sulphonic acid, for magnesium, 71
2 : 6-Dibromoquinone chloroimide,
  for phenols, 408
    phosphatase, 250, 256
2 : 4 - Dichlorbenzenediazonium chloride, for vitamin B_1, 359
2 : 6 - Dichlorphenolindophenol, for vitamin C, 290
Diethylamine, for carbon disulphide, 388
5 : 5-Diethyl-barbituric acid, physical data, for, 190
4 : 4′ - Dihydroxy-α : β - diethyl stilbene, 335
p-Dimethylaminobenzaldehyde,
  for atropine, 292
    ergot alkaloids, 302
    procaine, 316
    sulphonamides, 270
    tryptophan, 277
p-Dimethylamino - benzal - rhoda - nine,
  for gold, 50
    mercury, 81
    silver, 101
p-Dimethylamino - benzylidine - rhodanine,
  for gold, 50
    mercury, 81
    silver, 101
1 : 5 - Dimethyl - 5 - (1 - *cyclo*hexenyl-barbituric acid, physical data for, 190
Dimethylglyoxime, for nickel, 90
Dimethyl-α-naphthylamine, for sulphonamides, 269
asym. Dimethyl - *p* - phenylenediamine,
  for chlorine, 392
    cystine and cysteine, 211
    phenols, 408
    sulphides, 174
o - Dimethoxy - benzene, for lactic acid, 243
Di - β - naphthylthiocarbazone, for mercury, 87
m-Dinitrobenzene, for androsterone and 17-ketosteroids, 334
3 : 5-Dinitrobenzoic acid, for creatine an dcreatinine, 210
Dinitrochlorbenzene, for nicotinic acid and its derivatives, 343
2 : 4-Dinitryphenylhydrazine,
  for phenolpyruvic acid, 247
    pyruvic acid, 268
Dionin, 299

Diphenylamine,
for fructose, 216
nitrates, 145
Diphenylamine sulphonic acid, for nitrates, 145
Diphenylbenzidine, for nitrates, 145
Diphenyl-carbazide,
for chromium, 38, 40
mercury, 81
Diphenyl-carbazone, for mercury, 81
Diphenylthiocarbazone,
for copper, 34
lead, 64, 68
thallium, 106
zinc, 121
5 : 5-Dipropyl-barbituric acid, physical data for, 191
αα′-Dipyridyl,
for iron, 53, 56
synthesis of, 57
Disodium phenylphosphate, for phosphatase, 250
Dithio-oxamide, for copper, 46, 49
Dithiol, for tin, 109
Dithizone, see Diphenylthiocarbazone
Dormin, 190
Dry extract,
of belladonna, solanaceous alkaloids in, 296
nux vomica, strychnine in, 324
stramonium, solanaceous alkaloids in, 296
Dulcin, 395
Dyestuffs,
arsenic in, 22, 23
iron in, 64

EGGS, vitamin B_1 in, 369
Egg products, vitamin B_1 in, 363
Ehrlich's diazo reagent, for bilirubin in blood, 193
Eldoral, 190
Eosin, for quinine, 319
Ephedrine, 299
Epihydrinaldehyde, 417
Epoxytropine tropate, 298
Ergometrine, 301
in ergot, 303
liquid extract of ergot, 307
Ergometrinine, 302
Ergonovine maleate, 302
Ergot of Festuca grass, 302, 309
Ergot of rye, ergometrine and ergotoxine in, 303
Ergotamine, 302
Ergotaminine, 302
Ergotinine, 302
Ergotocin, 302
Ergotoxine, 301
in ergot, 303
liquid extract of ergot, 307
Ergotoxine ethanesulphonate, 303
Eriochrome cyanine, for aluminium, 7
Erythroquinine reaction, 318
Ethocaine, 316
Ethyl alcohol, 214
in blood, 214
urine, 215
5-Ethyl-5-allyl-barbituric acid, physical data for, 190
5-Ethyl-5-*iso*amyl-barbituric acid, physical data for, 190
5-Ethyl-barbituric acid, physical data for, 192
5-Ethyl-5-butyl barbituric acid, physical data for, 192
5-Ethyl-5-*sec*butyl-barbituric acid, 190
5-Ethyl-5-(1-*cyclo*hexenyl)-barbituric acid, physical data for, 191
5-Ethyl-5-hexyl-barbituric acid, 191
5-Ethyl-5-(1-methyl-1-butenyl)-barbituric acid, 190
5-Ethyl-5-(1-methylbutyl)-barbituric acid, 191
5-Ethyl-5-(1-methylbutyl)-2-thiobarbituric acid, 191
5-Ethyl-5-*cyclo*pentenyl barbituric acid, 191
5-Ethyl-5-phenyl-barbituric acid, physical data for, 191
5-Ethyl-5-(1-piperidyl)-barbituric acid, 190
5-Ethyl-5-*iso*propyl-barbituric acid, 191
Ethyl tropate, 298
Etoval, 190
Eunarcon, 190
European peppermint oil, distinction from American and Japanese oils, 401
Evipal, 190
Evipan, physical data for, 190
Extract of stramonium, solanaceous alkaloids in, 296

FÆCES, hydrogen sulphide in 178
Fats, rancidity of, 417

Fats, rancidity of,
by Pyke's method, 417
Walters, Muers and Anderson's method, 418
Fenton's test, for tartrates, 178
Ferric chloride, for salicylic acid, 162
Ferron, 53, 57
Ferrous tartrate, for tannin and gallic acid, 421
Fibrin, 261
Fibrinogen, 260
Fish, sulphides in, 175
Fish pastes, *p*-hydroxybenzoic acid in, 142
Fixed oils, rancidity of,
by Pyke's method, 417
Walters, Muers and Anderson's method, 418
Flour,
benzoyl peroxide in, 384
β-carotene in, 341
vitamin, B_1 in, 364, 369
Fluorescein, for bromides, 196
Fluorides, 127
Folin and Ciocalteu's reagent, 414
for stilbœstrol, 335
Folin and Wu's method for glucose, 219
Folin's sugar tube, 221
Foodstuffs,
arsenic in, 22, 24
cobalt in, 45
dulcin in, 396
iron in, 64
metals in, references to bibliographies on, 6
nicotinic acid in, 345
saccharin in, 420
table, showing β-carotene content of, 342
table, showing vitamin B_1 content of, 369
tin in, 110
Formaldehyde, 397
by hydrochloric and nitric acids, 398
phenylhydrazine hydrochloride, 397
Schiff's reagent, 398
for diamorphine, 298
gold, 50
methyl alcohol, 404
tryptophan, 277
Fouadin,
arsenic in, 22
lead in, 62
Fructose, 216
in blood, 217
urine, 217
Fruit, benzoic acid in, 133
Fruit cordials, benzoic acid in, 131
Furfuraldehyde, 400
for ascorbic acid, 290
urea, 279
in peppermint oil, 401
vinegar, 400

GALL and opium ointment, morphine in, 312
Gallic acid, 421
in coffee, 424
galls, 422
hops, 424
myrobalans, 422
tea, 423
Gallotannin, 421
Galls, tannin and gallic acid in, 422
Gardenal, 190
Gelatin, sulphur in, 178
Germanin, 275
Gland extracts, adrenaline in, 326
Globulin, 260
Glucose, 218
apparatus for determination of, in blood, 221
by Benedict's method, 222
Folin and Wu's method, 219
Milton's method, 224
Gold, 50
by formaldehyde, 51
o-tolidine, 52
in cyanide solutions, 51
organic materials, 52
Gold chloride, for vitamin E, 371
Gold, sol, preparation of, 202
Glycerin of belladonna, solanaceous alkaloids in, 295
Glyceryl trinitrate, 402
determination as ammonia, 402
determination as nitrate, 402
Glyoxylic acid, for tryptophan, 277
Graphite, 386
Grass,
β-carotene in, 338
manganese in, 80
vitamin B_1 in, 369
Green extract of belladonna, solanaceous alkaloids in, 296
Green galls, 423
Guaiacol, for lactic acid, 243
Gunmetal, bismuth in, 35
Gutzeit method, for arsenic, 16

H ACID, for procaine, 316
Haden scale, 231
Hæmatoxylin, for aluminium, 7, 9
Hæmin, 231
Hæmoglobin, 226
by alkaline hæmatin technique, 230
conversion to carboxyhæmoglobin, 227
direct colour measurement, 233
cell for direct measurement of, 233
iron content, 234
Haldane scale, 228
Ham, vitamin B_1 in, 369
Harrison cell, 234
Hay, β-carotene, in, 342
Heberal, 191
Heroin, 298
Herring, vitamin B_1 in, 369
Hexanastab, 191
Hexobarbitone, 192
Hexobarbitonum, 192
Histidine, in urine, 238
Homatropine, 298
Hops, tannin and gallic acid in, 424
Hormones, 289
Hydrogen cyanide, industrial toxic gas, 4
Hydrogen peroxide,
for molybdenum, 88
titanium, 112
vanadium, 118
Hydrogen sulphide, 175
apparatus for quantitative isolation, 176
industrial toxic gas, 4
Hydroquinone,
for lactic acid, 243
silicates, 165, 172
p-Hydroxybenzoic acid, 140
distinction from benzoic and salicylic acids, 143
esters of, 140, 143
in butter, 142
cordials, 141
fish pastes and canned fish, 142
meat pastes, 142
milk, 141
sausages, 142
wines, 141
8-Hydroxyquinoline,
for magnesium, 71
vanadium, 120
Hyoscine, 292
Hyoscyamine, 292
in belladonna leaf and root, 294
galenicals, 295
hypodermic tablets, 294
stramonium leaf, 295
Hypodermic tablets,
morphine in, 313
pilocarpine in, 315
procaine in, 317
solanaceous alkaloids in, in presence of morphine, 294
strychnine in, 321

ICTERUS index, 241
Idobutal, 191
Ilosvay's method for nitrites, 152
Indian belladonna, 297
Indican, 277
Injections,
ephedrine in, 300
morphine in, 313
pilocarpine in, 315
procaine in, 317
solanaceous alkaloids in, 294
strychnine in, 321
vitamin B_1 in, 360
Ink, 423
Inorganic salts, arsenic in, 20
Iodic acid, for morphine, 310
7 - Iodo - 8 - hydroxyquinoline - 5 - sulphonic acid, for iron, 53, 57
Ipral, 191
Iron, 53
by $\alpha\alpha'$-dipyridyl, 56
ferron, 57
sodium salicylate, 57
thiocyanate, 53
thioglycollic acid, 55
in zinc, 58
manganese in, 75
sulphides in, 174
Iron compounds, arsenic in, 21
Isolation of alkaloids from viscera, 287
Isonal, 191
Isotonic sodium sulphate solution, 181

JAPANESE peppermint oil, distinction from European and American oils, 401, 402
Jaundice, 193, 195, 241, 249
Jonescu's test, for benzoic acid, 129
Jorissen's test, for salicylic acid, 162

KAY and Graham's method fo phosphatase, 252
Kerocain, 318
17-Ketosteroids, 334
Kidneys, manganese in, 80
Kreis value, 418

LACTIC acid, 243
Lactoflavine, 290
Lævulose, 216
Lead, 60
antimony in, 12, 15
by diphenylthiocarbazone, 68
sulphide, 60
in biological materials, 69
calcium phosphate, 63, 67
dyestuffs, 64
food, 64
organic materials, 64
tartar emetic, 62
zinc, 63
zinc compounds, 63
Lead dioxide, for manganese, 73
Lead electrode cell, for arsenic, 26
Liniment of belladonna, solanaceous alkaloids in, 295
Liquid extract,
of belladonna, solanaceous alkaloids in, 295
ergot, ergometrine and ergotoxine in, 307, 309
nux vomica, strychnine in, 324
stramonium, solanaceous alkaloids in, 295
Liver, manganese in, 80
Liver, extracts,
nicotinic acid and nicotinamide in, 343
vitamin B_1 in, 369
Liver oil, vitamin A in, 352
Liver tissue, creatine and creatinine in, 206
Lucerne, β-carotene in, 342
Luminal, physical data for, 191
Lung tissue, silicates in, 166, 172

MAND B. 693, 272
M and B. 760, 272
Magnesium, 70
Magneson I, 70
Magneson II, 70
Maize germ oil, 373
Malonal, 191
Mal vinegar, 401
Manganese, 72
by lead dioxide, 72
periodate, 73, 77
persulphate, 75
sodium bismuthate, 74
in animal tissue, 78
iron, 73
milk, 79
organic material, 76, 78
plant tissues, 78
soil, 77
steel, 73
Manganese steel, nickel in, 92
Margarine, vitamin A in, 354
Meat,
formaldehyde in, 399
nitrates in, 147
sulphides in, 175
Meat extract,
creatine and creatinine in, 209
distinction from yeast extract, 210
Meat pastes, *p*-hydroxybenzoic acid in, 142
Mebaral, 191
Medinal, 191
Mentha arvensis, furfuraldehyde in, 401
Mercurous chloride, for gold, 50
Mercury, 81
in organic material, 81
urine, 87
Mercury cathode cell,
for aluminium, 10
arsenic, 28
Methæmoglobin, 226
Methexenyl sodium, 191
Methyl alcohol, 404
p-Methylaminophenol sulphate, for phosphates, 156
5-Methyl-barbituric acid, physical data for, 192
4-Methyl-1:2-dimercaptobenzene, for tin, 109
1-Methyl-5-phenyl-5-ethyl-barbituric acid, physical data for, 191
Milk,
benzoic acid in, 131
formaldehyde in, 397
p-hydroxybenzoic acid in, 141
manganese in, 79, 80
phsphatase in, 250, 252
vitamin B_1 in, 363

Milton's method for glucose, 224
Molybdenum, 87
Molybdenum blue test, for calcium, 37
Morphine, 310
  in aromatic powder of chalk with opium, 312
    camphorated tincture of opium, 311
    gall and opium ointment, 312
    hypodermic tablets, 313
    injections, 313
    powder of ipecacuanha and opium, 312
    tincture of chloroform and morphine, 312
Muscular tissue, barbiturates in, 186
Myrobalans, tannin and gallic acid in, 422

NAIROBI coffee, 424
α-Naphthol,
  for chloroform, 395
  in β-naphthol, 375
β-Naphthol,
  for chloroform, 394
    sulphonamides, 269
  α-naphthol in, 375
N-(1-naphthyl)-ethylenediamine dihydrochloride, for sulphonamides, 270
Narconumal, 191
National wheatmeal, vitamin B_1 in, 365
Natural waters, vanadium in, 118, 120
  zinc in, 124
Nembutal, 191
Neonal, 191
Nessler's reagent,
  for ammonia, 382
    saccharin, 420
  preparation of, 382
Nickel, 90
  in cobalt salts, 96
    cobalt steel, 92
    manganese steel, 92
    plain steel, 91
    silicate rocks, 94
Nickel alloys, beryllium in, 32
Nickel salts, cobalt in, 44
Nicotinamide, 343
  in liver extracts, 343
    urine, 345
Nicotinic acid, 343
  in foods, 345
    liver extracts, 343
    urine, 345
Nipagin A, 140
Nipagin M, 140
Nipasol M, 140
p-Nitraniline,
  for α-naphthol, 375
    phenols, 408
    quinine, 318
Nitrates, 145
  arsenic in, 21
  by phenoldisulphonic acid, 145
    2 : 4-xylen-1-ol, 149
  in meat, 147
    potable waters, 148
    soil, 146
Nitrites, 152
  by Ilosvay's method, 152
    Shinn's method, 153
p-Nitroazobenzene,
  for coumarin, 376
    vanilla extract, 376
o-Nitrobenzaldehyde, for acetone, 377
Nitrobenzene, industrial toxic gas, 4
p-Nitrobenzene-azo-resorcinol, for magnesium, 70
Nitroglycerin, 402
  determination as ammonia, 402
  determination as nitrate, 403
1-*p*-Nitrophenol-3-methyl-4-nitro-5-pyrazolone, for calcium, 37
5-Nitroquinaldinic acid, for zinc, 126
α-Nitroso-β-naphthol,
  for cobalt, 43
    œstrogens, 289
Nitroso-R salt,
  for cobalt, 43, 44
    potassium, 96
p-Nitrosodimethylaniline, for palladium, 6
p-Nitrosodiphenylamine, for palladium, 6
Nitrous acid, for morphine, 310
Nitrous fumes, industrial toxic gas, 4
Noctal, 191
Non-protein nitrogen, 245, 264
Nostal, 191
Novocain, 318
Nux vomica and its preparations, strychnine in, 321

ŒSTRADIOL, 289
Œstriol, 289
Œstrone, 289
Ointment of,
belladonna, solanaceous alkaloids in, 296
gall and opium, morphine in, 312
Orange Juice, vitamin B_1 in, 369
Organic matter,
arsenic in, 20, 22
bismuth in, 35
cobalt in, 45
copper in, 49
gold in, 52
iron in, 64
manganese in, 76, 78
mercury in, 81
tin in, 110
Ortal, 191
Osmium tetroxide, for tannin and gallic acid, 421, 423
Overvoltage, 25
Oxalates, 155
Oxalic acid, 155
Oxygen, in water, 406

PALLADIUM, 5
Palm oil, β-carotene in, 341
Paregoric, morphine in, 311
Pasteurisation, 254
Pear-tree leaves, in tap water, 410
Pentabromacetone, 127
Pentenal, 191
Pentobarbital, 191
Pentobarbital sodium, 192
Pentobarbitalum Sodicum, 192
Pentothal, 191
Peppermint oil, furfuraldehyde in, 401
Pernocton, 191
Pernoston, 191
Peroxide-free ether, preparation of, 188
Persulphates, for chromium, 41
Phanodorm, 191
Phanodorn, 191
Phemitone, 192
Phemitonum, 192
p-Phenetyl-carbamide, 395
Phenobarbital, 191
Phenobarbitone, 192
Phenobarbitonum, 192
Phenoldisulphonic acid, for nitrates, 145
Phenol - indo - 2 : 6-dichlorophenol, for vitamin C, 290
Phenolphthalein, 164
for cyanides, 137, 138
Phenols, 408
by diazo reaction, 409
Folin and Ciocalteu's reagent, 409, 414
indophenol reaction, 411
in blood, 408, 415
urine, 408, 414
water, 408
m-Phenylenediamine,
for gold, 50
nitrites, 152
Phenylhydrazine hydrochloride,
for formaldehyde, 397
gold, 50
Phenylketonuria, 248
Phenyl-phenol, for lactic acid, 243
Phenylpyruvic acid, 247
Phloroglucinol for rancidity of fixed oils and fats, 417
Phosgene,
industrial toxic gas, 4
in chloroform, 375
Phosphatase, 249
by Aschaffenburg and Neave's method, 256
Kay and Graham's method, 252
in blood, 250
butter, 259
cheese, 259
cream, 258
milk, 250, 252
Phosphates, 155
by 1 - amino - 2 - naphthol - 4 - sulphonic acid, 158
ammonium molybdate, 165
p - methylaminophenol sulphate, 156
stannous chloride, 161
in blood, 158
urine, 158
Phosphomolybdo-tungstic acid, for mercury, 81
Phosphoric acid, 155
Phosphotungstic acid,
for cystine and cysteine, 211
quinine, 318
Physiological saline solution, 242
Picric acid,
for creatine and creatinine, 204
cyanides, 137
Picrolonic acid, for calcium, 37
Pilocarpine, 315
Pimento berry oil, clove oil in, 402
Planocaine, 318

Plant ash, chromium in, 41
Plant material,
  boron in, 136
  furfuraldehyde in, 400
  manganese in, 78
  vitamin B_1 in, 363
Platinum group, 5
Pork, vitamin B_1 in, 369
Potassium, 96
  by Eden's method, 98
    Sideris's method, 97
    silver cobaltinitrite, 99
    sodium cobaltinitrite, 97, 98
Potassium dichromate, for icterus index, 241
Potassium dithio-oxalate, for nickel, 90
Potassium ferricyanide, for vitamin B_1, 360
Potassium guaiacol sulphonate, for testosterone, 290
Potassium iodide, for bismuth, 32
Potassium metabisulphite, for silicates, 173
Potassium periodate, for manganese, 77
Potassium persulphate, for adrenaline, 326
  for manganese, 73, 75
Potassium thiocarbonate, for nickel, 90
Potassium thiocyanate, for molybdenum, 88
Potatoes,
  manganese in, 80
  vitamin B_1 in, 369
Powder of ipecacuanha and opium, morphine in, 312
Powdered nux vomica, strychnine in, 324
Powdered belladonna leaf, solanaceous, alkaloids in, 295
Pregnancy test, 241
Prepared ergot, 309
Procaine, 316
  trade names for, 318
Prominal, physical data for, 191
Proponal, physical data for, 191
*iso*Propyl alcohol, 378
5-*iso*Propyl-barbituric acid, physical data for, 192
5 - *iso*Propyl - 5 - (2 - bromallyl) - barbituric acid, 191
Protein nitrogen, 383
Proteins, 260
  by biuret method, 262
Proteins,
  by wet oxidation, 260
  in blood, 260, 262, 264
    cerebrospinal fluid, 260, 263
    urine, 260, 263, 266
Pyke's method for rancidity of fixed oils and fats, 417
Pyridine,
  for carbon tetrachloride, 389
    chloroform, 394
Pyridoxine, 290
Pyrogallol, 421
Pyruvic acid, 267

QUINALIZARIN,
  for aluminium, 7
  for beryllium, 31
    borates, 135
Quinine, 318
  for bismuth, 33

RABBIT tissue, creatine and creatinine in, 206
Rancidity of fixed oils and fats, 417
  by Pyke's method, 417
    Walters, Muers and Anderson's method, 418
Rectidon, 191
Reinsch reaction, 12
Riboflavin, 290
Resorcinol, for fructose, 216
Road dressings, phenol from 409
Roasted galls, tannin and gallic acid in, 422
Rocks,
  chromium in, 41
  nickel in, 94
  titanium in, 113
Rosaniline, for formaldehyde, 397
Rosemary oil, in light camphor oil, 402
Rubeanic acid, for copper, 46, 49
Rutonal, physical data for, 191

SACCHARIN, 419
  Salicylates, 162
Salicylic acid, 162
  by ferric chloride, 163
    Jorissen's test, 164
  distinction from benzoic and *p*-hydroxybenzoic acids, 143
  in acetylsalicylic acid, 163
Sandoptal, physical data for, 191

Sawdust in tap water, 410
Sausages, *p*-hydroxybenzoic acid in, 142
Sea water, gold in, 50
Schiff's reagent,
  for formaldehyde, 397
    methyl alcohol, 404
Scopolamine, 292
Seconal, 191
Serine, 183
Sevicaine, 318
Sewage,
  chlorine in, 393
  hydrogen sulphide in, 178
Shinn's method, for nitrites, 153
Silage, β-carotene in, 342
Silicate rocks, nickel in, 94
Silicates, 165
  by ammonium molybdate, 165
    King's method, 172
    reduction, 165, 172
  in aluminium, 166, 170
    aluminium alloys, 166, 170
    animal tissue, 172
    lung tissue, 172
Silicosis, 166
Silicic acid, 165
Silver, 101
Silver cobaltinitrite, for potassium, 99
Skatole, for fructose, 217
Sodium, 102
  in blood, 105
Sodium alizarin sulphonate,
  for aluminium, 7
    fluorides, 127
Sodium bismuthate, for manganese, 72, 74
Sodium bisulphite, constitution of, 22
Sodium cobaltinitrite, for potassium, 97
Sodium diethyl-dithio-carbamate, for copper, 46
Sodium β-glycerophosphate, for phosphatase, 249
Sodium metabisulphite, constitution of, 22
  for silicates, 173
Sodium metavanadate,
  for oxalates, 155
    tartrates, 178
Sodium molybdate, for arsenic, 29
Sodium 1:2-napthoquinone-4-sulphonate for cystine and cysteine, 211
Sodium nitroprusside, for cystine and cysteine, 211
Sodium-1-nitroso-2-hydroxy-naphthalene-3:6-disulphonate, for cobalt, 43, 44
Sodium periodate, for manganese, 77
Sodium salicylate, for iron, 53, 57
Soil,
  carbon tetrachloride in, 390
  chromium in, 41
  cobalt in, 45
  manganese in, 77
  nitrates in, 146
Solbrol, 140
Soluble saccharin, 421
Somonal, 191
Soneryl, 191
Soya bean meal, for urea, 281
Sphagnum moss in tap water, 410
Spinach,
  β-carotene in, 342
  vitamin B_1 in, 369
Spinal cord, barbiturates in, 188
Stannous chloride,
  for gold, 50
    phosphates, 156, 159
Steam distillation apparatus for isolation of chloroform and carbon tetrachloride, 390
Steel,
  carbon in, 386
  chromium in, 39, 40
  manganese in, 73
  molybdenum in, 89
  nickel in, 91, 92
  titanium in, 114
  vanadium in, 119
Stibophen,
  arsenic in, 22
  lead in, 62
Stilbœstrol, 335
  in oil, 336
    tablets, 336
  qualitative colour test for, 337
Stilbœstrol dipropionate, 336
Stramonium leaf, solanaceous alkaloids in, 295
Straw, in tap water, 410
Strychnine, 321
  in galenicals, 324
    hypodermic tablets, 323
    injections, 323
    nux vomica seeds, 324
Sulphadiazine, 272
Sulphaguanidine, 272

Sulphanilamide, 269
bedside method for, 273
for nitrites, 152
Sulphanilic acid, for phenols, 408
for nitrites, 152
Sulphapyridine, 272
Sulphathiazole, 272
Sulphates, in blood, 273
Sulphides, 174
for lead, 60
in fish, 175
meat, 175
sewage, 178
Sulphonamides, 269
bedside method for, 273
p-Sulphophenyldiazonium chloride, for bilirubin in blood, 193
Sulphur dioxide, industrial toxic gas, 4
Sulphurous acid, 174
Suramin, 275
Syncaine, 318

TABLET of phenobarbitone and theobromine, 188
Tablets, vitamin B_1 in, 360
Tannic acid,
for carboxyhæmoglobin, 237
molybdenum, 88
Tannin, 421
in coffee, 424
galls, 422
hops, 424
myrobalans, 422
tea, 423
Tapioca, manganese in, 80
Tar in water, 411
Tartar emetic,
arsenic in, 22
lead in, 62
Tartrates, 178
Tea, tannin and gallic acids in, 423
Testosterone, 290
1 : 2 : 5 : 8 - Tetrahydroxy - anthraquinone,
for aluminium, 7
beryllium, 31
Thallium, 106
Thiamin, 359
Thiochrome method for vitamin B_1, 290, 360
Thiocyanates, for iron, 53
Thioglycollic acid,
for iron, 53, 55
tin, 109
β-Thiol-α-aminopropionic acid, 211
Thiophen, for lactic acid, 243
Thiourea, for bismuth, 33
Thymol,
for sulphonamides, 269
titanium, 112, 116
tryptophan, 277
Tin, 109
apparatus for isolation of, 111
in food, 110
organic materials, 110
Tincture,
of belladonna, solanaceous alkaloids in, 295
chloroform and morphine, morphine in, 312
nux vomica, strychnine in, 324
Titan yellow, for magnesium, 70
Titanium, 112
by hydrogen peroxide, 113
thymol, 116
in cast iron, 115
clay, 116
rocks, 113
steel, 114
α-Tocopherol, 371
in blood serum, 372
concentrates, 372
wheat germ oil, 371
o-Tolidine,
for chlorine, 391
gold, 50
Tomatoes, β-carotene in, 342
Tomato purée, copper in, 49
Toxic gases in industry, leaflets on, 3, 4
Toxicological specimens, arsenic in, 22
Trigonelline, 343
in foods, 349
urine, 348
Tropæolin OO, for magnesium, 70
Tropine mandelate, 298
Tryptophan, 277
Tungsten, 89
Turf in tap water, 410
Turnips, β-carotene in, 342

URANYL zinc acetate, for sodium, 102
Urea, 278
in blood, 279
urine, 280
Urease, for urea, 279

Uric acid, 283
in blood, 283
urine, 285
Urine,
amino-acid nitrogen in, 184
barbiturates in, 187
creatine and creatinine in, 206
ethyl alcohol in, 215
fructose in, 217
histidine in, 238
mercury in, 87
nicotinic acid and nicotinamide in, 345
phenols in, 408, 414
phenylpyruvic acid in, 247
phosphates in, 158
protein in, 260, 263, 266
sulphanilamide in, 269, 271
sulphonamides in, 269, 271
urea in, 278, 280
uric acid in, 283, 285
vitamin B_1 in, 366
vitamin B_2 in, 290
Urine analyses, 182
Urobilin, for zinc, 121

VANADIUM, 118
by hydrogen peroxide, 118
by 8-hydroxyquinoline, 120
in natural waters, 120
steel, 118
Vanadium oxychloride, for capsaicin, 376
Vanillin, for tryptophan, 277
Vegetables, vitamin B_1 in, 363
Vegetable drugs, continuous extraction apparatus for, 304
Vegetable foods, benzoic acid in, 131
Vegetable material, furfuraldehyde in, 400
Vegetable tannins, distinction from tar phenols, 410
Veratrole, for lactic acid, 243
Veronal, 191
Vinegar, furfuraldehyde in, 400
Viscera, isolation of alkaloids from, 287
Vitamin A, 349
conversion factor for, 352
in butter, 354
concentrates, 353
cocoa and chocolate, 355
liver oils, 352
margarine, 354
Vitamin B_1, 359
by diazotisation method, 360
thiochrome method, 360
in bread, 363
egg products, 363
milk and dried milk, 363
National Wheatmeal, 365
urine, 366
vegetables and plant tissu 363
white flour, 364
yeast extract, 363
yeast and related compound 361
loss of, on baking, 368
table showing content of, in foods, 369
Vitamin B_2, 290
Vitamin B_6, 290
Vitamin C, 290
Vitamin E, 371
in blood serum, 372
concentrates, 371
wheat germ oil, 371
Vitamins, 290

WALPOLE comparator, 93
Walters, Muers and Anderson's method for rancidity in fixed oils and fats, 418
War gases, arsenical contamination of food, 24
Water,
ammonia in, 382
chlorine in, 391
cyanides in, 138
dissolved oxygen in, 406
hydrogen sulphide in, 178
nitrates in, 148, 150
phenols in, 408
vitamin E in, 371
White flour, vitamin B_1 in, 364
White galls, 423
Wines, *p*-hydroxybenzoic acid in, 141
Wurster's red, 392

XANTHOPHYLL, 338
Xanthoproteic reaction, for stilbœstrol, 335
Xanthydrol, for urea, 279
2:4-Xylen-1-ol, for nitrates, 145, 149

YEAST, vitamin B_1 in, 361
Yeast extract,
distinction from meat extract, 210
vitamin B_1 in, 363

ZINC, 121
in biological material, 122
Zinc,
in natural waters, 124
iron in, 58
lead in, 63
Zinc compounds, lead in, 63
Zinc-copper alloy, for arsenic, 24
Zinc-copper couple, for nitrates, 145, 150

PRINTED IN GREAT BRITAIN BY WILLIAM CLOWES AND SONS, LIMITED, LONDON AND BECCLES