See Notices, p. 78

THE ANALYST

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THE ANALYST

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

A JOINT Meeting of the Society with the Food Group of the Society of Chemical Industry was held at 7 p.m. on Wednesday, December 1st, 1948, in the Lecture Room of the Institution of Civil Engineers, Great George Street, Westminster, London, S.W.1. The President, Mr. Lewis Eynon, was in the chair.

The general subject of the meeting was "Food Standards and Labelling," and the following contributions were given:—"Introduction," by C. A. Adams, C.B.E., B.Sc., F.R.I.C., Barrister-at-Law; "The View-point of the Manufacturer," by L. H. Lampitt, D.Sc., F.R.I.C., M.I.Chem.E.; "The View-point of the Public Analyst," by H. E. Monk, B.Sc., F.R.I.C.; a general discussion followed.

NEW MEMBERS

James Cassidy, A.R.I.C.; Geoffrey Bernard Courtier, B.Sc. (Lond.), D.I.C., F.R.I.C.; Hugh Francis Edward Donnelly, B.A., B.Sc. (Oxon.), A.R.I.C.; Kenneth Arthur Lees; John Thomas Martin, B.Sc. (Birm.), D.Sc. (Lond.), F.R.I.C.; Nanduri Sambasiva Rao, M.Sc. (Andra Univ.), A.R.I.C.; Wilfred Ebenezer White; Leslie Henry Williams, A.M.C.T., A.R.I.C.

PHYSICAL METHODS GROUP

THE Fourth Annual General Meeting of the Group was held at 6 p.m. on Tuesday, November 30th, 1948, in the Chemistry Lecture Theatre, Imperial College of Science and Technology, London, S.W.7. Dr. J. G. A. Griffiths, the Chairman of the Group, presided. The Group Officers and elected Members of Committee for the forthcoming year are as follows:—*Chairman*—Dr. J. G. A. Griffiths. *Vice-Chairman*—Mr. B. S. Cooper. *Hon. Secretary*—Dr. J. E. Page, Glaxo Laboratories Ltd., Greenford, Middlesex. *Members of Committee*—Messrs. W. Cule Davies, W. F. Elvidge, L. A. Haddock, R. A. C. Isbell, J. A. C. McClelland and H. E. Monk. *Hon. Auditors*—Messrs. C. A. Bassett and D. C. Garratt (re-appointed).

Votes of thanks were accorded to the three retiring members of Committee, Messrs. J. R. Edisbury, J. Haslam and D. M. Smith, for their services to the Group during the past two years, to the Hon. Auditors and to the Hon. Secretary for their work during the past year.

The Annual General Meeting was followed by the Seventeenth Ordinary Meeting of the Group, at which Mr. R. Donaldson, M.A., of the National Physical Laboratory, delivered a lecture on "The Measurement of Colour."

BIOLOGICAL METHODS GROUP

THE Fourth Annual General Meeting of the Group was held at 6.15 p.m. on Friday, December 17th, 1948, at Gas Industry House, Grosvenor Place, London, S.W.1. At this meeting Dr. E. C. Wood, who had served the Group as Hon. Secretary since its inception, resigned this post, and Mr. S. A. Price was elected to succeed him. The Group Officers and elected Members of Committee for the forthcoming year are as follows:—*Chairman*—Dr. A. J. Amos. *Vice-Chairman*—Mr. N. T. Gridgeman. *Hon. Secretary*—Mr. S. A. Price, The Research Laboratories, Vitamins Ltd., 23 Upper Mall, London, W.6. *Elected Committee Members*—Messrs. A. L. Bacharach, W. A. Broom, H. O. J. Collier, R. L. Edwards, H. E. Monk and H. Pritchard. *Hon. Auditors*—Messrs. D. M. Freeland and J. H. Hamence (as last year).

The Annual General Meeting was followed by an Ordinary Meeting at which the following papers were read and discussed: "The Assay of Aneurine by the Plate Method," by Miss A. Jones and Dr. S. Morris; "The Extraction of Growth Factors from Natural Products prior to Microbiological Assay," by Mr. J. S. Harrison.

NORTH OF ENGLAND SECTION

AN Ordinary Meeting of the Section was held in Manchester on Saturday, December 4th, 1948. The Chairman of the Section, Mr. C. H. Manley, presided, and the attendance was 37. A film, depicting incidents at the Summer Meeting at Windermere, was shown by Mr. J. F. Clark. The following paper was read and discussed: "The Determination of the Meat Content of Sausages," by R. W. Sutton, B.Sc., F.R.I.C., and J. Markland, B.Sc., F.R.I.C.

The Twenty-fourth Annual General Meeting of the Section was held in Manchester on Saturday, January 29th, 1949. The Chairman, Mr. C. H. Manley, presided over an attendance of 30. The Hon. Secretary presented the Report and Financial Statement, which were adopted. Appointments for the forthcoming year were made as follows:—*Chairman*—Mr. J. G. Sherratt. *Vice-Chairman*—Mr. A. A. D. Comrie. *Hon. Secretary*—Mr. Arnold Lees, 87 Marshside Road, Southport, Lancs. *Elected Committee Members*—Messrs. J. F. Clark, C. H. Manley, J. Markland, R. K. Matthews, J. E. Sands and H. Weatherall. *Hon. Auditors*—Messrs. C. J. House and J. R. Walmsley.

The retiring Chairman, Mr. C. H. Manley, presented his "Chairman's Address," after which a number of members contributed to a discussion on several matters of professional interest.

SCOTTISH SECTION

THE Fourteenth Annual General Meeting of the Section was held in Glasgow on January 27th, 1949, and the following office bearers were elected for the coming year:—*Chairman*—Dr. J. Sword. *Vice-Chairman*—Mr. H. C. Moir. *Hon. Secretary and Treasurer*—Mr. R. S. Watson, City Analyst's Department, 20 Trongate, Glasgow, C.1. *Elected Committee Members*—Messrs. F. J. Elliott, A. N. Harrow, M. Herd, J. Malcolm, R. O. Scott and R. G. Thin. *Hon. Auditors*—Messrs. A. R. Campbell and W. M. Cameron.

MICROCHEMISTRY GROUP

THE Fifth Annual General Meeting of the Group was held on Friday, January 29th, 1949, at the Imperial College of Science and Technology, South Kensington, London, S.W.7. It was reported that the number of members of the Group is now 258, an increase of 48 during 1948. The following Officers and Committee Members were elected for 1949:—*Chairman*—Mr. Ronald Belcher. *Vice-Chairman*—Dr. Cecil L. Wilson. *Hon. Secretary*—Mr. Donald F. Phillips. *Hon. Treasurer*—Mr. Clifford Whalley. *Elected Committee Members*—Messrs. A. F. Colson, A. E. Heron, G. Ingram, C. E. Spooner, D. W. Wilson and G. H. Wyatt.

A paper entitled "The Rapid Micro-analytical Determination of Carbon and Hydrogen in Organic Compounds" was read by Dr. A. F. Colson. This was followed by an open discussion on B.I.O.S. Final Report No. 1606: "Progress in Microchemistry in Germany."

Analytical Methods Committee

Tragacanth Sub-Committee

REPORT No. 2

The Evaluation of Flake Tragacanth

THE Analytical Methods Committee has received the following Report from the Tragacanth Sub-Committee and its publication has been duly authorised.

The Sub-Committee is composed as follows:—

Norman Evers, B.Sc., Ph.D., F.R.I.C. (*Chairman*).
D. C. Garratt, B.Sc., Ph.D., F.R.I.C.
C. A. Hallas, B.Sc., F.R.I.C.
J. S. Hamburger (*Hon. Secretary*).
R. S. Higginbotham, B.Sc.
R. H. Marriott, D.Sc., F.R.I.C.
W. Mitchell, B.Sc., Ph.D., F.R.I.C.
G. A. Mittler, Ph.D., F.R.I.C.
W. M. Seaber, B.Sc., F.R.I.C.
S. G. E. Stevens, B.Sc., F.R.I.C.

Introduction

In Report No. 1 (The Analyst, 1948, **73**, 368–377) a method was given for the evaluation of powdered tragacanth. The present report deals with the application of this method to the flake or ribbon gum and with the determination of ash and volatile acidity.

Determination of viscosity

It is known that the process of grinding tragacanth may have the effect of lowering the viscosity of mucilages prepared from it, but as a uniform mucilage suitable for the determination of viscosity cannot be produced from the whole gum within a reasonable time, it was considered necessary to powder the sample for the test. The problem before the Sub-Committee, therefore, was to find a method of grinding the whole gum to a degree of fineness suitable for the preparation of a uniform mucilage with a minimum loss of viscosity.

Preliminary experiments suggested that in order to obtain a satisfactory mucilage, it was necessary to use a powder not coarser than that passing a No. 30 mesh sieve. The rejection of the finer powder made little or no difference to the viscosity of the mucilage.

The first series of tests was conducted on two samples of flake tragacanth "A" and "B". Members were asked to carry out the following methods—

(a) take a 50-g. sample, grind to pass a No. 30 mesh sieve, and carry out the method recommended in Report No. 1 except that the mucilage, after the addition of the water, is allowed to stand for 24 hours before being heated.

(*b*) take a 50-g. sample, grind to pass a No. 60 mesh sieve, and carry out the method recommended in Report No. 1, *i.e.*, without allowing the mucilage to stand for twenty-four hours before the heating.

Table I

Flake Gum "A"

Member	Method	C_{100}	C_{100} on commercial powder
1	(*a*)	0·53	0·64
	(*b*)	0·54	
2	(*a*)	0·52	0·57
	(*b*)	0·54	
3	(*a*)	0·56	0·63
	(*b*)	0·55	
5	(*a*)	0·57	0·60
	(*b*)	0·52; 0·58	
7	(*a*)	0·57	0·63
	(*b*)	0·61	
9	(*a*)	0·51	0·61
	(*b*)	0·58	
10	—	—	0·58
Mean	(*a*)	0·543	0·609
	(*b*)	0·560	

Flake gum "B"

Member	Method	C_{100}	C_{100} on commercial powder
1	(*a*)	0·22	0·28
	(*b*)	0·23	
2	(*a*)	0·19	0·27
	(*b*)	0·20	
3	(*a*)	0·23	0·29
5	(*a*)	0·21; 0·22	0·27; 0·28
	(*b*)	0·20; 0·24	
7	(*a*)	0·22	0·29
	(*b*)	0·23	
9	(*a*)	0·21	0·27
	(*b*)	0·21	
10	—	—	0·26
Mean	(*a*)	0·213	0·276
	(*b*)	0·218	

The results are shown in Table I. C_{100} indicates the concentration necessary to give a mucilage with an efflux time of 100 seconds in the Redwood viscometer. Parallel tests were also carried out by the method recommended in Report No. 1 on powders from the samples of the same gums ground under commercial conditions to pass a No. 120 mesh sieve.

Conclusions—There would seem to be little or no real difference between the results obtained by the two methods (*a*) and (*b*). The results obtained indicate, however, that grinding the flake in the laboratory produces a powder which, when dispersed in water, possesses a viscosity higher than that of the mucilage produced from the commercially ground material. It was decided that the results were sufficiently encouraging to warrant a more extended trial of method (*a*) but it was found that the 24-hour period of standing before heating was unnecessarily long, and the period was reduced to 1 hour.

A second series of tests of the five samples of flake gum "C," "D," "E," "F" and "G" was therefore carried out by the following method—

RECOMMENDED METHOD—

After sifting out any accompanying powder, grind a representative sample, preferably not less than 50 g., until the whole passes through a No. 30 mesh sieve. The grinding may be effected in a coffee mill or a laboratory disintegrator of the Christy and Norris type or by any other method in which the process of grinding is not prolonged and does not appreciably heat the gum. Determine the moisture in the powder by the method given in Report No. 1. Weigh out a quantity of the powdered gum equivalent to the required weight of dry gum into a dry 500-ml. conical flask and add 5 ml. of 95 per cent. alcohol. Ensure that the gum is completely wetted and dispersed evenly over the inner surface of the flask. Add 195 ml. of cold distilled water as quickly as possible and shake. Allow to stand for 1 hour, swirling frequently. Connect the flask to a reflux condenser and place in a vigorously boiling water-bath, so that the surface of the water is about 1 inch above the surface of the mucilage. Complete the determination by the method as described in Report No. 1.

TABLE II

	C_{100}				
Member	"C"	"D"	"E"	"F"	"G"
1	0·226	0·410	0·440	0·73	0·91
2	0·235	0·440	0·450	0·82	0·94
3	0·246	0·490	0·460	0·81	0·90
5	0·226	0·440	0·400	0·83	0·90
7	0·240 0·236	0·413 0·439	0·452	0·74	0·98
8	0·236	0·370 0·380	0·440	0·82	0·96
9	0·246	0·386	0·448	0·77	0·94
Mean	0·236	0·419	0·441	0·790	0·933

These results are regarded as sufficiently concordant for the method described above to be recommended.

NOTE ON THE CORRECTION OF THE REDWOOD VISCOMETER—

It has been pointed out to the Sub-Committee by Dr. G. Barr* of the National Physical Laboratory that the use of the method of correction recommended in Report No. 1 for Redwood Viscometers giving different rates of flow with water may lead to serious errors. The correction applied by the Sub-Committee's recommended method may amount to 10 per cent. whereas differences between instruments certified by the National Physical Laboratory will rarely amount to 2 per cent. at 100 seconds.

Some of the viscometers used have been checked by determining the efflux time at 20° C. of an aqueous solution containing 72·4 per cent. of glycerol and having an uncorrected specific gravity at 25°/25° C. of 1·1886 ± 0·0001. This solution should have an efflux time at 20° C. of 100 seconds. The instruments tested gave efflux times of 100 ± 1 seconds with this solution.

* Private communication

Suspending power

It should be made clear that, although viscosity is one of the factors that determine the suspending power of gum tragacanth, the latter property is dependent on several other factors. It has been shown from experiments by members of the Sub-Committee that mucilages prepared from different gums and having the same efflux time in the Redwood viscometer vary considerably in their suspending power for bismuth carbonate. Viscosity cannot therefore be regarded as a measure of suspending power. Since suspending power is known to be dependent on the physical properties of the suspended particles, it does not seem to be practicable to prescribe a standard method for its determination. It is recommended therefore that when gum tragacanth is being purchased for use as a suspending agent, the purchaser should carry out a form of test using the concentration of tragacanth normally employed in his process and using all the materials that he desires to suspend.

The determination of ash*

The percentage of ash is apparently unrelated to the quality of tragacanth, at any rate within the limits encountered in genuine samples, although a high ash content may indicate crude adulteration of the powdered gum.

Preliminary experiments on the determination of ash by direct ignition gave results that did not show satisfactory agreement. Attention was therefore given to the determination of sulphated ash and better concordance was obtained.

The method recommended is as follows—Weigh accurately about 1 g. of the powdered gum into a platinum dish, and ash at about 300° C., preferably over an Argand burner. Moisten the ash with sulphuric acid (50 per cent. by volume) and heat gently until the sulphuric acid is removed, then more strongly until a white ash is obtained. Again moisten with sulphuric acid and ignite at a bright red heat (about 850° C.). The sulphated ash should be expressed as a percentage of the dry gum.

The results on a sample of flake gum "C" and a sample of powdered gum "H" are given in Table III. They are regarded as sufficiently satisfactory to validate the method described above.

Table III

Results for the determination of sulphated ash expressed as a percentage of the dry gum

Member	Sulphated ash %: Flake gum "C"	Sulphated ash %: Powder "H"
1	—	4·62
		4·66
		4·66
		4·68
2	4·89	4·41
	4·91	4·47
		4·51
		4·57
3	4·67	4·53
	4·73	4·45
5	4·93	4·45
	4·97	4·49
6	4·93	4·47
7	4·74	4·37
	4·63	4·27
	4·64	
8	4·74	4·59
	4·74	4·61
9		4·29
		4·32
		4·58
Mean	4·793	4·500

* This method is applicable to powdered tragacanth without further grinding.

THE DETERMINATION OF VOLATILE ACIDITY*

The determination of volatile acidity is of value in detecting adulteration of tragacanth with Karaya gum (also known as Indian tragacanth) from *Sterculia* species and with Gum Ghatti (Indian gum) from *Anogeissus latifolia.*

The method used was that described in "*Methods of Analysis of the Association of Official Agricultural Chemists,*" 6th Edition, 1945, p. 709. The details are as follows—

Treat 1 g. of the whole or powdered sample in a 700-ml. round-bottomed long-necked flask in the cold with 100 ml. of water and 5 ml. of phosphoric acid (85 per cent. by weight) for several hours or until the gum is completely swollen. Boil gently for 2 hours under a reflux condenser. A very small quantity of cellulose substance will remain undissolved. Tragacanth yields a practically colourless solution. Karaya gum gives a pink or rose solution. This reaction may be used as a preliminary test for detection of Karaya gum. Distil the hydrolysed product with steam, using a scrubber to connect the distillation flask with the condenser. Continue the distillation until the distillate amounts to 600 ml. and the acid residue to about 20 ml. To avoid scorching of the residue, do not permit concentration of the contents of the distilling flask to less than 20 ml. Titrate the distillate with 0·1 *N* sodium hydroxide, using 10 drops of phenolphthalein indicator. Correct the results by a blank determination and express as "volatile acidity" the number of ml. of 0·1 *N* sodium hydroxide required to neutralise the volatile acid obtained.

The results on two samples of tragacanth "C" and "G" are given in Table IV. The results are calculated as ml. of 0·1 *N* sodium hydroxide per 1 g. of gum as received.

TABLE IV

Member	ml. of 0·1 *N* sodium hydroxide for 1 g.	
	Gum "C"	Gum "G"
1	3·55; 3·45	3·50; 3·50
2	3·55	3·85
3	3·60	3·75
5	3·55; 3·75	3·50; 3·70
7	3·60	3·80
9	3·90; 3·75	3·90; 3·10

These figures are regarded as sufficiently concordant to justify recommending the method for adoption. Figures obtained by a member using this method on 28 samples of tragacanth gave a range of 2·3 ml. to 4·0 ml. with a mean of 3·46 ml. of 0·1 *N* sodium hydroxide. One sample of Indian gum (Gum Ghatti) gave 12·75 ml. and a sample of Karaya gum 22·2 ml.

The Determination of Steroids in Urine

By S. L. TOMPSETT

(*Read at a Meeting of the Scottish Section at Glasgow, on November* 28, 1947)

STEROID substances are found in the urine of both sexes and are considered to be related to the steroid hormones elaborated by the suprarenal cortex, ovary and placenta—see Tables I and II†. For the most part these are excreted as water-soluble conjugates with glucuronic and sulphuric acids. As a result urine shows but little hormonal activity until the conjugates have been decomposed by acid hydrolysis. It should be noted that the same set of hormones is produced in both the male and the female, but that the hormonal balance is obviously different. In many conditions due to increased or decreased activity of an organ this balance is altered. In the female, changes occur both during the menstrual cycle and in pregnancy.

The isolation and identification of particular steroids has occupied the attention of many workers. Much of this work has been only qualitative although the object in many cases was to attempt to associate the presence of a particular steroid with a particular condition or disease. On the quantitative side, methods have been worked at for the estimation of

* This method is applicable to powdered tragacanth without further grinding.

† For the basic ring structure of the steroids and the numbering of their carbon atoms, see Appendix, p. 17. In the formulae α-substituents are denoted by dotted linkages and β-substituents by plain linkages.

TABLE I

ORIGIN OF THE PRINCIPAL STEROID HORMONES

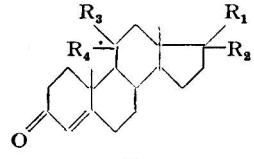
Sex	*Tissue*	*Hormones*
Male	Suprarenal cortex	Corticoids Oestrogens Progesterone Androgens
	Testes	Androgens
Female	Suprarenal cortex	as for male
	Ovary	Oestrogens Progesterone
	Placenta	Oestrogens Progesterone

oestrogens, 17-ketosteroids and pregnanediol. The steroids known to be present in urine may be divided into two groups (see Table III): (1) "phenolic" steroids and (2) neutral steroids. The former contain a phenolic ring in their structure and hence are extractable from solution in organic solvents by aqueous solutions of alkalis. The latter are fully saturated or at least contain no more than one double bond in any 6-membered ring.

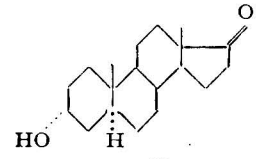
It is probably safe to say that in the past the chemical and physiological study of the steroids has been almost entirely an academic pursuit.

TABLE II

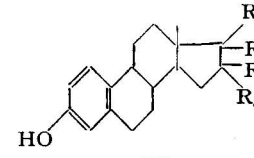
THE PRINCIPAL NATURAL STEROIDS IN MAN



I



II



III

(Key formulae)

ANDROGENS—promote male sex activity:

1. Testosterone—**I**: $R_1 = OH$; $R_2 = R_3 = R_4 = H$. The most active; regarded as the hormone.
2. Androsterone—**II**.

OESTROGENS—promote female sex activity; connected with menstruation, pregnancy and development of the mammae.

1. α-Oestradiol—**III**: $R_1 = OH$; $R_2 = R_3 = R_4 = H$. The most active; regarded as the hormone.
2. Oestrone—**III**: $R_1 + R_2 = :O$; $R_3 = R_4 = H$
3. Oestriol—**III**: $R_1 = R_3 = OH$; $R_2 = R_4 = H$

} Probable metabolites of α-oestradiol.

PROGESTERONE—**I**: $R_1 = .COCH_3$; $R_2 = R_3 = R_4 = H$. Connected with menstruation and pregnancy.

CORTICOIDS—produced by the suprarenal cortex:

1. Corticosterone—**I**: $R_1 = .COCH_2OH$; $R_3 = OH$; $R_2 = R_4 = H$
2. Desoxycorticosterone—**I**: $R_1 = .COCH_2OH$; $R_2 = R_3 = R_4 = H$
3. 11-Dehydrocorticosterone—**I**: $R_1 = .COCH_2OH$; $R_2 = H$; $R_3 + R_4 = :O$
4. 17-Hydroxycorticosterone—**I**: $R_1 = .COCH_2OH$; $R_2 = R_3 = OH$; $R_4 = H$
5. 17-Hydroxy-11-dehydrocorticosterone—**I**: $R_1 = .COCH_2OH$; $R_2 = OH$; $R_3 + R_4 = :O$
6. 17-Hydroxydesoxycorticosterone—**I**: $R_1 = .COCH_2OH$; $R_2 = OH$; $R_3 = R_4 = H$

The above are concerned with

1. electrolytic balance
2. carbohydrate metabolism
3. ability to combat stress.

TABLE III

CLASSES OF STEROIDS PRESENT IN HUMAN URINE

Phenolic steroids: α-oestradiol, oestrone, oestriol. (See Table II.)

Neutral steroids:

Ketones:

17-Ketosteroids. (See Table X.)
α-Alcoholic, *e.g.*, androsterone
β-Alcoholic, *e.g.*, *iso*androsterone
Non-alcoholic, *e.g.*, androstenone-17
20-Ketosteroids, *e.g.*, allopregnanol-3(β)-one-20. (See Table X.)
3 : 20-Ketosteroids, *e.g.*, 17-hydroxycorticosterone. (See Table II.)

Alcohols: (See Table XIII.)
α-Alcohols, *e.g.*, Pregnanediol-3(α):20(α)
β-Alcohols, *e.g.*, Androstenetriol-3(β):16:17.

It is now recognised that a study of the urinary steroids is necessary in hospital routine as a diagnostic aid and in the control of certain forms of treatment. Techniques used in hospital biochemical laboratory routine work should conform as far as possible to the following standards. They should—

(1) be as rapid as possible;
(2) permit of more than one estimation being undertaken at the same time;
(3) not require too high a standard of technical skill;
(4) be of an accuracy sufficient for the purpose to which the result is put;
(5) not require too much apparatus, in particular special apparatus difficult to obtain, and take up too much laboratory space.
(6) yield results of clinical value.

During the past 18 months the writer has been carrying out an investigation concerning suitable and useful methods for urinary steroid analysis. The following represents an account of results so far obtained.

HYDROLYTIC PROCEDURES

As a preliminary to any examination, it is necessary to separate the steroids from other materials in the urine. In general three types of procedure have been employed, *viz.*,

(1) extraction of the conjugates with butyl alcohol followed usually by acid hydrolysis,
(2) acid hydrolysis of short duration followed by extraction of the free steroids with an organic solvent,
(3) simultaneous hydrolysis by acid and extraction with a comparatively high-boiling solvent, *e.g.*, toluene or carbon tetrachloride.

There has been much discussion in the literature about the relative merits of various hydrolytic procedures; whatever process is employed, there is some destruction of the steroids, resulting usually in partial desaturation. A completely satisfactory method of hydrolysis is probably an impossibility owing to the large number of different steroids present and the different ways in which they are conjugated.

THE OESTROGENS

Oestrogens are found in male and female urine mainly as water-soluble conjugates. Except in pregnancy the outputs are very low, the measurements being made in micrograms. The oestrogenic content of urine in pregnancy is approximately several hundred times that found in other conditions. (See Table IV.)

TABLE IV

OESTROGENIC CONTENT OF HUMAN URINE (ROBSON[16])

Males	..	5 to 15 micrograms per day
Females (non-pregnant)	..	10 » 36 » » »
» (pregnant)	..	20,000 » » »

Previous to examination, the urine is hydrolysed with acid and the liberated steroids are extracted with an organic solvent. Owing to their phenolic nature, oestrogens may be separated from the neutral steroids by treatment of the extract with aqueous solutions of alkali.

Numerous colorimetric tests have been suggested for the determination of either total or individual oestrogens (see Table V). These are applicable to the higher concentrations

TABLE V

CHEMICAL TESTS FOR THE OESTROGENS

	Reactants	Reference
Total oestrogens	Phenol and sulphuric acid	Kober[10] (1931); Venning *et al.*[19] (1937)
	β-Naphthol and sulphuric acid	Kober[11] (1938)
	Sulphuric acid	Cohen and Bates[3] (1947)
Oestrone	*m*-Dinitrobenzene and potassium hydroxide	Talbot *et al.*[17] (1940)
Oestriol	Sodium *p*-phenolsulphonate and phosphoric acid.	Bachman[2] (1939)

encountered in the urine of pregnancy, but within the more normal range difficulties arise. The main difficulty is the occurrence of relatively high blank values. Where these occur, biological assay, employing ovariectomised rats or mice, is generally used.

There is evidence that deviations from the normal, the measurement of which may be of diagnostic importance, occur in many conditions. Unfortunately such deviations are only measurable in micrograms, as with the normal amounts. Biological assay of the oestrogens is unsatisfactory both in point of accuracy and in the time required—hence a satisfactory chemical method for the determination of minute amounts of the oestrogens is urgently required. It has been found that when oestrogens are injected into human beings only 1 to 10 per cent. can be recovered in the urine as conjugates of α-oestradiol, oestrone and oestriol. The main oestrogen metabolites are therefore unknown. The solution of this problem might lead to more satisfactory methods of following oestrogenic secretion.

THE 17-KETOSTEROIDS

This group, which has received the greatest attention, includes all those neutral steroids that have a ketone group in position 17 (see Appendix). They are found in male and female urine as water-soluble conjugates. Differences between normal and diseased

TABLE VI

THE URINARY EXCRETION OF 17-KETOSTEROIDS—MG. PER DIEM (DORFMAN,[7] 1948)

Normal	Male	8 to 22	average 14
	Female	5 to 15	average 9
On the basis of average figures it is assumed that 9 mg. are derived from the suprarenal cortex and 5 mg. from the testes.			
Addison's disease	Male	up to 5	
	Female	almost 0	
Castration or primary agonadism			
	Male	9 mg. or less	
	Female	"	
Adreno-genital syndrome			
	Male	increased excretion; 200 mg. or even more may be encountered	
	Female		
Simmond's disease (panhypopituitarism)			
	Male	almost 0	
	Female		

NOTE—Low values are encountered in both sexes before puberty and in many chronic diseases.

are shown in Table VI. In the main, the free steroids have androgenic, *i.e.*, male sex hormone, activity. In spite of this, however, the variations in the urinary output are in general, representative of adrenal cortical activity.

THE DETERMINATION OF THE TOTAL 17-KETOSTEROIDS IN URINE—

A method has recently been published by Tompsett and Oastler[18] (1946). 250 Ml. of the urine are heated under reflux with 25 ml. of concentrated hydrochloric acid for 10 minutes, after which the liquid is cooled rapidly. Steroids are extracted with peroxide-free ether, and oestrogens, etc., are removed by treating the ether extract with aqueous sodium hydroxide

solution followed by water. The ether is removed and the residue dissolved in aldehyde-free alcohol. Two colorimetric procedures:

(1) the Zimmermann reaction,

(2) the Pincus reaction,

are available for the final estimation of the 17-ketosteroid content. In the former, equal parts of the alcoholic solution of steroid, an alcoholic solution of *m*-dinitrobenzene and an aqueous solution of potassium hydroxide are allowed to react at 25° C. for 45 minutes. A purple colour is produced and may be evaluated after suitable dilution with absolute or aqueous alcohol. The test reacts to any compound containing the $-CH_2CO-$ grouping. In the Pincus reaction, the dried steroid residue is heated in a boiling water bath with a solution of antimony trichloride in glacial acetic acid and acetic anhydride. The mixture is diluted with 95 per cent. acetic acid and the colour, which is blue, allowed to develop after standing in the dark for 1 hour at room temperature.

There has been much controversy as to the most suitable hydrolytic and extraction process. Three methods have been compared, *viz.*,

(1) hydrolysis with acid for 10 minutes followed by extraction with ether;

(2) simultaneous hydrolysis with acid and extraction with carbon tetrachloride for 1½ hours;

(3) simultaneous hydrolysis with acid and extraction with toluene for 15 minutes.

All three methods have been found to give similar results when the total 17-ketosteroid content was estimated by the Zimmermann test—the greatest difference was 7 per cent. For single estimations the first procedure has been adopted, but when for any particular purpose large volumes of urine need to be extracted the third procedure is used.

The Zimmermann test is reputed to react more or less equally with all the known urinary 17-ketosteroids. This has been confirmed for the two available 17-ketosteroids, *viz.*, androsterone and dehydro*iso*androsterone. With the Spekker absorptiometer, using green filters and 1-cm. cells, quantities of steroid ranging from 0·00 to 0·20 mg. in 10 ml. gave drum readings (less reagent blanks) ranging, in proportion, from 0·00 to 0·40 for androsterone and from 0·00 to 0·43 for dehydro*iso*androsterone. The Zimmermann test gives but little reaction with the non-ketonic fraction of the urinary steroids.

The Pincus reaction gives somewhat different results. With dehydro*iso*androsterone it produces little colour compared with that given with androsterone. In our own experiments the colour produced by 0·4 mg. of dehydro*iso*androsterone is equivalent to that produced by 0·15 mg. of androsterone. In addition, the non-ketonic fraction of the urinary steroids gives some measurable colour. It has been suggested that comparisons of the results obtained with the two reactions may be a useful indication of the amount of dehydro*iso*androsterone present, but this would, strictly speaking, necessitate a preliminary separation of ketonic and non-ketonic steroids. Whereas the Zimmermann reaction follows Beer's law down to very low levels, the Pincus reaction does not do so below 0·1 mg. of androsterone. With the Spekker absorptiometer, using red filters and 1-cm. cells, the working graph for quantities of androsterone from 0·4 mg. down to 0·1 mg. in 10 ml. was a straight line with drum readings from 0·465 down to 0·075; for quantities of androsterone below 0·1 mg. the graph proceeds to the origin at a reduced slope. Recent work by Jaffe *et al.*[9] (1947) indicates that the Pincus reaction depends on the presence of hydroxyl rather than ketone groupings.

Results obtained by the two reactions on the same urinary steroid extract have been compared (see Table VII). The Zimmermann reaction tends to give slightly higher results, and on general considerations it has been adopted.

TABLE VII

THE 17-KETOSTEROID CONTENT OF URINE—MG. PER DIEM

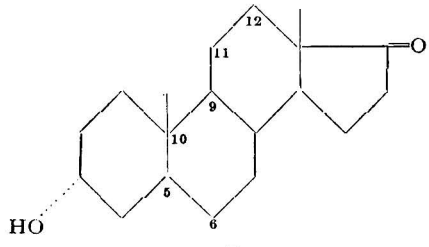
Zimmermann reaction	Pincus reaction
5·9	4·2
3·2	3·8
8·0	7·5
112·0	112·0
8·7	6·1
3·2	2·1

THE DETERMINATION OF α- AND β-ALCOHOLIC AND NON-ALCOHOLIC 17-KETOSTEROIDS IN URINE—

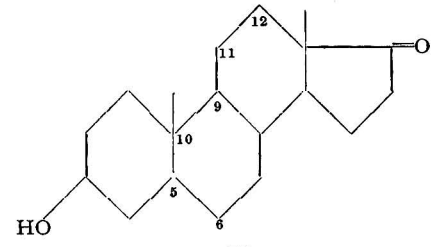
The individual 17-ketosteroids that have been isolated from urine are shown in Table VIII.

TABLE VIII

17-KETOSTEROIDS THAT HAVE BEEN ISOLATED FROM URINE



I



II

(Key formulae)

α-ALCOHOLIC KETONES—not precipitable by digitonin:

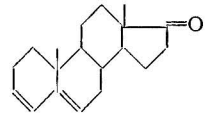
Androsterone, *cis*-Androsterone, Androstane-3(α)-ol-17-one— **I**: α-H at C_5
Aetiocholane-3(α)-ol-17-one, 3(α)-Hydroxycholane-17-one— **I**: β-H at C_5
Androstane-3(α):11-diol-17-dione, 11-Hydroxyandrosterone— **I**: α-H at C_5; OH at C_{11}
Δ^{11}-Androstene-3(α)-ol-17-one— **I**: α-H at C_5; double bond at C_{11}-C_{12}
Aetiocholanol-3(α)-dione-11:17— **I**: β-H at C_5; :O at C_{11}
Δ^{9}-Aetiocholanol-3(α)-one-17— **I**: β-H at C_5; no CH_3 at C_{10} but double bond at C_9–C_{10}

β-ALCOHOLIC KETONES—precipitable by digitonin:

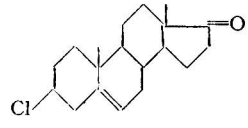
*Iso*androsterone, *Trans*androsterone, Androstane-3(β)-ol-17-one— **II**: α-H at C_5
Dehydro*iso*androsterone, *Trans*dehydroandrosterone, Δ^{5}-Androstene-3(β)-ol-17-one— **II**: double bond at C_5–C_6

NON-ALCOHOLIC KETONES—

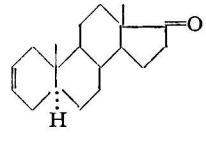
$\Delta^{3,5}$-Androstadiene-17-one—



3-Chlorodehydroandrosterone—



Androstenone-17—



REFERENCES—Pincus and Pearlman[15] (1943), Dobriner *et al.*[6] (1947), Mason[13] (1945), Liebermann and Dobriner[12] (1946), Hirschmann and Hirschmann[8] (1947), Mason and Kepler(1947).[14]

It will be noted that they are divided into three groups, *viz.*:

(1) α-alcohols;
(2) β-alcohols;
(3) non-alcohols.

The non-alcohols, which usually constitute about 15 per cent. of the total, are in all probability derived from the alcohols owing to the destructive effects of acid hydrolysis. This is especially likely since ketosteroids cannot be extracted from urine with organic solvents prior to acid hydrolysis. The α- and β-alcohols represent those with a hydroxyl group attached to the carbon atom 3 and are distinguishable by the fact that the latter but not the former are precipitated from alcoholic solution by digitonin. Normally the β-fraction constitutes

about 10 per cent. of the total. High 17-ketosteroid excretion is characteristic of both adrenal cortical hyperplasia and adrenal cortical tumour. There is evidence that in the former, the percentage of β-alcohols is unchanged, but in the latter is much increased as compared with the normal. This is obviously a useful diagnostic test.

For the differential determination of urinary 17-ketosteroids, the following procedure has been found to be satisfactory. Three separate determinations are required:

1. The total 17-ketosteroid content.
2. The non-alcoholic 17-ketosteroid content.
3. The α- and non-alcoholic 17-ketosteroid content after precipitation of the β-alcohols with digitonin.

Then—

Non-alcoholic ketosteroids	= 2
α-Ketosteroids	= 3 minus 2
β-Ketosteroids	= 1 minus 3

NON-ALCOHOLIC KETOSTEROIDS—

An alcoholic solution of urinary steroids is evaporated to dryness in an 8 in. by 1 in. Pyrex test tube. An amount corresponding to about 10 mg. of total 17-ketosteroid should be taken. To the dry residue are added 0·5 g. of succinic anhydride and 2 ml. of pyridine and the tube is plugged with cotton wool. The tube and its contents are placed in a boiling water bath for 2 hours. Then 10 ml. of water are added and the heating is continued for a further ½-hour. After cooling, the contents of the tube are extracted 3 times with 15-ml. portions of ether. The ether extract contains the alcohols as half succinate esters and the non-alcohols unchanged. The ether solution is then extracted with three 20-ml. portions of 0·1 *N* potassium carbonate in 5 per cent. sodium chloride solution. The esterified alcoholic ketosteroids are removed by this process, the non-alcoholic ketosteroids being left in the ether. The ether solution is washed twice with 10 ml. of water and evaporated to dryness. The residue is dissolved in 2 ml. of aldehyde-free alcohol and the 17-ketosteroid content determined in 0·2 ml. by the method of Tompsett and Oastler[18] (1946).

Five-mg. and 10-mg. lots of both androsterone and dehydro*iso*androsterone were submitted to this treatment and the residual ketosteroid content of the ether was determined. It amounted to from 0·5 to 3·5 per cent. of the original amount of steroid taken.

ALPHA ALCOHOLIC AND NON-ALCOHOLIC KETOSTEROIDS

An alcoholic solution of urinary steroids containing about 5 mg. of total 17-ketosteroids is transferred to a 50-ml. centrifuge tube. The volume is adjusted to 2 ml. either by evaporation or by dilution with alcohol. Three ml. of a 2 per cent. solution of digitonin in 83 per cent. alcohol are added and the mixture is allowed to stand overnight. Next day, 10 ml. of ether are added and the mixture is centrifuged. The supernatant fluid is removed and the residue treated with three 10-ml. portions of ether. The combined ether extracts, which contain the α-alcohols and the non-alcohols, are washed with three 25-ml. portions of water and then evaporated to dryness. The residue is dissolved in 10 ml. of aldehyde-free alcohol and the 17-ketosteroid content determined in 0·2 ml. according to the method of Tompsett and Oastler.

Mixtures of androsterone and dehydro*iso*androsterone were submitted to the above treatment. The results are shown in Table IX.

TABLE IX

RECOVERY OF ANDROSTERONE FROM A MIXTURE OF ANDROSTERONE AND DEHYDRO-*iso*ANDROSTERONE AFTER PRECIPITATION OF THE DEHYDRO*iso*ANDROSTERONE WITH DIGITONIN

Androsterone (mg.)	Dehydro*iso*androsterone (mg.)	Recovery (mg.)
4·5	0·5	4·4
4·0	1·0	3·9
3·0	2·0	2·8

Chromatographic analysis

There are indications that individual variations in the quality of the 17-ketosteroids may be of significance in diagnosis, possibly in malignant disease. The isolation of individual ketosteroids is well beyond the scope of the routine laboratory but fluid chromatography has been used by Dobriner *et al.*[5] (1944) and Dingemanse *et al.*[4] (1946). This appears to have possibilities.

The 3 : 20-ketosteroids

For some years it has been recognised that extracts of human urine are capable of maintaining the life of adrenalectomised animals in the same way as the normal hormones of the suprarenal cortex. The active substances are extractable by organic solvents without previous hydrolysis; in fact, treatment with acid has a destructive effect. This is the only steroid fraction that is known to be excreted in an unconjugated state. Although none has yet been isolated and identified, they would appear to be 3 : 20-ketosteroids as the cortical hormones. Increased excretions are noted after stresses, *e.g.*, injury, burns and Cushing's disease. Variations in their amount then have a diagnostic importance, especially as they do not necessarily follow the urinary excretion of 17-ketosteroids. This group has been referred to as:

(1) the 11-oxycorticosteroids;
(2) the total reducing ketonic steroids;
(3) the corticoids.

Tompsett and Oastler[18] (1947) have developed a chemical method for their estimation. This involves extraction with chloroform, separation as a Girard-T complex, liberation of the free ketones and a colorimetric determination of their reducing power towards an alkaline copper reagent. Such substances are typified by reducing alkaline copper reagents in the same way as sugar. The 3 : 20-ketosteroid content of normal urine is of the order of 0·2 to 0·4 mg. per day.

Pregnanediol

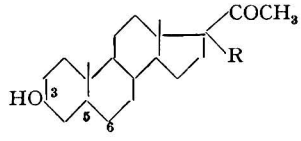
Pregnanediol is the only steroid alcohol that has received much attention. The term "pregnanediol" refers specifically to pregnanediol-3 (α) : 20 (α). It is believed to be a metabolite of progesterone, a hormone elaborated by the corpus luteum of the ovary and the placenta in women and by the suprarenal cortex of both sexes. It is excreted as a water-soluble conjugate with glucuronic acid and is inactive. It occurs in measurable quantity only in female urine. In the non-pregnant female it is excreted in amounts up to 8 mg. per day, depending on the stage of the menstrual cycle. In pregnancy as much as 100 mg. per day may be excreted in the later stages. Tompsett and Oastler[18] (1948) have used a modification of the method of Astwood and Jones[1] (1941). The daily output of urine is simultaneously hydrolysed with hydrochloric acid and extracted with toluene. The toluene extract is washed with aqueous sodium hydroxide solution and then with water and evaporated to dryness The residue is dissolved in 10 ml. of alcohol, 40 ml. of hot 0·1 *N* sodium hydroxide are added and the mixture is allowed to stand overnight in the ice chest. The next morning the precipitate is filtered off. It is dissolved in 10 ml. of alcohol, 40 ml. of hot water are added, and the mixture is allowed to stand overnight in the ice chest. The next morning, the precipitate is filtered off, dried and weighed. This is reckoned as "pregnanediol." This method is based on the observation that under the above conditions pregnanediol is precipitated in filtrable condition, whereas other steroids form colloidal solutions. The estimation seems to have some application in obstetrics and gynaecology.

The determination and quantitative fractionation of the neutral steroids of urine

The quantitative aspects of steroid metabolism, so far as urine is concerned, have been confined mainly to estimations of the 17-ketosteroid, corticosteroid and pregnanediol contents. Isolation studies have shown that other steroids or steroid groups may be present, *e.g.*, 20-ketosteroids and steroid alcohols other than pregnanediol. Members of these series that have been isolated from human urine are shown in Tables X and XI. Attempts have, therefore, been made to determine quantitatively the various steroid fractions that do or might occur in human urine. Since so little information was available, this was largely of an exploratory nature and urines from a wide variety of clinical states were examined. It was

TABLE X

20-KETOSTEROIDS THAT HAVE BEEN ISOLATED FROM HUMAN URINE



(Key formula)

Pregnanol-3(α)-one-20— α-OH at C_3; β-H at C_5; R = H
Allopregnanol-3(β)-one-20— β-OH at C_3; α-H at C_5; R = H
Allopregnanol-3(α)-one-20— α-OH at C_3; α-H at C_5; R = H
Pregnanediol-3(α):17-one-20— α-OH at C_3; β-H at C_5; R = OH
Δ^5-Pregnenediol-3(β):17(β)-one-20— β-OH at C_3; double bond at C_5-C_6; R = OH

REFERENCES—Pincus and Pearlman[15] (1943), Liebermann and Dobriner[12] (1945), Hirschmann and Hirschmann[8] (1947).

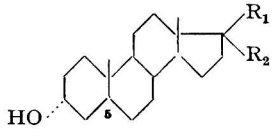
necessary to confine oneself to quantities of urine no greater than that passed during 24 hours, as otherwise important variations might be missed.

The following method was adopted for obtaining crude extracts of the urinary neutral steroids.

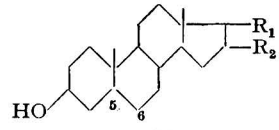
The complete 24-hour sample of urine, together with one-tenth of its volume of re-distilled toluene, was heated to boiling under a reflux condenser. Then one-tenth of its volume of concentrated hydrochloric acid was added and the boiling continued for a further 15 minutes. The mixture was cooled and the toluene removed in a separating funnel. The hydrolysed urine was further extracted with two similar quantities of toluene. The combined toluene extracts were washed twice with aqueous 10 per cent. sodium hydroxide solution and twice with water. The toluene extracts were evaporated to dryness, preferably *in vacuo*. The dry residue contained the free steroids, which were invariably mixed with pigment.

TABLE XI

ALCOHOLS THAT HAVE BEEN ISOLATED FROM HUMAN URINE



I



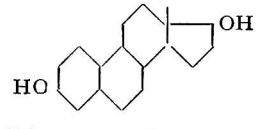
II

(Key formulae)

α-ALCOHOLS—
Pregnane-3(α):17:20-triol— **I**:β-H at C_5; R_1 = .CHOH.CH_3; R_2 = OH
Pregnanediol-3(α):20(α)— **I**:β-H at C_5; R_1 = .CHOH.CH_3; R_2 = H
Allopregnanediol-3(α):20(α)— **I**:α-H at C_5; R_1 = .CHOH.CH_3; R_2 = H
Pregnanol-3 (α)— **I**:β-H at C_5; R_1 = .CH_2CH_3; R_2 = H

β-ALCOHOLS—
Cholesterol
Δ^5-Pregnenediol-3(β):20(α)— **II**: double bond at C_5–C_6; R_1 = .CHOH.CH_3; R_2 = H
Δ^5-Androstenediol-3(β):17(α)— **II**: double bond at C_5–C_6; R_1 = OH; R_2 = H
Allopregnanediol-3(β):20(α)— **II** : α-H at C_5; R_1 = .CHOH.CH_3; R_2 = H
Δ^5-Androstenetriol-3(β):16:17— **II**: double bond at C_5–C_6; R_1 = R_2 = OH

UNCLASSIFIED—
Oestranediols—



REFERENCES—Pincus and Pearlman[15] (1943), Hirschmann and Hirschmann[8] (1945), Mason and Kepler[14] (1945).

The quantitative separation and determination of the ketonic and non-ketonic steroids was attempted. For this purpose, Girard's reagent T was employed. Under appropriate

conditions this reagent forms with ketones, but not with non-ketones, a compound soluble in water but insoluble in organic solvents. Such compounds are, however, readily converted into free ketones in the presence of mineral acids at room temperature.

The dry steroid residue was dissolved in ether and an aliquot containing approximately 10 to 15 mg. of steroid introduced into an 8 in. by 1 in. Pyrex test tube. The solvent was evaporated and 0·1 g. of Girard reagent T (kept in a vacuum desiccator over concentrated sulphuric acid) and 0·5 ml. of glacial acetic acid were added. The tube was plugged with cotton wool and placed in a boiling water bath for 20 minutes. The tube and its contents were cooled in a mixture of ice and water. Thirty ml. of ice-cold water and 3 ml. of 10 per cent. sodium hydroxide solution were added, the latter neutralising nine-tenths of the acetic acid initially present. The mixture was transferred to a 50-ml. glass-stoppered cylinder and the non-ketones were extracted with three 20-ml. quantities of ether. The combined ether extracts were transferred to a separating funnel and washed once with 20 ml. of 0·1 *N* sodium hydroxide and twice with 20-ml. quantities of water. The washed ether extract was transferred to a weighed beaker, the solvent evaporated off and the quantity of non-ketonic material determined gravimetrically.

Three ml. of concentrated hydrochloric acid were added to the aqueous solution remaining and the mixture was allowed to stand overnight at room temperature. On the following day, the freed ketones were extracted with three 20-ml. quantities of ether. The combined ether extracts were washed once with 20 ml. of 0·1 *N* sodium hydroxide and twice with 20-ml. quantities of water. The washed ether extract was transferred to a weighed beaker, the solvent evaporated off and the quantity of ketonic material determined gravimetrically.

Both the ketonic and non-ketonic residues contained pigment. The 17-ketosteroid content was determined by means of the Zimmermann reaction (Tompsett and Oastler[18] (1946)).

Pure ketonic steroids have been subjected to the above treatment and their recoveries are shown in Table XII. In view of the small quantities used, reasonable recoveries would appear to be possible.

TABLE XII

RECOVERY OF KETONIC STEROIDS AFTER SEPARATION AS COMPLEXES WITH GIRARD REAGENT T

	Ketonic steroid used (mg.)	Ketonic steroid recovered (mg.)
Androsterone	5	5·3
	6	5·9
	10	9·6
Dehydro*iso*androsterone..	2·5	2·2
	5·0	4·3
	10·0	9·6

Samples of urine from a wide variety of clinical cases have been examined. Some typical results are shown in Table XIII.

TABLE XIII

THE TOTAL KETONE AND 17-KETOSTEROID CONTENT OF THE NEUTRAL STEROID FRACTION OF HUMAN URINE—SOME TYPICAL RESULTS

	Total ketone neutral steroids (mg./diem)	17-Ketosteroids (mg./diem)
1	2·1	1·5
2	3·1	2·0
3	3·6	3·5
4	10·6	8·3
5	10·0	9·4
6	17·7	16·8
7	19·5	17·5
8*	65·8	8·5
9*	56·0	13·3

* Females—8th month of pregnancy.

In many instances close agreement existed between the 17-ketosteroids and the total ketone content. This would suggest the absence, or extremely low concentrations, of 20-ketosteroids. The chief exception was encountered in urines from cases of late pregnancy.

It would appear that in this condition very large quantities of 20-ketosteroids are excreted. Although pregnanolenes have been isolated from human pregnancy urine, no quantitative results have previously been recorded. Recently Venning (1946) suggested that in late pregnancy approximately 80 mg. of pregnonolone per day was excreted—this was based on a difference in the 17-ketosteroid content as determined by the Zimmermann and Pincus reactions. Dr. Oastler and I have obtained evidence that 20-ketosteroids may be excreted in small quantities in certain other clinical states—these are being recorded elsewhere.

The total ketone and non-ketone contents of urine in some typical cases are shown in Table XIV. It will be noted that the non-ketonic fraction of the neutral steroids of human urine are of the same order of magnitude as the ketonic fraction. Oastler and I have observed significant variations in the magnitude of this fraction in some clinical conditions—these have been recorded elsewhere (Tompsett and Oastler,[18] 1948).

TABLE XIV

THE KETONIC AND NON-KETONIC FRACTIONS OF THE NEUTRAL STEROIDS OF HUMAN URINE

	Total ketones mg./diem.	Non-ketones mg./diem.
1	2·1	5·0
2	3·6	2·4
3	1·2	2·7
4	10·6	7·7
5	14·7	14·0
6	18·5	17·4
7	17·7	23·0
8	25·0	24·0

The nature of the non-ketonic fraction has received some investigation. Steroid alcohols react with succinic anhydride to form half succinate esters. When this reaction was applied to the non-ketonic fraction, it was found that approximately two-thirds reacted to form succinate esters. It is considered therefore that variations in the non-ketonic fraction reflect quantitatively the variations in the alcoholic fraction of the urinary neutral steroids. By application of the well known reaction with acetic anhydride and sulphuric acid, cholesterol was found to be present in only negligible proportions.

Owing to the complexities and inter-relationships of steroid hormone metabolism, estimation of a single substance or group of substances gives only limited information. A more complete picture may be given by the following determinations:

(1) Oestrogens.
(2) 17-ketosteroids.
(3) Total ketones—indicative of presence of the 20-ketosteroids.
(4) The non-ketones.
(5) Pregnanediol-3 (α) : 20 (α).
(6) The corticosteroids.

It is, of course, possible that with increasing knowledge, the number of estimations may be limited in the examination of any particular case. For example, at present it is known that pregnanediol-3 (α) : 20 (α) is related to the metabolism of progesterone and hence has little value in the investigation of the male.

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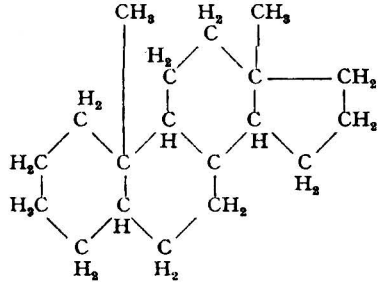
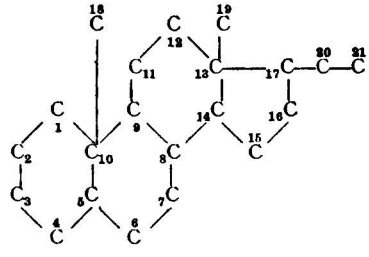
APPENDIX

Basic ring structure of the steroids.

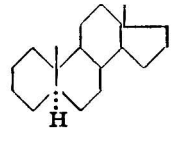


Numbering of the carbon atoms.

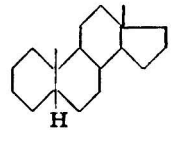


Mother Substances

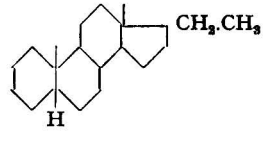
Androstane



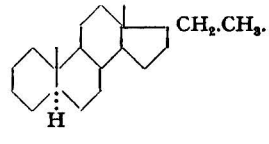
Aetiocholane



Pregnane



Allopregnane



INSTITUTE OF PATHOLOGY
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GLASGOW

The Determination of Aneurine (Vitamin B_1) in Uncooked Wheat Products

By H. N. RIDYARD

THE methods described below do not differ essentially from the classical thiochrome method of Jansen,[1] improved by Harris and Wang[2,3] and adapted by Booth[4] (see also Nicholls *et al.*[5]) for use with the Spekker Fluorimeter. They do, however, include a great number of refinements that experience and detailed investigation have shown to be of the utmost importance for the attainment of high accuracy and speed and also for ease of approach for workers new to this estimation. It has been found that junior workers previously unacquainted with aneurine or this kind of work can obtain results to within ± 5 per cent. on National flour with this written account as their only guide.

REAGENTS REQUIRED—

Sodium hydroxide solution, 30 per cent.—30 g. of purest sodium hydroxide dissolved in water with stirring, allowed to cool and diluted to 100 ml.

Potassium ferricyanide solution, 5 per cent.—5 g. of potassium ferricyanide (purest) dissolved in 100 ml. water.

Methyl alcohol, pure, free from fluorescence.

Ethyl alcohol, pure, free from fluorescence, absolute or rectified (96 to 100 per cent).

Iso*butyl alcohol*, pure—This should be steam distilled in all-glass apparatus, using distilled water, the first and last fractions to distil being returned to residues. After use it may be recovered by removing the aqueous layers in a separating funnel, washing three times with distilled water to remove lower alcohols, and steam distilling (see Note 1, page 22). It is then cooled to room temperature, preferably by standing overnight or longer, to deposit excess of water, and is used water-saturated but not cloudy. This and the other reagents should be shown to be free from fluorescence affecting the estimation in the manner described later.

Hydrochloric acid, approximately 0·2 *N*—Twenty ml. of purest hydrochloric acid (36 per cent. w/v) are diluted to 1 litre.

PREPARATION OF EXTRACTS—

(*a*) *Flour*—10 g. of the flour are weighed into a 100-ml. conical flask. 50 ml. of approximately 0·2 *N* hydrochloric acid are placed in a measuring cylinder, from which about 30 ml. are added to the flour and shaken vigorously to form a smooth suspension. The flask may be corked during shaking if the cork is quite clean and kept for this purpose only. The cork and the interior of the flask are washed down with the remainder of the acid and the mixture is allowed to stand overnight.

(*b*) *Wholemeal flour*—5 g. are treated as above.

(*c*) *Bran, etc.*—2 or 3 g. are placed in a 100-ml. flask and 50 ml. of 0·2 *N* acid added without shaking and allowed to stand overnight.

(*d*) *Wheat*—20 g. of whole wheat are weighed and passed through a hand mill (coffee mill) with precautions against contamination or loss. The mill should be prepared for use by grinding a small portion of wheat, turning the mill until no more material is ejected, and rejecting this material. The ground material is placed in a 250-ml. conical flask; 200 ml. of 0·2 *N* hydrochloric acid are measured out, about half is added to the flask, which is then agitated, and the remaining acid is used to wash down the flask, which is allowed to stand overnight (see Note 2).

It is advisable to prepare extracts in duplicate.

In every case the extracts are well shaken the following morning, and allowed to settle, and the liquids poured into centrifuge tubes or test tubes. These are allowed to stand for 1 hour or more to settle, or centrifuged until fairly clear. It is best to pour off bran or wholemeal extracts through a pad of glass wool to remove floating particles, as these may give a high colour with alkali—and so affect the result.

PREPARATION OF STANDARDS—

0·100 g. of pure crystalline aneurine hydrochloride (see Note 3) is weighed in a watch glass, washed through a funnel into a 500-ml. measuring flask with 0·2 *N* hydrochloric acid

and made up to 500 ml. with similar acid. Of this solution 25 ml. are taken and made up to 500 ml., giving a solution containing 10 μg. per ml. Quantities of this solution ranging from 5 to 55 ml. in 5 ml. steps are taken and made up to 500 ml., giving a series of standards ranging from 0·1 up to 1·1 μg. per ml. in 0·1 μg. steps. For all the dilutions 0·2 *N* hydrochloric acid is used; the solutions keep well for months in well-stoppered bottles. It is essential to make up other solutions independently for use as checks on accuracy (see Note 4).

Mixing is a serious problem with such dilute solutions; if they are made up in measuring flasks they should be well shaken, then emptied by inverting the flask into a dry beaker, the solution being returned to the flask and again emptied into the beaker, five times in all. Where determinations are a routine it is preferable to find the weight of the 500 ml. of acid, and to weigh all the successive volumes in stoppered tared flasks of about 700 ml. capacity in which the solutions can be shaken thoroughly. Flasks with standard ground necks, tared to the same weight, when unstoppered, by means of a lead strip soldered round the neck, are of great assistance.

PREPARATION OF THE QUININE SOLUTION—

0·08 g. of quinine bisulphate is weighed in a watch glass, washed into a flask and made up to 500 ml. with 0·1 *N* sulphuric acid. Of this solution 5 ml. are diluted to 1000 ml. with the same acid, giving a solution containing 0·8 μg. per ml. If batches of this solution are made up at later dates, they may be adjusted to give the same curve by appropriate addition of 0·1 *N* sulphuric acid or more concentrated quinine solution as necessary, thus compensating for moisture or other variations in the solid quinine bisulphate.

METHOD OF OXIDATION OF EXTRACTS AND STANDARDS—

Test-tubes, 150 × 25 mm., in sufficient number to accommodate all the extracts under examination, together with at least four standards, are placed in a rack, which is conveniently circular, holding about twenty tubes and with accommodation for each tube of extract behind its appropriate test-tube. Two standards, 0·8 μg. per ml., may begin the set, which may be terminated by standards of 0·6 and 0·4 μg. per ml. Two ml. of standard solution or extract are placed in each tube and, when all have been measured, 2 ml. of pure methyl alcohol added to each. While a current of clean air to cause violent agitation is passed into the first tube through a 5-mm. glass tube drawn to a jet, 1 ml. of 30 per cent. caustic soda solution is added, followed about 6 seconds later by 0·25 ml. of 5 per cent. potassium ferricyanide solution. This effects the oxidation to fluorescent thiochrome. About 30 seconds later, 25 ml. of *iso*butyl alcohol (Note 5) are added to extract the thiochrome, and agitation is continued for 60 seconds longer. It is convenient to agitate four tubes at once and oxidise and extract the tubes in pairs, timing being thus simplified. When all the tubes are oxidised, the set is allowed to stand for 10 minutes or longer. During all this work the tubes must be shielded from strong daylight, as thiochrome is photosensitive. (Notes 6 and 7.)

After settling, 1 ml. of ethyl alcohol is added to each tube, and (after all the additions have been made) mixed with the *iso*butyl alcohol layer by bubbling for a second, care being taken that the bubbles do not disturb the surface of the aqueous layer (see Note 8). Each *iso*butyl extract in turn is then used to fill one cell of the Spekker fluorimeter, the other cell being filled with the quinine sulphate solution containing 0·8 μg. per ml. The cells are filled to within about 1/16 inch of the top, the glass lid is placed in position, and all faces are thoroughly wiped. No trace of the bottom aqueous layer in the tubes may be allowed to enter the cell, since it strongly absorbs the active light. It is easier to avoid this if, after the cell has been nearly filled, the tube is restored to the vertical position and rotated on its axis through 90° and the filling of the cell than completed. A background of grey or pale blue blotting paper is a help in the rather dim light that must be maintained.

USE OF SPEKKER FLUORIMETER (see Note 9)—

The quinine cell is placed on the left-hand side of the sliding carrier of the instrument (A, Fig. 1) and need be changed only every second day. The following filters should be placed in the fluorimeter. Before the right-hand photo-cell (B) – Wratten 47 (blue) filter (C) (Note 10). On the right-hand side of the lamp, close to drum support (D), Wood's glass (ultra-violet transmitting) filter (E); on the left, Wood's glass (F) nearest the lamp, then a small circular cell (G) containing the quinine sulphate solution 0·08 g. per 500 ml., followed by neutral glass and coloured or plain glass filters (H, K). The nature and number of these is

adjusted in the following manner. With the cell containing the standard quinine solution (0·8 μg. per ml.) in position in front of the right-hand photo-cell (B), the rotating drum (L) of the fluorimeter is adjusted until the fixed arrow points to 0, the light shutter (M) is closed and the zero mark adjusted or marked according to the type of galvanometer and scale, the shutter opened and the iris diaphragm (N) moved to the fully "open" position. The filters are then adjusted until a small movement of the iris diaphragm brings the galvanometer spot back to zero. This will give the maximum sensitivity over the range of normal flour extracts.

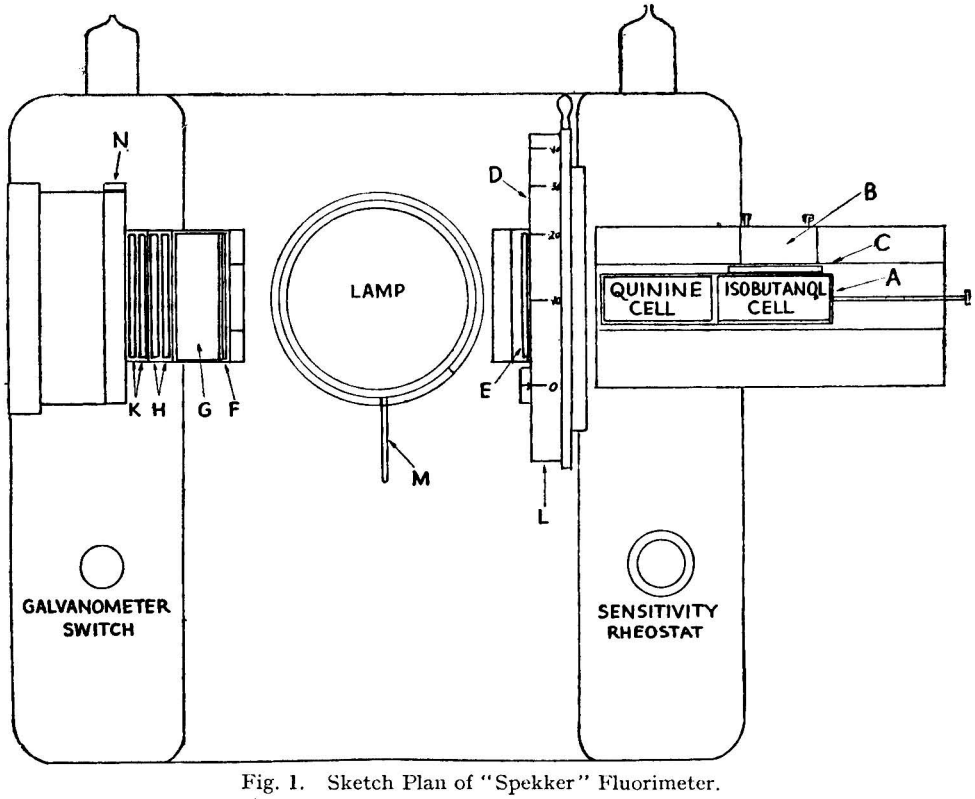

Fig. 1. Sketch Plan of "Spekker" Fluorimeter.

The cell containing the first *iso*butyl extract is gripped by two diagonally opposite vertical edges, using finger and thumb, and placed on the right-hand side of the carrier, which is then moved so that this cell is in the optical beam; spring-loaded stops indicate the correct position (Note 11). The rotating drum of the instrument is set so that the fixed mark is at 0. The light shutter is closed, the galvanometer zero adjusted, the shutter opened and closed twice, with adjustment of the zero if necessary, and the shutter then left open continuously during the examination of the whole run of tubes. The iris diaphragm is now closed until the galvanometer spot is again at zero, and the fluorimeter carrier moved until the quinine cell is in the optical beam. The rotating drum is then moved until once more the galvanometer spot is at zero, whereupon the drum reading is noted and recorded against the standard or extract concerned. Great care must be exercised that no parts are moved at any time during a reading except as mentioned. The remaining tubes are examined in the same manner.

STANDARD CURVES—

A standard curve is prepared by using the whole range of standards from 0·1 to 1·1 μg. per ml., preferably each in duplicate. It will be found that this curve moves slightly from day to day, or even from run to run, so that after a number of standardisations a family of curves (Fig. 2) will be obtained. After this has been done, four standards in each run will suffice to select the appropriate curve for that run. The first, or sometimes the second, tube in each run may be uncertain, owing to the photo-cell taking a little time to reach its correct response

under the conditions of working, but this is minimised by the method of zero adjustment described above. It is for this reason that two standards are placed at the end of the set of tubes.

If only one or two samples are to be examined, their extracts may well be included in the run of standards. But at least two runs done at different times will be necessary in this event to justify any confidence in the results.

The curve having been selected in this way, the drum reading of each extract is used to determine the aneurine content in μg. per ml. The curve is conveniently drawn on linear-log paper, with μg. of aneurine on the linear scale, and drum readings on the log scale. Each curve thus obtained is slightly sigmoid, but nearly straight in the middle portion, and the extreme curves of the family are separated by about 12 per cent. of the microgram readings. One or other of these extremes may not be reached for months at a time, but wider departures than mentioned above are usually a sign of dirty electrical contacts or failing photo-cells.

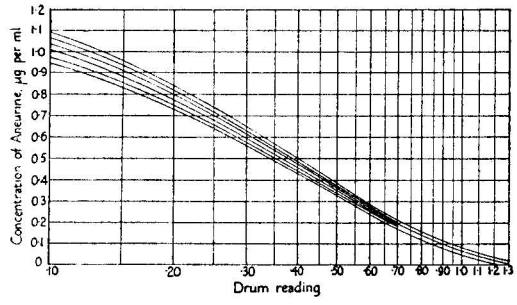

Fig. 2. Standard Curves.

A reproducibility to within $\pm$ 0·02 μg. per ml. can be regarded as usual in good work (Note 12).

A blank determination, on 2 ml. of extract, following the same procedure as above, but omitting the potassium ferricyanide, is carried out on all samples until the usual level for the material concerned has been established by many estimations; then it may be found more accurate as well as speedier to take a mean value (*e.g.*, 0·05 μg. per ml. for white flour, 0·02 for wheats). This blank is deducted from the value of the oxidised sample as representing the fluorescence of matter other than thiochrome (see Note 13). It is convenient to determine blanks next to the oxidised sample; they must in any case be determined in the same run. A careful watch on the reagents should be kept by means of blank tubes in which 2 ml. of 0·2 *N* hydrochloric acid are oxidised and extracted in the usual way. Such blanks should be very low (below 1·2 on the Spekker drum).

CALCULATION OF RESULTS—

Results are still officially reported in international units (i.u.) per g., 1 i.u. being equal to 3·125 μg. of crystalline aneurine. Thus:

$$\frac{(\mu\text{g. aneurine/ml.} - \text{blank } \mu\text{g./ml.}) \times \text{total ml. of extract}}{\text{wt. of material taken} \times 3{\cdot}13} = \text{i.u./g.}$$

With the more coloured extracts derived from brans, etc., there is some interference due to absorption of active light in the cells and other causes. This is shown by the fact that somewhat different results are obtained by using extracts of different concentrations, *e.g.*, 2 and 3 g. per 50 ml. The weaker concentration usually gives the higher result. Adsorption methods are available for overcoming this,[6] but a commonly recommended procedure, which is also a valuable check on the accuracy of the whole method, is the addition of pure aneurine to the extracts. This is worse than useless unless carried out with the greatest precision, and the following scheme gives excellent results with homogeneous material. Five portions of material (5 g. of flour or wheat, the latter preferably ground after weighing; 2 g. of bran) are weighed into 100-ml. conical flasks. To each of the first two (labelled "+ 0") are added 50 ml. of 0·2 *N* HCl, to the third (labelled "+ 0·4") 50 ml. of 0·4 μg. per ml. standard aneurine solution, and to the last two (labelled "+ 0·6") 50 ml. of 0·6 μg. per ml. standard, all preferably employing the same 0·2 *N* HCl. Shaking, washing down and standing overnight

are carried out as described earlier for the particular material, as is also the subsequent treatment of the extracts. These results give five aneurine concentrations, which when plotted as a graph against aneurine additions (Fig. 3) should give a straight line with divergencies of the order of the experimental error ± 0·02 μg. per ml. in practised work.

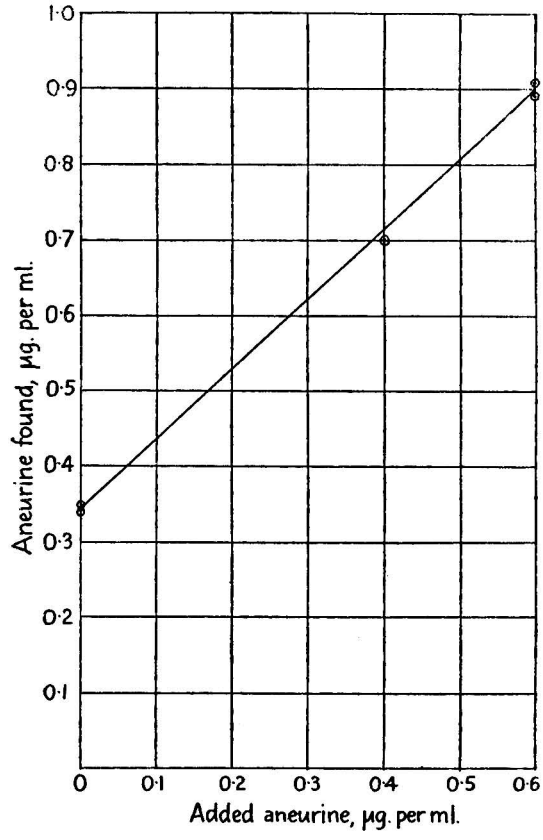

Fig. 3. Recovery of aneurine added to extracts of an 85% extraction flour.

The slope of the line will be nearly 45° with flour—*i.e.*, the "recovery factor":

$$\frac{\text{Aneurine found}}{\text{Aneurine added}} = 98 \text{ to } 102 \text{ per cent.}$$

The recovery is lower with wheats and still lower with brans—possibly as low as 70 per cent. Good recovery is to be regarded as a criterion of reliable results and "corrections" for low recovery must be regarded with suspicion. The causes of low recovery may be complex and this subject will be treated in greater detail in a later publication (Note 14).

Notes

Note 1—Distilled water is invariably used for washing and steam distillation, as serious trouble was experienced on one occasion from the accumulation of volatile material in the *iso*butyl alcohol, which rapidly reduced ferricyanide and gave erratic results. This appeared to be derived from chlorine in town water and was possibly chloramine. Other volatile fluorescent substances have been found to accumulate in operating on scutellum-rich materials if the first fraction to distil is not returned to residues.

Note 2—This procedure was adopted as giving highly reproducible results. Grinding before weighing, particularly grinding in a disintegrator, was found to result in segregation, which subsequent mixing could not overcome. Weights less than 20 g. may be used, with resulting decrease in accuracy owing to losses in the mill, etc. With cereal products segregation is a very serious problem; errors as great as 20 per cent. can easily arise with coarse

wholemeals and similar mixtures. Variation in moisture content within the sample has also been a cause of erratic results.

Note 3—Crystalline aneurine hydrochloride contains approximately one molecule of water of crystallisation. Samples of the National Standard with memorandum may be obtained from the Medical Research Council, Department of Biological Standards, National Institute for Medical Research, Hampstead, London, N.W.3. Larger supplies should be obtained from other sources for use as laboratory standards. 3·125 μg. of the National Standard is stated to be equivalent to 1 international unit.

Note 4—Standards and extracts made up with 0·1 *N* hydrochloric acid have been found to deteriorate with keeping, owing to the growth of organisms. This has not occurred with acid more concentrated than 0·2 *N*. It has frequently been stated that strong solutions of various vitamins keep better than those that are very dilute. This would be contrary to mass action considerations in most cases, and in fact the writer has found with riboflavine as well as aneurine that very dilute solutions keep better than strong if protected from the growth of organisms and, in the case of riboflavine, from light (*cf.* Memorandum on National Standard, see Note 3).

Note 5—Any burette tap or other apparatus used in the estimation must be perfectly free from grease or any material, such as cork or rubber, that can yield fluorescent substances to extracts at any stage.

Note 6—The effect of the variation of every detail has been studied independently of other workers' findings. The accumulated data may be published separately.

Note 7—Special rapid pipettes greatly increase speed, accuracy and safety with this method when many determinations have to be made. An account of these is being published (see this vol, p. 24).

Note 8—The *iso*butyl alcohol extracts of thiochrome may stand for an hour or more without noticeable change if covered by a dark cloth to exclude light. After the addition of the ethyl alcohol they should be examined on the fluorimeter at once, as they become cloudy again on standing. A slight turbidity in the *iso*butanol extract does not influence the result. Hence the liquids in the tubes, after settling at the end of the run, may be poured back into the fluorimeter cell for a check on any particular reading, and this will be found to have changed only slightly to give a higher drum reading.

Note 9—(The adjustments described below should only be undertaken by highly skilled workers.)

Various fittings of the Spekker fluorimeter as delivered were found to cause slight variations in results in such highly sensitive work as aneurine estimation. For this reason the whole of the internal wiring, plug connections, sensitivity resistance and galvanometer switch were removed and replaced by metal-sheathed insulated conductors throughout, joined by the minimum number of carefully soldered and insulated joints, and the outer sheaths bonded. The galvanometer switch, etc. were omitted. The instrument is kept in a warm room, as it was found that some troubles disappeared under these conditions; apparently they were due to traces of condensation in the photo-cell circuit when a humid period followed a cold one. The photo-cell contacts may need careful cleaning when first used, or after standing for some time without use. The mercury lamp is run all day when determinations are being made, the galvanometer lamp being switched on only as required. A constant voltage transformer in the lamp circuit improved steadiness and reproducibility. Needless to say, if an instrument is used in which these alterations have not been made, the sensitivity has to be at a maximum for this work and the galvanometer switch pressed for each galvanometer reading.

Note 10—The Wratten No. 47 filter has been found to be the best commercial filter so far tried for thiochrome. No. 39 was used for a considerable time but in one instance was found to absorb less well a small fluorescence not due to thiochrome.

Note 11—The optical beam should be exactly central in the cell and not touching the glass at any point. If it is not the makers should be consulted.

Note 12—Failure to obtain reproducible results on the *same extract* indicates failure in apparatus or procedure, the most usual being the failure to agitate briskly when adding alkali, or delay after this in adding ferricyanide, which gives low and irregular results. Failure to obtain reproducibility between different extracts may be due to the above causes or to sampling errors, the estimation of aneurine being a severe test of mixing. It was found impossible to mix ground (particularly disintegrator-ground) wheat with sufficient accuracy for

the most refined comparison, hence the procedure recommended above. A proportion of commercial flours, particular fortified flours, cannot be mixed to give uniformity. An examination of routine results on National Wheatmeals at one time showed the following divergencies between duplicates (mostly done by different workers in different runs).

Divergency	Proportion of samples per cent.
Less than ± 0·02 i.u. per g.	69
" " 0·03 "	85
" " 0·04 "	93

Most of those showing greater divergency than this failed to give better results on repeated examination.

Note 13—It can be shown that with wheat products the greater part of this blank disappears on oxidation.[6]

Note 14—It should be noted, that for bread, prepared foods and some cereals, digestion[2,3,7] is essential; the oxidation procedure described above can then serve only for examination of extracts prepared less simply than has been described, the accuracy of the whole estimation being lower. Methods recently developed appear likely to give accurate results with these materials.

SUMMARY

The estimation of aneurine (vitamin B_1) in wheat products by the thiochrome method has been used extensively with a steadily increasing refinement and speed. The fullest details of present procedure are described in the attempt to give a greater certainty and ease of approach to those less fully occupied in this field of work.

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January, 1948

High Accuracy and High Speed Pipettes and Associated Apparatus

BY H. N. RIDYARD

A PIPETTE which was designed originally for the accurate measurement of such solutions as bromine in benzene, by combining the principles of the Jakowkin pipette and the pyknometer,[1] was modified shortly afterwards to give still higher accuracy with solutions containing a very volatile constituent that is to be kept constant in concentration (chlorine water originally). More recently the principles employed have been applied to the design of high speed pipettes with an accuracy suitable for many measurements, especially when frequently repeated. Safety with corrosive reagents is another feature of these instruments.

TYPE II, HIGH-ACCURACY PIPETTE FOR VOLATILE SUBSTANCES

The original form of this pipette (Type I), described in 1928,[1] has been modified by dividing the "waste" bulb into two parts by a septum A, having a hole, B, in the upper part. A small bulb, D, above the setting capillary, E, facilitates the setting of the liquid volume before running out the pipette. After the pipette has been filled (conveniently, as originally recommended, by suction at a piece of cycle valve rubber tubing), it is brought into a horizontal position to allow the surplus liquid to fall into the second half of the "waste" bulb, into which is sealed a small air jet, C. After setting the level of the liquid to any point in the narrowest part of the capillary E (a definite mark is unnecessary) by means of a filter

paper applied to the tip of the pipette, the liquid can be run out, the incoming air bubbling through the waste liquid at C and so becoming pre-saturated with vapour and in equilibrium with the pipette contents. This device enables extremely volatile substances to be measured.

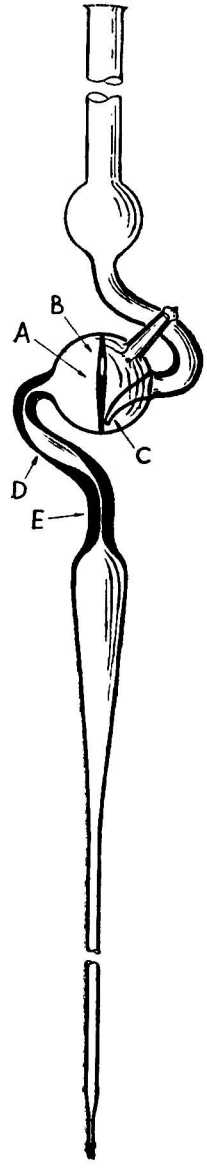

Fig. 1. High accuracy pipette, Type II.

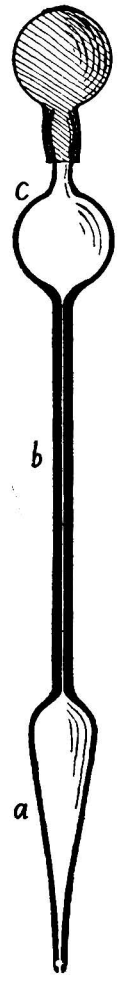

Fig. 2. High speed pipette, Type III.

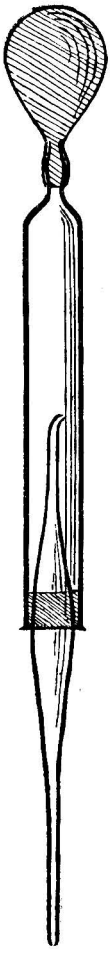

Fig. 3. High speed pipette, Type IV.

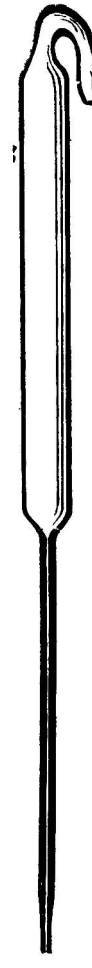

Fig. 5. Inoculation pipette.

The weight of liquid delivered, as stated in the description of the first type, is constant to 1 part in 5000 for water and 1 part in 3000 for benzene for a 2 ml. pipette.

This pipette may have the jet ground to fit a small socket connected to a reservoir so that filling exposes to the atmosphere only that portion of liquid which is taken into the waste bulb.

High speed pipettes

A demand for great numbers of determinations of aneurine (vitamin B_1) in wheat products, each of which requires six separate volume measurements, led to the development of the rapid pipettes (types III, IV and V) described below. These were so successful that they

reduced the time of oxidation of a run of 20 tubes from about 35 to 15 mins., and enabled two workers to oxidise the contents of 10,000 tubes per annum.

TYPE III, HIGH-SPEED PIPETTE—

The pipette (Fig. 2) consists of a bulb (*a*) drawn in a uniform taper to a jet at one end, and narrowing sharply from the widest point to a fine capillary (*b*), 3 to 5 inches long, with a bulb (*c*) above, ending in a tubulure to fit a rubber teat or small bulb. As with the earlier pipettes it is most important in the smaller sizes that the capillary and bulb should be drawn from one wide tube, for even the most perfect joint leaves undulations that impede drainage. Pipettes of 0·5-ml. capacity or less may be blown from capillary tube and drawn to shape; in those of 5 ml. or over the error caused by a good glass joint is relatively insignificant. The size of the capillary is of great importance in relation to the liquid for which it is designed, being greater for liquids of higher viscosity. Final adjustment of capacity can if necessary be made by shortening the jet. The pipette is filled by pressing to the required degree on the bulb, allowing to fill until the liquid reaches the fine capillary, whereupon the speed of filling is greatly reduced, giving time for the pipette to be removed from the liquid and its contents ejected into another vessel. It is immaterial to what particular point in the capillary the liquid rises. A practised worker can make 15 measurements per minute. The accuracy of this is shown by the following measurements, which were made by pipetting successive portions of water, etc. into a series of weighing bottles. (All figures are in grams, uncorrected for temperature.)

PIPETTE 1

DESIGNED FOR USE WITH METHYL ALCOHOL (FINE CAPILLARY). NOMINAL CONTENT 2 ML.

	Weights delivered g.		Deviation from mean g.
	2·0293		+ 0·0006
	2·0302		+ 0·0015
	2·0306		+ 0·0019
	2·0214		− 0·0073
	2·0325		+ 0·0038
	2·0280		− 0·0007
	2·0360		+ 0·0073
	2·0260		− 0·0027
	2·0314		+ 0·0027
	2·0212		− 0·0075
	2·0300		+ 0·0013
	2·0278		− 0·0009
Mean	2·0287	Mean	0·0031
		Max.	0·0075

PIPETTE 2

DESIGNED FOR USE WITH CHLOROFORM

Weight delivered Water	Deviation	Weight delivered Chloroform	Deviation
0·495	− 0·019	0·729	− 0·026
0·510	− 0·004	0·746	− 0·009
0·512	− 0·002	0·753	− 0·002
0·520	+ 0·006	0·748	− 0·007
0·519	+ 0·005	0·749	− 0·006
0·513	− 0·001	0·753	− 0·002
0·515	+ 0·001	0·756	+ 0·001
Mean, excluding 1st result:		0·762	+ 0·007
0·514	0·003	0·761	+ 0·006
Max. excluding 1st result:			
0·520	0·006		
		1 hr. later	
		0·754	− 0·001
		0·759	+ 0·004
		0·756	+ 0·001
		0·759	+ 0·004
		0·752	− 0·003
		0·758	+ 0·003
		Mean, excluding 1st and 2nd results:	
		0·755	0·004
		Max., excluding 1st and 2nd results:	
		0·762	0·007

A third pipette designed to deliver 5 ml. of a bacteriological medium gave with nine successive deliveries the following deviations:

	Delivery g.	Mean deviation	Max. deviation
Water: 1st day ..	4·908	± 0·005	− 0·015
2nd day ..	4·921	± 0·011	+ 0·039
Water + glycerol ..	5·364	± 0·028	± 0·087

With this pipette 36 tubes were charged in 3½ minutes.

TYPE IV, HIGH-SPEED PIPETTE—

The above type of pipette proved unsuitable for flour extracts owing to the froth formed when suction is applied, and another type was developed (Fig. 3) in which the first portion entering the pipette is rejected, this serving to remove froth and to wash out the pipette with changing material. This is capable of considerable accuracy with careful use, with a mean

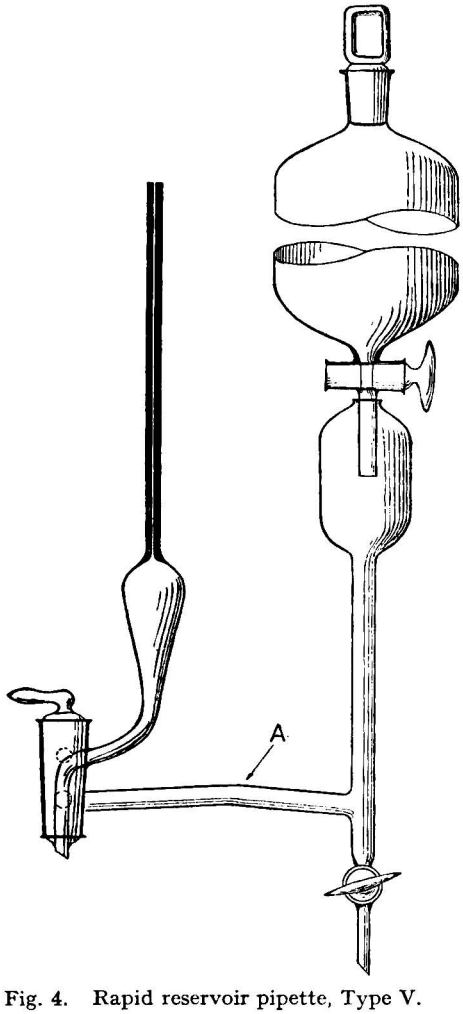

Fig. 4. Rapid reservoir pipette, Type V.

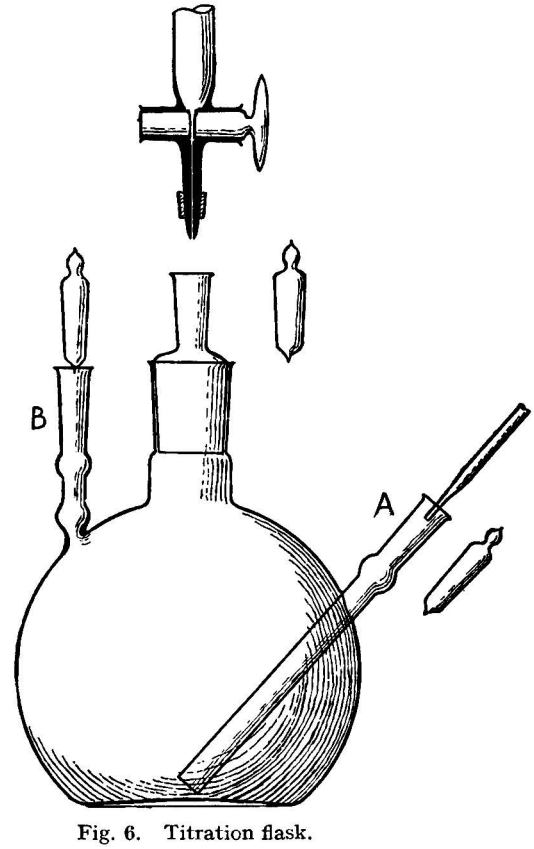

Fig. 6. Titration flask.

deviation of 0·002 on a 2·0 ml. pipette, and a maximum of 0·004, but has not been completely reliable for rapid working. It is hoped to overcome this with modifications which may also improve both speed and accuracy in other types.

(In view of the development of these pipettes originally partly from the pyknometer, it is interesting to notice the considerable degree of similarity of this form with the pyknometer of Cripps,[2] which only recently came to the notice of the present writer.)

TYPE V, RAPID RESERVOIR PIPETTE—

The same form of bulb was used in a reservoir pipette for delivering larger volumes, and this has been made in two slightly different forms. The pipette is shown in Fig. 4. The hollow bore stopcock is so designed that when turned to the "off" position the bulb is filled from the reservoir, *via* two holes drilled in the outer shell of the key; when the stopcock is turned to the "on" position the reservoir is cut off and the contents of the pipette drain out through the inner tube of the key. In the most accurate form the tap is connected to a constant level device as illustrated, so that the liquid rises to the same point in the capillary each time. If the pipette bulb is large, or a lower accuracy sufficient, the pipette may be joined directly to a reservoir at the point A, and then the level of liquid in the capillary will vary with that in the reservoir. Delivery time with this pipette is 20 secs. if it is allowed to drain completely, or 15 if the tap is closed after the continuous stream has ceased and four drops have fallen. With a certain pipette, by the first method, the delivery of water-saturated *iso*butanol (mean of six measurements) was 20·53 g. with a maximum deviation of 0·07 and a mean deviation of 0·036; by the second method 20·54 g. with a maximum deviation of 0·11 and a mean deviation of 0·04. The determinations were made on different days and with different alcohol.

INOCULATION PIPETTE—

Another pipette (Fig. 5) was designed for, and proved of great value in, the inoculation of tubes for microbiological estimations. This consisted of a bulb about 12 cm. long and of 1-cm. bore, drawn out at the top and bent downwards. To the lower end is sealed a length of 0·5 to 1 mm. capillary drawn to a fine jet at the lower end and about 12 to 14 cm. in over-all length. The tubulure at the upper end is packed with a small plug of cotton wool. The whole may conveniently be placed in a glass tube and this plugged with cotton wool and sterilised and left until required. When needed for use the upper end is withdrawn from the sterilising tube and a short length of rubber tube fitted with a screw clip pressed over. The pipette is now completely withdrawn and filled with the saline suspension of the bacteria. It is mounted in a burette stand, and the screw clip adjusted so that drops fall from the lower end of the pipette at such a rate that an operator can take a tube of medium from a rack on his left hand, remove the plug, catch a drop of inoculum in the tube, heat the mouth of the tube, replace the plug, fire and place the tube in a second cage in the correct order. A gross of tubes could be inoculated in about 30 minutes.

TITRATION FLASKS

In order fully to utilise for the titration of volatile materials the high accuracy of the original pipette (Type I), or the first one mentioned in this paper (Type II), a special titration flask was employed.

This had the form shown in Fig. 6. The flask had a neck about 25 mm. in diameter, fitted with a carefully ground stopper, itself carrying a ground neck, of about 7-mm. internal diameter at the top, fitted with a stopper. The flask itself had a stoppered 7-mm. bore tube A sealed through one side and reaching to the bottom, and also carried a small stoppered absorption tube, B. Its use for the estimation of bromine dissolved in volatile solvents will serve as a sufficiently typical example. A few ml. of strong potassium iodide solution are placed in the flask, and a few crystals of solid potassium iodide in the absorption tube, moistened with water. The flask is stoppered, and the small socket of the stopper pressed over a small piece of rubber tube on the jet of the burette to be used. The leg of the pipette containing the liquid to be examined is slipped into the side tube of the flask and the liquid run out beneath the potassium iodide solution, no vapour being lost. The pipette is withdrawn, the side tube stoppered, and the flask gently agitated to aid partition of the bromine. The thiosulphate may now be run in gradually from the burette with agitation, until nearly all the free iodine is removed. The flask is removed from the burette, the contents of the absorption tube are washed into the flask, a few drops of starch solution added, all stoppers are replaced and the flask is vigorously shaken. The last few drops of thiosulphate are added, with vigorous shaking, in this way. Highly reproducible results are obtained.

The high-accuracy pipette type II and the titration flask were designed and employed in the Chemical Department, University of London, King's College, and the author is grateful to Professor A. J. Allmand, D.Sc., F.R.S., for agreeing to their inclusion in this publication.

SUMMARY

Pipettes are described which were designed for high speeds or for measuring volatile materials with high accuracy. A titration flask for use with the latter, and an inoculation pipette are also described.

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ST. ALBANS, HERTS

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The Assay of Folic Acid with *Streptococcus faecalis* NCTC 6459

BY A. JONES AND S. MORRIS

VARIOUS names have been applied to substances having folic acid potency, such as factor SLR, *L. casei* factor and vitamin B_c. These factors have at various times been identified with pteroylglutamic acid, although Hall[1] has recently queried this assumption. They all appear to be available to micro-organisms without preliminary treatment.

The vitamin B_c conjugate, or according to Pfiffner,[2] pteroyl heptaglutamic acid, appears to be split into free, or available, folic acid and an inactive fraction, by the action of hog kidney enzyme. Results have been obtained by Other workers who have used this enzymic treatment for microbiological assay, and have obtained results that compare favourably with bio-assay methods (Bird, Bressler, Brown, Campbell and Emmett).[3]

The folic acid available to the test organism (*S. faecalis*) without preliminary digestion with the conjugase (to liberate folic acid from the conjugated material) has been called "available folic acid" in these experiments.

The present paper deals with a study of the assay of folic acid, available and in the conjugate form. The folic acid used as standard throughout the experiments was supplied by Ashe Laboratories in ampoules containing 2 μg. per ampoule.

The preliminary stages of this work, including all the experiments on the media for growth and subculturing of *S. faecalis*, were carried out in conjunction with Miss M. Meiklejohn, of the Ashe Laboratories.

EXPERIMENTAL

1. MEDIUM USED FOR ASSAY OF FOLIC ACID—

Streptococcus faecalis (*S. lactis R*) was used in the assay of folic acid in preference to *L. casei* for several reasons—

(1) The production of acid by *S. faecalis* is sufficiently rapid to allow of the titration after 16 to 18 hours' incubation, although Tepley and Elvehjem[4] used a 30- to 72-hour incubation period. With an 18-hour incubation period, titration values of 8 ml. of 0·1 *N* sodium hydroxide were found at a 10-mμg. folic acid level, whereas at 72 hours a value of 12 ml. of 0·1 *N* sodium hydroxide was recorded. The assay values obtained with both incubation periods, however, using the same samples, were in all cases in complete agreement, within the limits of the experiment.

(2) The growth of *S. faecalis* may be determined either by titration or by turbidimetric measurements.

(3) *L. casei* is known to be a rather unsatisfactory organism with which to work.

The assay method used was essentially that of Tepley and Elvehjem[4] with very slight modifications. The composition of the basal medium is shown in Table I.

Preparation of the peptone—50 g. of peptone (Difco Bactopeptone) are dissolved in 200 ml. of water, the *p*H is adjusted to 3·0 with concentrated hydrochloric acid and the liquid stirred with 5·0 g. of activated charcoal for 1 hour. After filtration, the volume is adjusted to 500 ml. This solution is stored in the refrigerator under toluene and *renewed at fortnightly intervals*.

Salts B are as for liver tryptone agar.

2. MEDIA USED FOR GROWTH AND SUB-CULTURING OF *S. faecalis*—

Using a 16- to 18-hours incubation period for the assay, the method of maintenance of the stock culture of *S. faecalis* was found to be of critical importance. When the stock culture was maintained in stab culture on Difco yeast extract (or bakers' yeast extract) – sodium acetate – glucose agar and sub-cultured weekly, the response was good for the first four to six weeks. With further sub-culturing, the organism showed little or no response to folic acid.

Although these later sub-cultures of the organism failed to respond to the standard folic acid solution, good growth could always be obtained in an assay containing treated yeast samples. It would appear that the treated yeast samples contained a stimulatory substance or substances required during the first 16- to 18-hours growth, as it was always possible to obtain a good response to the standard folic acid solution when a longer incubation period, 72 hours, was used for the assay.

TABLE I

COMPOSITION OF THE BASAL MEDIUM FOR *S. faecalis*

Casein hydrolysate	5·0 g.
Glucose	20·0 »
Sodium citrate (dihydrate)	25·0 »
Dipotassium hydrogen phosphate	2·5 »
L-cystine	0·2 »
DL-tryptophan	0·4 »
Asparagine	0·1 »
DL-alanine	0·2 »
Adenine	0·01 »
Guanine	0·01 »
Uracil	0·01 »
Xanthine	0·01 »
Biotin	0·4 µg.
Aneurine hydrochloride	200 »
Riboflavine	200 »
Nicotinic acid	600 »
Pyridoxine	1200 »
Calcium pantothenate	400 »
p-Aminobenzoic acid	10 »
Peptone treated with charcoal	2·0 ml.
Inorganic salts B	5·0 »
Glass-distilled water to	500 »

The organism was sub-cultured weekly on the bakers' yeast medium during a period of six months, and tested at intervals. Although the majority of the sub-cultures showed no response to folic acid with the 16 hours incubation period, a good response was occasionally obtained similar to that found with the earliest sub-cultures. No satisfactory explanation has been found for this inconsistent behaviour.

In view of the results obtained by Nymon and Gortner[5] with stock cultures of *L. casei* and *L. arabinosus*, it was decided to alter the medium used for making the sub-culture for *S. faecalis*. The culture was transferred from the bakers' yeast extract agar to liver tryptone broth and from this broth to liver tryptone agar slopes. After the first sub-culture through these two media, the response of the organism to folic acid was regained.

A culture was also transferred from the bakers' yeast extract agar to a takadiastase-treated brewers' yeast agar. In this case, also, the response of the organism was regained in a single sub-culture. Further sub-culturing through liver tryptone broth and agar, or through takadiastase-treated brewers' yeast agar over a period of nine months continued to give satisfactory results.

The results obtained with these sub-culturing media are shown in Fig. 1.

The composition of the media are—

(1) *Liver tryptone broth*—Tryptone (Difco) 1·0 g., K_2HPO_4 0·5 g., glucose 0·2 g., yeast extract (Difco) 0·2 g., liver extract 10 ml. and glass-distilled water to 100 ml.

(2) *Liver tryptone agar*—Tryptone (Difco) 1·0 g., glucose 1·0, K_2HPO_4 0·2 g., $CaCO_3$ 0·3 g., agar 2·0 g., liver extract 10 ml., salts A 0·5 ml., salts B 0·5 ml. and glass-distilled water to 100 ml.

The liver extract is prepared by suspending one pound of ground fresh liver in 2 litres of water, heating for 60 minutes on a steam bath and then filtering through cheese cloth. The

filtrate is neutralised to *p*H 7·0, heated again for 15 minutes, filtered through coarse filter paper and stored under toluene in a dark bottle in the refrigerator.

(3) *Salts A*—K_2HPO_4 25 g., KH_2PO_4 25 g. and glass-distilled water to 250 ml.

(4) *Salts B*—$MgSO_4.7H_2O$ 10·0 g., $MnSO_4.4H_2O$ 0·5 g., $FeCl_3.6H_2O$ 0·03 g., concentrated HCl 5 drops and glass-distilled water to make 250 ml.

(5) *Takadiastase-treated brewers' yeast medium*—Pressed brewers' yeast (25 per cent. solids) 50 g., 1 per cent. sodium acetate buffer solution of *p*H 4·5 500 ml., takadiastase 1·0 g.

The mixture is incubated for 18 hours at 37° C., neutralised to *p*H 7·0 and finally autoclaved for 15 minutes at 15 lb. pressure. After filtration the volume of the solution is adjusted to 500 ml. and the following are added, glucose 2 g., salts B 5 ml. and agar 10 g. The

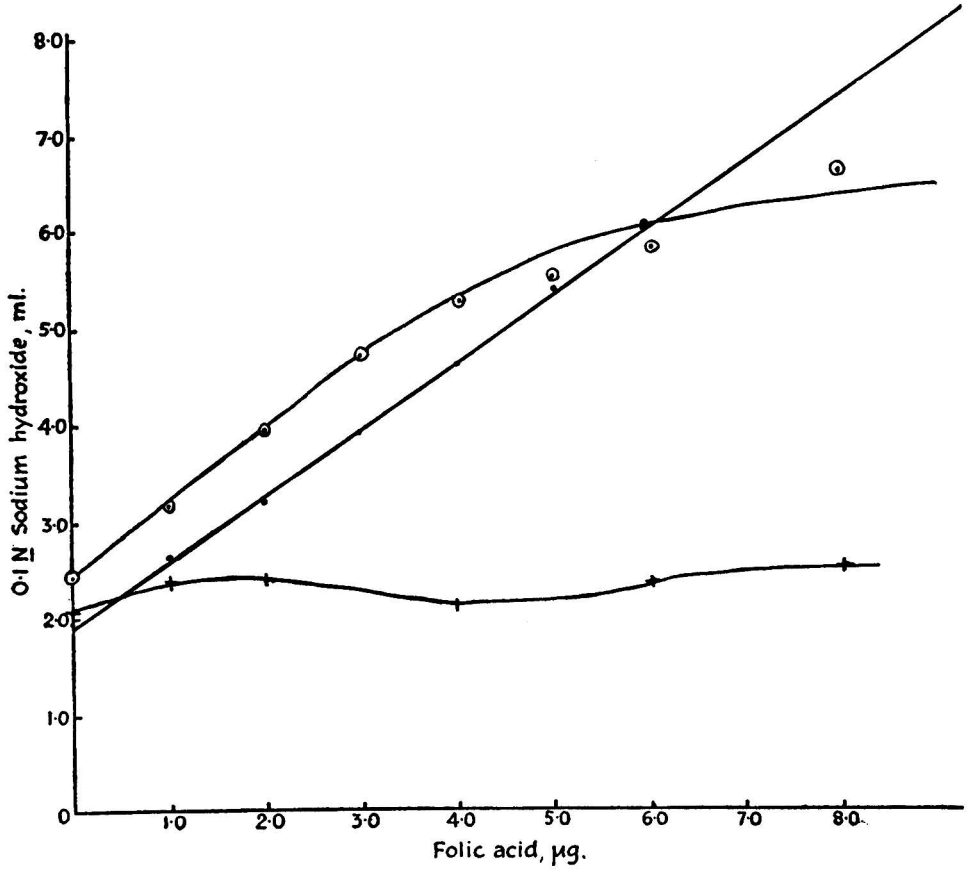

Fig. 1. The effect of the sub-culturing medium on the efficiency of *S. faecalis* for folic acid assay.

⊙ Bakers' yeast medium—1st sub-culturing.
\+ Bakers' yeast medium—6th sub-culturing.
● Liver tryptone broth and agar.

whole is steamed to dissolve the agar, and finally autoclaved 15 minutes at 15 lb. pressure after distribution in the tubes.

3. EFFECT OF TAKADIASTASE IN THE PREPARATION OF SAMPLES FOR ASSAY—

When assaying yeast samples for available folic acid content, using taka diastase according to the method of Cheldelin, Eppright, Snell and Guirard,[6] very irregular results were obtained. It was therefore decided to investigate the effect of takadiastase on the standard folic acid solution.

To 0·2 µg. of folic acid in 8·0 ml. of an acetate buffer solution, *p*H 4·5 to 4·6, were added 0·02 g. of takadiastase and a few drops of toluene. The solution was incubated at 37° C. for 16 to 18 hours. As a control, 0·2 µg. of folic acid in the same buffer solution was treated

in the same manner, but without the enzyme. After adjusting the *p*H to 7·0 the solutions were steamed for 15 minutes to inactivate the enzyme and finally cooled and diluted for assay.

The results are shown in Table II.

TABLE II

EFFECT OF TREATING STANDARD FOLIC ACID SOLUTION WITH TAKADIASTASE

	Folic acid added mμg.	Folic acid found mμg.	Found %
Control	2·0	1·8	90
	4·0	4·2	105
Takadiastase-treated standard solution.	2·0	0·5	25
	4·0	1·3	33
		3·0	75
	6·0	2·6	43
		4·2	70
	8·0	4·5	56
		5·6	70
	10·0	6·9	69

These results show that there may be a loss of 25 to 75 per cent. of folic acid on treatment with takadiatase, whilst the incubated control shows little or no loss.

In order to show whether this effect was due to the action of the inactivated enzyme on the folic acid during the incubation period of the test, 0·02 g. of takadiastase was incubated in the acetate buffer solution, neutralised, steamed and diluted to one quarter of the previous volume. Two ml. of this solution were added to a series of tubes containing folic acid, and these assayed for folic acid content in the usual manner. From Table III it is clear that the inactivated enzyme, when added at four times the normal strength to folic acid dilutions in the test itself, does not lead to any loss of folic acid activity.

TABLE III

EFFECT OF ADDING A CONSTANT AMOUNT OF INACTIVATED TAKADIASTASE TO FOLIC ACID IN THE TEST

Folic acid added, mμg.	0	1·0	2·0	3·0	4·0	5·0	6·0
Folic acid found, mμg.	0	1·1	2·0	3·1	4·2	5·3	6·0

A sample of dried yeast was then incubated in the acetate buffer with takadiastase, and also with addition of 0·2 μg. of folic acid. These samples were neutralised, steamed and diluted for assay. The results are shown in Table IV.

TABLE IV

EFFECT OF TREATING A YEAST SAMPLE WITH TAKADIASTASE

Dried yeast 1 : 1000	Test solution taken, ml.	Folic acid added, mμg.	Folic acid found, mμg.	Folic acid, μg. per g. of yeast	Maximum % found
Yeast alone	1·0	0·0	0·8	0·8	
	2·0	0·0	1·4	0·7	
	3·0	0·0	2·0	0·67	
	4·0	0·0	2·5	0·63	
Yeast + Folic acid.	0·5	1·0	0·9		66
	1·0	2·0	1·5		55
	2·0	4·0	3·5		65
	3·0	6·0	5·5		68

From all the results, both with the folic acid and with dried yeast samples, it is clear that the use of takadiastase is unsatisfactory in the preparation of samples for assay purposes. The noticeable "drift" in the results for yeast alone would indicate that the takadiastase is interfering with the assay.

4. EFFECT OF 1 PER CENT. SODIUM ACETATE SOLUTION AND OF 0·1 *N* HYDROCHLORIC ACID IN THE PREPARATION OF SAMPLES FOR ASSAY—

In view of the unsatisfactory results obtained with takadiastase to extract the available folic acid, yeast samples were prepared for assay by autoclaving at 15 lb. pressure from

15 to 60 minutes either in 1 per cent. sodium acetate solution, *p*H 4·0 to 4·2, or 0·1 *N* hydrochloric acid or by steaming for 15 minutes in 0·1 *N* hydrochloric acid. Values obtained after treatment of samples by these methods are shown in Table V.

TABLE V

VALUES OBTAINED FOR AVAILABLE FOLIC ACID CONTENT OF A DRIED YEAST SAMPLE TREATED WITH 1 PER CENT. SODIUM ACETATE SOLUTION AND WITH 0·1 *N* HCl

Treatment of yeast	Time of autoclaving, mins.	Folic acid, μg. per g.
Sodium acetate ..	15	1·18
	30	1·51
	45	1·81
	60	2·21
0·1 *N* HCl ..	15	2·0
	30	2·26
	45	2·25
	60	2·05
0·1 *N* HCl steamed ..	15	0·83

From these experiments, it can be seen that the treatment with 0·1 *N* hydrochloric acid gives higher values for available folic acid from the dried yeast. When folic acid itself was treated in the same manner, there was a considerable loss, a recovery of 35 to 45 per cent. being recorded.

TABLE VI

EFFECT OF PRIOR TREATMENT WITH TAKADIASTASE, 0·1 *N* HCl AND SODIUM ACETATE ON THE AVAILABLE FOLIC ACID CONTENT OF VARIOUS FOODSTUFFS

	Folic acid content in μg. per g. of sample treated with		
Food	Takadiastase	0·1 *N* HCl by autoclaving for 15 min. at 15 lb. pressure	Sodium acetate by autoclaving for 15 min. at 15 lb. pressure
Dried yeast	0·86	3·95	1·25
Dried yeast tablets	0·73	1·00	0·77
Sacc. cereviseae with bacteria (acetone dried sample).	2·00	2·80	2·60
Dried whole milk	nil	nil	nil

On the other hand, the yeast sample, when treated with acetate buffer, showed with increasing time of autoclaving an increase in the values for available folic acid, which may indicate a gradual release of the conjugated material to a form available to the test organism. Folic acid treated in a similar manner gave a recovery of 90 to 105 per cent.

The effect of the three different forms of treatment on various foodstuffs is shown in Table VI. From the results, it is obvious that there is no reliable method at present for the assay of available folic acid in foodstuffs such as yeast, in which the folic acid is, in part at least, held inside the cell wall. The agents that can be used to destroy the cell wall and liberate the folic acid appear to breakdown the conjugate. For this reason it has been decided to ignore the available folic acid and assay the total folic acid, available and conjugated. Justification for such a procedure lies in the fact that both forms appear to be equally available and equally utilised in the animal body.

5. EFFECT OF LIGHT ON FOLIC ACID—

The effect of artificial light and of ultra-violet light on folic acid was studied by exposing a solution of folic acid containing 0·2 μg. per ml. to the rays from either a 100-watt lamp for 4 hours, or to ultra-violet light (1849 to 4500 A.) for 2 hours. All solutions were exposed directly in layers 10 mm. in depth in crystallising dishes, at a distance of 30 cm. from the light source and, after exposure for the given time, diluted for assay.

The solutions exposed to the ultra-violet light showed a loss of folic acid of 33 per cent. after 30 minutes, 40 per cent. after 60 minutes, 55 per cent. after 90 minutes, and 72 per cent. after 120 minutes exposure. The solutions exposed to the rays of the 100-watt lamp showed no loss of potency after 4 hours exposure.

Thus ordinary electric light seems to have little or no effect on the folic acid solution containing 0·2 μg. per ml. after a fairly long exposure, but some loss will occur after a comparatively short exposure to ultra-violet light.

6. EXTRACTION OF THE FOLIC ACID FROM THE VITAMIN B_c CONJUGATE—

In order to liberate the folic acid from its conjugated form for assay with *S. faecalis*, the method of Bird *et al.*[3] with slight modifications, was used. Dried hog kidney was prepared by mincing one or more fresh kidneys, adding five volumes of acetone, filtering, washing with acetone and finally air-drying. The dried kidney was ground as finely as possible and stored in the refrigerator.

The weight of dried kidney required to liberate the maximum amount of folic acid from the conjugate in 18 hours at 40° C. was found by taking a constant amount (0·1 g.) of a dried yeast, adding various amounts (0·01 to 0·10 g.) of dried kidney and incubating in 8·0 ml. of 1 per cent. sodium acetate solution, *p*H 4·0 to 4·2, with addition of a few drops of toluene. After incubation, the samples were neutralised to *p*H 7·0, steamed for 15 minutes and, after cooling, diluted for assay. From the results recorded (Table VII) it is clear that 0·03 g. of dried kidney is sufficient to liberate the folic acid from its conjugated form in 18 hours from 0·1 g. of dried yeast. Similar results were obtained with other samples of dried kidney.

TABLE VII

AMOUNTS OF FOLIC ACID LIBERATED FROM A DRIED YEAST BY VARIOUS AMOUNTS OF DRIED KIDNEY IN 18 HOURS AT 40° C.

Dried kidney, g.	μg. Folic acid, per g. of dried yeast
0·01	31·6
0·02	38·5
0·04	35·5
0·06	34·1
0·08	38·4
0·10	39·5

The blank value for 0·03 g. of the dried kidney was comparatively low, and remained constant for the same batch during four months' storage.

The maximum amount of folic acid appeared to be liberated in 18 hours at 40° C. with 0·03 g. of kidney, as no increase in the folic acid content of dried yeast was found with longer incubation periods of 24, 48, or 72 hours. When folic acid alone was treated in the same manner with 0·03 g. of dried kidney for 18 hours at 40° C. a recovery of 95 to 105 per cent. was always obtained.

7. A COMPARISON OF VALUES OBTAINED BY THE USE OF *S. faecalis* AND *L. casei* AS TEST ORGANISM FOR THE ASSAY OF FOLIC ACID—

Although *S. faecalis* is frequently used as test organism for the assay of folic acid, statements have been made that *L. casei* is preferable, yielding more accurate results. *L. casei* is certainly much more sensitive to folic acid, more especially at the lower concentrations. It was felt, however, that this difference in sensitivity of *L. casei* would only become apparent and significant when dealing with trace quantities of folic acid in the test material. In view of the contradictory statements on this subject it was decided to compare the results obtained using the two organisms on the same series of test solutions.

A series of preliminary experiments with *L. casei* showed that it was impossible to use the same assay medium as was used with *S. faecalis*. The failure of this medium was due to the presence in the vitamin-free casein hydrolysate of some substance or substances which could replace folic acid for *L. casei*. That it was not folic acid was seen from the fact that it did not act as a growth factor for *S. faecalis*. In view of this, an amino acid basal medium was prepared, similar to that proposed by McMahan and Snell[7] for use with *L. casei* and *L. arabinosus* in the assay of valine and arginine. A strepogenin concentrate was prepared according to the method of Sprince and Woolley[8] and added to the medium. Choline chloride and inositol were found to be unnecessary and were omitted from the medium.

The organisms for the inoculum were maintained on liver tryptone agar, *L. casei* as a stab culture, *S. faecalis* on slopes. For the inoculum, *L. casei* was grown in 10 ml. of liver tryptone broth for 18 hours at 37° C., washed twice with 0·9 per cent. salt solution, suspended

in 20 ml. of salt solution and finally diluted by adding 2 ml. to 50 ml. of salt solution. One drop of this suspension was added to each assay tube.

Adequate washing of the organisms is essential, otherwise a response may possibly be obtained even in absence of any added folic acid. Further, the volume of the inoculum added to the basal medium is very important. When more than one drop of the final diluted suspension of organisms was added, maximum growth and acid production was frequently found when no folic acid was added. It is possible that the extreme sensitivity of *L. casei* for folic acid found by other workers may be due to inadequate washing of the organisms and inadequate care in the addition of the inoculum to the basal medium or to a lack of knowledge of the importance of these factors.

The results of a comparison of *S. faecalis* and *L. casei* for the assay of folic acid are given in Table VIII.

TABLE VIII

COMPARISON OF THE ACTIVITY OF *S. faecalis* AND *L. casei* FOR THE ASSAY OF FOLIC ACID

Material	Folic acid (μg. per g.) as assayed by *S. faecalis*	*L. casei*
Hog kidney	10·0	10·0
"	8·7	8·4
Dried milk	nil	nil
"	"	"

With the materials used it is clear that both organisms are effective for assay purposes. It is fully realised that there are certain other factors, the deficiency or presence of which may affect the growth of one or other of these organisms, but it would appear that, as regards the assay of folic acid *per se*, either organism may safely be used.

At this stage, a further precaution in the assay of folic acid with *S. faecalis* must be noted.

Test solutions assayed after storage for five days at 4° C. were not infrequently found to give higher values than when assayed immediately after preparation. All solutions for folic acid assay must be tested immediately on preparation and not stored, otherwise unreliable results may be obtained. Obviously some other growth factor capable of replacing folic acid is being formed slowly by the interaction or breakdown of the compounds originally present.

8. RECOMMENDED METHOD OF ASSAY OF FOLIC ACID WITH *S. faecalis*—

(*a*) *Treatment of the sample*—Weigh 0·1 g. of the sample containing 10 to 50 μg. of total folic acid per gram and 0·03 g. of desiccated hog kidney into a 50-ml. conical flask; add 8·0 ml. of acetate buffer (1 per cent. sodium acetate solution, *p*H 4·0 to 4·2) and a few drops of toluene and incubate at 40° C. for 18 hours. After neutralisation to *p*H 7·0 with sodium hydroxide solution, steam the suspension for 15 minutes, cool, dilute to 10 ml. and filter. A suitable dilution of the filtrate, estimated to contain approximately 2 mμg. of folic acid per ml. is taken for assay.

(*b*) *Method of assay*—Into a series of test-tubes measure 5·0 ml. of the basal medium, *p*H 6·8, given in Table I, followed by suitable aliquots of the test material, four levels in triplicate, in quantity such that the amount of folic acid per tube ranges from 1·0 to 8·0 mμg.

Set up in triplicate a series of tubes for the standard, containing 0, 1·0, 2·0, 4·0, 6·0, 8·0 and 10·0 mμg. of folic acid.

After making up the contents of all the tubes, both test and standard, to 10 ml. with water, autoclave at 10 lb. pressure for 10 minutes.

Inoculate each tube with one drop of a twice washed *S. faecalis* culture re-suspended in 20 ml. of 0·9 per cent. salt solution, and then incubate at 30° C. for 18 hours.

Titrate the acid produced with 0·1 *N* sodium hydroxide to *p*H 8·5, adding thymol blue to each tube as indicator.

9. ASSAY OF VARIOUS FOODSTUFFS—

The results of assays of different samples of brewers' yeast, milk and other foods are given in Table IX. It will be noted that the foods, other than yeast, contained only minimal quantities of folic acid.

Samples tested from weekly batches of pressed brewers' yeast, all containing approximately 25 per cent. of total solids, were found to vary considerably in folic acid content. Similar variations from week to week were also found in the pyridoxine content.

TABLE IX

THE TOTAL FOLIC ACID CONTENT OF VARIOUS FOODSTUFFS

	No. of samples	Folic acid, μg. per g.
Dried yeast tablets	4	5·0
Dried yeast (brewers')	10	35·5
Saccharomyces cereviseae acetone-dried (pure culture)	2	20·9
Saccharomyces + bacteria, acetone dried	2	27·5
Pressed yeast (calculated on dry weight)		
(i)	2	21·2
(ii)	2	17·0
(iii)	2	18·8
(iv)	2	7·4
(v)	2	26·5
(vi)	2	23·6
(vii)	2	17·5
Dried full-cream milk	5	Nil to trace
Fresh cows' milk	2	"
Poultry mash	2	0·78
Dairy nuts	2	0·85
Wheat	2	trace
Maize meal	2	"
Oats	2	"

SUMMARY

1. A study has been made of the method of assay of total folic acid using *S. faecalis* NCTC 6459.

2. No method has been found so far, for the assay of free folic acid, owing either to the reaction of added material with the folic acid, as when takadiastase is used, or to the partial splitting of the conjugate, as in autoclaving with 0·1 *N* hydrochloric acid.

3. Ordinary artificial light has little or no effect on folic acid. Some loss occurs after a comparatively short exposure to ultra-violet light.

4. A method is given for the maintenance of the culture of *S. faecalis*, consisting of serial sub-culture through liver tryptone broth and agar.

5. A comparison of assay results using *S. faecalis* and *L. casei* has shown that, with the materials assayed, and when certain precautions are taken, there is little or no difference in the results.

6. Assays of brewers' yeast, dried milk and various foods by the recommended method are included.

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A Study of the Stokes Method for the Assay of Vitamin B_6 with *Neurospora sitophila* M.299

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Several methods for the assay of vitamin B_6 have been advocated at different times, but only two are in general use, a gravimetric method using the mould *Neurospora sitophila M.299* and a turbidimetric method using the yeast *Saccharomyces carlsbergensis*. In both, the total vitamin B_6 activity is assayed, whereas, by using *Lactobacillus casei* and *Streptococcus faecalis* (Snell and Rannefeld[1]) the pyridoxal and pyridoxamine fractions respectively are determined. The use of *S. faecalis* is considered extremely unsatisfactory on account of the very variable response of the assay organism.

The turbidimetric method has a short incubation period and there is no necessity to remove aneurine present (Atkin, Schultz, Williams, and Frey[2]). It has, however, the disadvantages of requiring a readily controllable shaker and a sensitive photo-electric colorimeter, which are not available in every laboratory. Further, the degree of accuracy obtainable is by no means high, and the duplication of results not infrequently poor.

The gravimetric method, using *N. sitophila*, has an incubation period of five days and aneurine must be removed. Nevertheless the method has the advantage of being gravimetric and relatively simple.

Various methods of extracting pyridoxine from different materials, in a form readily available to the assay organism, have been suggested. Rubin, Scheiner, and Hirschberg[3] used 0·055 *N* sulphuric acid or treatment of the sample with an enzyme preparation, for extraction of the vitamin B_6. Since *Saccharomyces carlsbergensis* was used as assay organism, it was unnecessary to remove the aneurine present in the extract. Rabinowitz and Snell[4] also used 2 *N* sulphuric acid as the extractant in a series of differential assays with *Sacch. carlsbergensis*, *L. casei*, and *Strept. faecalis*.

With *Neurospora sitophila* as assay organism, acid hydrolysis of the test sample must be followed by further treatment to remove any aneurine present. Stokes[5] has advocated the use of sodium sulphite followed by hydrogen peroxide to destroy the aneurine. This treatment has not, however, given either accurate or reproducible results. A study has, therefore, been made of this method and the modifications necessary to obtain accurate results. The effects of the age of the mould culture, of visible and ultra-violet light and of aneurine on the assay are included, together with the results of a series of assays of different foods.

Throughout, the term vitamin B_6 refers to the complex containing pyridoxine, pyridoxal, and pyridoxamine.

Experimental

1. Medium used for growth and sub-culturing of *N. sitophila*—The resting spores of the mould were maintained in a 10 per cent. cane sugar medium and transferred to a solid medium three days or more before a culture was required for assay purposes. This solid medium consisted of maltose 15·20 g., Bactopeptone Eupepton 3·20 g., malt extract (70 per cent. solids) 1·14 g., agar 8·00 g., and water to make up 400 ml.

A slope was incubated for three days at 30° C. and a loopful of the culture added to 70 ml. of sterile water for use as inoculum.

2. Medium used for the assay of pyridoxine—The composition of the basal medium used in the assays was based on that advocated by Stokes, Larson, Woodward, and Foster.[6] It consisted of sucrose 7·50 g., ammonium tartrate 2·50 g., potassium dihydrogen phosphate 1·25 g., sodium dihydrogen citrate 1·00 g., inorganic salt solution A 10 ml., inorganic salt solution B 10 ml., biotin 2 μg., and water to make up 250 ml.

Inorganic salt solution A consisted of 2·5 g. of $MgSO_4 \cdot 7H_2O$ and 0·01 g. of $ZnSO_4.7H_2O$ per 100 ml. of water and solution B of 0·5 g. of NaCl, 1 g. of $CaCl_2.6H_2O$ and 0·167 g. of $FeCl_3$ per 100 ml. of water.

3. Effect of the age of the culture of *N. sitophila*—With certain organisms, such as *S. faecalis*, sub-culturing on the same medium for a comparatively short period of time leads to a partial failure of the organism to respond to a vitamin normally essential for

growth. Nymon and Gortner[7] altered the growth medium for *L. casei* and obtained an increased response of the organism in riboflavine assays. A similar change of medium has been found essential with *S. faecalis* in the assay of folic acid (Jones and Morris[8]).

It was, therefore, decided to investigate the length of life of *N. sitophila* for pyridoxine assay. A series of cultures of *N. sitophila* was taken from the original 10 per cent. cane sugar culture to a solid malt extract – peptone medium at intervals of approximately one month.

These sub-cultures were stored in the refrigerator until tested. Five such sub-cultures, varying in age from one to five months, were examined and compared with a fresh culture.

The results, shown in Table I, show that little or no change in activity takes place as a result of maintaining a culture over a fairly long period (five months) on the malt-extract – peptone medium. The variations between one series of results and another bear little or no relationship to the age of the culture and are the normal daily variations that might be expected with the same culture. For this reason it is essential and customary to arrange a series of control experiments with each series of test solutions.

The results obtained warrant the assumption that, unlike certain bacterial cultures such as *S. faecalis*, the mould *N. sitophila* retains its activity for the assay of pyridoxine over fairly long periods.

TABLE I

EFFECT OF AGE OF CULTURE USED IN ASSAY

Pyridoxine, μg.	Weight of mould mycelium in mg.					
	144 days old	126 days old	98 days old	67 days old	44 days old	14 days old
0·1	6·8	6·6	5·5	6·6	6·2	7·2
0·4	15·7	16·6	16·6	16·9	20·9	17·3
0·8	32·2	34·7	35·8	36·1	36·6	33·1
1·6	37·4	41·8	37·1	46·3	41·2	42·6

4. EFFECT OF ANEURINE ON THE ASSAY OF PYRIDOXINE—Aneurine is known to affect the assay of pyridoxine with *N. sitophila*, but it has never been clearly shown whether aneurine is an essential nutrient for the mould. Thus, Barton-Wright[9] states that in presence of aneurine the mould no longer responds quantitatively to additions of pyridoxine.

A series of experiments was carried out to determine whether aneurine is an essential vitamin for the growth of *N. sitophila*, and whether the presence of aneurine in a pyridoxine solution gives an additive effect.

One ml. of an aqueous solution of pyridoxine containing 0·2 μg. was added to a series of tubes of basal medium and followed by different quantities of an aqueous solution of aneurine containing 0·2 μg. per ml. After diluting each to 10 ml. the tubes and contents were autoclaved at 15 lb. pressure for one hour, cooled and inoculated with *N. sitophila*. They were then incubated for five days and the mycelium weighed. Conjointly, two further tests were carried out, one in which aneurine alone was added to the basal medium, and the other in which pyridoxine alone was added.

Fig. 1 shows that there is a linear relationship between the amount of aneurine present in the medium and the growth response of the mould, as measured by the weight increase. The response with aneurine is, however, relatively small when compared with that found with pyridoxine. Further, there are occasions when the mould shows no response to addition of aneurine.

The results show that the presence of aneurine may interfere markedly with the assay of pyridoxine, possibly in an additive manner. It is obvious, therefore, that aneurine must be removed from any solution which has to be assayed for pyridoxine.

5. EFFECT OF SODIUM SULPHITE AND HYDROGEN PEROXIDE ON PYRIDOXINE—The method advocated for the destruction of aneurine, when present in solution together with pyridoxine, is by addition of sodium sulphite, any excess of sulphite being removed by means of hydrogen peroxide. The *p*H of the sulphite-treated material must be 8, and a slight excess of peroxide is necessary to destroy all the sulphite, otherwise the mould will not grow. It is known, however, that both sulphite and peroxide attack pyridoxine, although Cunningham and Snell[10] found that the loss of pyridoxine was minimal between *p*H 8 and 8·6.

In view of the possible source of error in this method of preparing extracts, a series of experiments was arranged in which solutions of pyridoxine in water were treated with sodium

sulphite at *p*H 8, and steamed at 100° C. for 30 minutes, and the sulphite finally removed with hydrogen peroxide. Any excess of peroxide was destroyed when the medium was

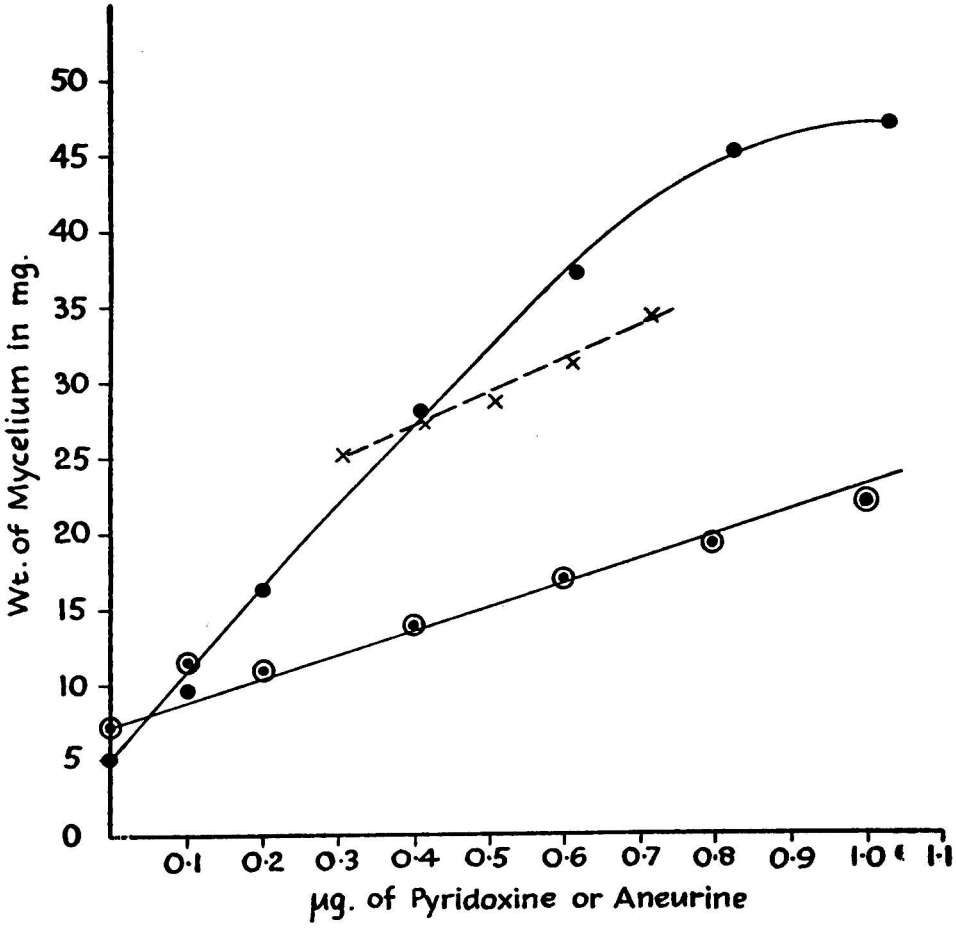

Fig. 1. Curves showing the effect of aneurine alone, pyridoxine alone and mixtures of aneurine and pyridoxine on the growth of *N. sitophila*.

● Pyridoxine alone.
⊙ Aneurine alone.
× Pyridoxine and aneurine mixtures.

sterilised. The results, an average of three separate experiments, are shown in Table II.

It is clear that, even when all precautions are taken, the sulphite – peroxide treatment affects the recovery of pyridoxine from pure solution. The results show an average loss, in the present series of experiments, of about 35 per cent.

TABLE II

EFFECT OF SODIUM SULPHITE AND HYDROGEN PEROXIDE ON THE ASSAY OF PYRIDOXINE

Pyridoxine added, µg.	Pyridoxine found, µg.	Percentage loss
0·1	0·07	30
0·2	0·15	25
0·4	0·28	30
0·6	0·43	28
0·8	0·50	38
1·0	0·59	41

With brewers' yeast as the test material, a series of pyridoxine assays was carried out in a similar manner, and the results (Table III) show a variation of the order of 100 per cent., clearly indicating that little or no reliance can be placed on the method of analysis.

TABLE III

ASSAY OF VITAMIN B_6 CONTENT OF BREWERS' YEAST COMPARING Na_2SO_3 AND NaOH FOR THE INACTIVATION OF ANEURINE

Amount of final diluted sample taken, ml.	Vitamin B_6 found, μg. per g.	
	Na_2SO_3 treatment	
	Yeast 1	Yeast 2
1	8	6
2	9	8
3	10·3	11
4	15	12·4

6. EFFECT OF SODIUM HYDROXIDE ON THE ASSAY OF PYRIDOXINE—In view of the results of the experiments in which sodium sulphite was used to remove the aneurine, this method for the assay of pyridoxine had to be abandoned. Fortunately, however, aneurine is particularly sensitive to the presence of alkali, whereas pyridoxine is stable. It is stated that none of the pyridoxine group of vitamins is affected by heat treatment in presence of *N* sodium hydroxide (Cunningham and Snell[10]).

A series of experiments was arranged to determine whether sodium hydroxide affected pyridoxine, pyridoxal, and aneurine. The pyridoxal used was a sample of the pure product supplied by Merck or a sample made according to the method given by Barton-Wright. In the first series pyridoxine was treated with *N* sodium hydroxide and assayed. Pyridoxal was treated in a similar manner. The results are given in Table IV. To investigate the effect of *N* sodium hydroxide on aneurine, different quantities of a solution of pyridoxine were added to 1 ml. of an aqueous solution containing 1 μg. of aneurine. The solution was autoclaved at 15 lb. pressure for 1 hour with *N* sodium hydroxide and assayed for pyridoxine. The results are given in Table V. Finally, 1 ml. of an aqueous solution of pyridoxine, containing 0·2 μg. was added to different quantities of an aqueous solution of pyridoxal. The mixtures were assayed before and after treatment with sodium hydroxide. The results are given in Table VI. The complete results show that, whereas aneurine is completely inactivated by heating in presence of sodium hydroxide, pyridoxine and pyridoxal are relatively unaffected.

TABLE IV

EFFECT OF *N* NaOH ON THE ASSAY OF PYRIDOXINE AND PYRIDOXAL

Pyridoxine		Pyridoxal	
Amount added, μg.	Amount found, μg.	Amount added, μg.	Amount found, μg.
0·10	0·08	0·18	0·20
0·20	0·21	0·36	0·36
0·40	0·37	0·54	0·55
0·60	0·60	0·72	0·66
0·80	0·84	0·90	0·86
1·00	1·10	—	—

TABLE V

EFFECT OF *N* NaOH ON THE ASSAY OF PYRIDOXINE IN THE PRESENCE OF ANEURINE

Pyridoxine added, μg.	Aneurine added, μg.	Pyridoxine found, μg.
0·10	1·00	0·08
0·20	1·00	0·22
0·30	1·00	0·33
0·40	1·00	0·41
0·50	1·00	0·54

TABLE VI

EFFECT OF *N* NaOH ON THE ASSAY OF MIXTURES OF PYRIDOXINE AND PYRIDOXAL

Pyridoxine added, μg.	Pyridoxal added, μg.	Total found, untreated, μg.	Total found treated with *N* NaOH, μg.
0·20	0·05	0·27	—
0·20	0·23	0·50	0·42
0·20	0·50	0·67	0·69
0·20	0·68	0·75	0·80

7. EFFECT OF DIFFERENT QUANTITIES OF SODIUM HYDROXIDE AND OF SODIUM HYDROXIDE FOLLOWED BY ACID ON THE EXTRACTION OF VITAMIN B_6 FROM YEAST—Rubin, Scheiner, and Hirschberg found that extraction with 0·055 *N* sulphuric or hydrochloric acid was essential to free all the B_6 vitamins. Following hydrolysis of the test sample with 0·1 *N* sodium hydroxide, in which only a small fraction of the B_6 group was extracted, a further treatment with 0·055 *N* sulphuric acid extracted all the vitamin B_6. For assay purposes they used *Saccharomyces carlsbergensis*.

The effect of different concentrations of sodium hydroxide on the extraction of vitamin B_6 was examined by autoclaving dried bakers' yeast at 15 lb. pressure for 1 hour with 0·1 *N*, *N*, and 2 *N* solutions. A further series of samples was treated as before and hydrochloric acid added after autoclaving to bring the final concentration of acid to 0·055 *N*. The solution was again autoclaved at 15 lb. pressure for 1 hour. In all cases 5 g. of yeast and 40 ml. of the sodium hydroxide solution were used. Concentrated hydrochloric acid was used to bring the final acid concentration to 0·055 *N* in a total volume of 50 ml.

The results, given in Table VII, show that the maximal amount of vitamin B_6 was extracted by sodium hydroxide treatment alone. Further treatment of the sample with 0·055 *N* hydrochloric acid appears to be unnecessary when *N. sitophila* is used as the assay organism and the alkali at least 1·0 *N* in concentration.

TABLE VII

EFFECT OF DIFFERENT CONCENTRATIONS OF NaOH AND OF NaOH FOLLOWED BY 0·055 *N* HCl ON THE EXTRACTION OF VITAMIN B_6 FROM DRIED BAKERS' YEAST

μg. Vitamin B_6 per g. of dried yeast					
0·1 *N* NaOH		*N* NaOH		2 *N* NaOH	
Alone	Followed by 0·055 *N* HCl	Alone	Followed by 0·055 *N* HCl	Alone	Followed by 0·055 *N* HCl
13	11	10	11·8	10	10
16·3	11	11·3	10·8	10	12

The greater variation shown with 0·1 *N* sodium hydroxide treatment seems to be due to incomplete destruction of the aneurine present. Treatment with 0·055 *N* hydrochloric acid appears to destroy either any residual aneurine or some other factor affecting the assay.

A further series of tests with yeast was carried out to test the efficiency of the treatment and to determine whether concordant duplicate results could be obtained. Table VIII gives the results obtained.

TABLE VIII

ASSAY OF VITAMIN B_6 CONTENT OF BREWERS' YEAST, USING *N* NaOH TO INACTIVATE THE ANEURINE

Amount of final diluted sample added, ml.	μg. of Vitamin B_6 per g.		
	Yeast 2	Yeast 3	Yeast 4
1	17	19	23
2	16	—	—
3	17	—	—
4	20	21	26

The maximum variation is very much lower with sodium hydroxide than by the use of sodium sulphite. The sodium hydroxide treatment for the inactivation of aneurine has, therefore, been adopted for the assay of vitamin B_6.

8. EFFECT OF LIGHT ON THE ACTIVITY OF PYRIDOXINE—Daylight and artificial light are known to affect and inactivate pyridoxine, but only recently have results been published showing the exact effect. Cunningham and Snell (1945) have shown that a major inactivation takes place when a pyridoxine solution is exposed to direct sunlight for 1 hour, whereas diffuse daylight or artificial light give minimal losses for the same time of exposure. The present experiments deal with the effect of artificial light and ultra-violet light on pyridoxine activity.

In the first series a solution of pyridoxine, containing 0·2 μg. per ml., was exposed to ultra-violet light (1849–4500 A.) for 30 to 120 minutes. The source of light was placed 30 cm. from the solution, which was exposed directly in a crystallising dish to the ultra-violet light. The solution was 0·5 cm. in depth. After treatment the solution was assayed and in all cases the pyridoxine was completely inactivated within the sensitivity of the test. Using a solution containing 20 μg. per ml. exposed for 1 hour to ultra-violet light, a loss of activity of only 35 per cent. was recorded. The rate of inactivation would appear to depend, in part at least, on the pyridoxine concentration of the solution irradiated, other factors being kept constant.

The effect of artificial light was studied by exposing a solution of pyridoxine, containing 20 μg. per ml., to the rays from a 100-watt lamp for 4 hours under exactly the same conditions as for the ultra-violet light exposure. Only 6 to 10 per cent. of the pyridoxine appeared to be inactivated.

From the results it may be concluded that artificial light has little or no effect on the assay of pyridoxine, whereas direct sunlight or ultra-violet light must be avoided.

9. RECOMMENDED METHOD OF TREATING SAMPLES—Weigh 5 g. of the sample to be assayed into a flask and add 40 ml. of *N* sodium hydroxide. Autoclave the whole for 1 hour at 15 lb. pressure, cool, dilute with water to 100 ml. Pipette 10 ml. of the solution into a 50-ml. conical flask and neutralise to *p*H 7 with concentrated hydrochloric acid. Adjust the *p*H to 4·6 with *N* hydrochloric acid, using external indicators, and finally dilute the solution to give a concentration of approximately 0·3 μg. of vitamin B_6 per ml., filtering if necessary.

The method of assay is, otherwise, similar to that published by Stokes.

10. ASSAY OF VARIOUS FOODSTUFFS—The results of assays on different samples of brewers' yeast, milk, and various foods are given in Table IX.

TABLE IX

VITAMIN B_6 ASSAY OF BREWERS' YEAST, MILK, AND VARIOUS CEREALS AND CEREAL MIXTURES

Material	Number of samples assayed	Pyridoxine per g. of material
Dried yeast A ..	3	135
Pressed yeast A ..	1	20
" " B ..	1	25
" " C ..	1	14
" " D ..	1	7
" " E ..	1	6
" " F ..	1	5·4
Dried whole milk ..	3	7
Fresh milk	2	6·5
Wheat	2	13
Maize meal	2	5·6
Oats	2	8·9
Dairy nuts	2	8·2
Poultry mash ..	2	9·6

The results obtained are considerably higher than the majority of previously published results by Stokes's original method, and this, it is felt, is due, in part at least, to the replacement of sodium sulphite and hydrogen peroxide by sodium hydroxide for the inactivation of the aneurine present in the samples. The considerable variation in the vitamin B_6 content of pressed brewers' yeast (all samples of which contained approximately 25 per cent. of total solids) may be due to the rather abnormal conditions at present existing in the

brewing industry. Samples tested from weekly batches of yeast were found to vary considerably in vitamin B_6 content, as can be seen in Table IX.

SUMMARY

1. A study has been made of several factors that might possibly interfere with the assay of vitamin B_6 by means of the mould *N. sitophila*. Of these factors, the age of the culture has no effect on the response found.

2. In the assay of vitamin B_6 it has been found that aneurine must be inactivated, but this cannot be carried out with sodium sulphite and hydrogen peroxide, or pyridoxine will also be affected.

3. A method has been evolved in which the aneurine is inactivated by heat treatment in presence of *N* sodium hydroxide, and several assays of brewers' yeast, dried milk, and various foods by this method are recorded.

4. The effect of ordinary artificial light on pyridoxine is slight but ultra-violet light may cause total inactivation.

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Phosphate Removal in Qualitative Analysis

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OF the several methods proposed for the removal of the phosphate ion in qualitative analysis, most require a modification of, if not a complete departure from, the usual systematic scheme for cation separation effected in absence of the phosphate; moreover, they frequently demand not a little manipulative skill on the part of the operator for their successful execution, skill that can only be acquired by continual use. For those procedures that do not require subsequent rearrangement of the group scheme, the remark about manipulation applies to an even greater degree. However, of the two types, the latter are the more attractive, and, inasmuch as their disadvantages are confined to the technique of phosphate removal their improvement would appear to be possible by an improvement of that technique.

Accordingly, two of these methods have been examined—one, where the phosphate is removed by stannic chloride, and the other where it is precipitated by means of soluble zirconium salts.

STANNIC CHLORIDE METHOD

The procedure originally devised by Gatterman and Schindhelm[1] entails the precipitation of the phosphate by addition of stannic chloride to a slightly acid solution and removal of the excess of tin by sulphide precipitation. The main objection lies in the bulk and nature of the precipitate formed, which is colloidal and very difficult to filter.

Experimental—The use of tannin as a flocculating agent is well known, and the tin–tannin complex (recently described by Holness and Schoeller[2] for the quantitative separation of tin from aluminium, iron, manganese, etc. in hydrochloric acid solutions) is precipitated under the conditions of the removal; hence it was to be expected that tannin might prove to be of value in eliminating this filtration difficulty. But

these expectations were only realised on addition of a considerable quantity of tannin, excess of which it is necessary to remove. Also, although complete removal of phosphate is not claimed by the authors of the method, it was observed that tannin actually increased the amount of residual phosphate, as detected by the molybdate test.

The use of solid stannic chloride pentahydrate, instead of the "solution" containing 5 g. of solid in 5 ml. of water,[3] also increased the amount of residual phosphate. Ammonium stannichloride, 5 g. of which were dissolved in the minimum of water (20 ml.), proved to be as efficacious as the stannic chloride, and it has the advantage of being non-hygroscopic. As with solid stannic chloride, the solid stannichloride failed to remove all of the phosphate in one operation. Addition of tannin had the same effect with it as with stannic chloride, *i.e.*, it caused even more incomplete removal than occurred without it.

The slow addition of the stannic chloride was tried, as recommended for zirconium phosphate (*infra*). Although this facilitated filtration to a certain extent, some phosphate remained in solution even after the operation had been repeated three times.

Discussion—The intractability of the precipitate and the variable extent of phosphate removal are serious factors operating against the applicability of this method. Reference to these drawbacks brings up the question of the constitution of the precipitate. It has been stated to be an adsorption complex of PO_4''' on stannic acid, and this is borne out by the fact, mentioned above, that tannin is able to break up this complex to a certain extent to form the tin–tannin complex. This is not to be expected of a definite compound. Considerable experimental evidence has been adduced in support of the general theory that tannin acts by flocculation of a disperse hydroxide,[4] and the observation that the phosphate is precipitated by the stannic chloride solution over the same acidity range as that at which tannin precipitates tin would suggest that the action of stannic chloride on the phosphate is the result of its hydrolysis. This further points to the formation of a hydroxide-phosphate complex.

Zirconium phosphate method

Of all the metallic phosphates, those formed by zirconium and hafnium are the least soluble in strong acids. Use of this property in phosphate separation was first made by Curtman and his co-workers,[5,6] who added a zirconyl chloride solution to an acid solution of the interfering phosphate. More recently, the use of zirconyl nitrate has been proposed.[7]

The chief disadvantages of the original method are:

(*a*) the precipitate formed is colloidal,
(*b*) there is a large loss of cations from solution,
(*c*) phosphate removal is often incomplete,
(*d*) excess of zirconium interferes with the subsequent tests for aluminium, and
(*e*) an extra separation is entailed—that of zirconium in Group IIIa.

One modification[8] involves the use of more dilute zirconium solutions but does not overcome (*a*), (*c*), (*d*), and (*e*). Reilly and O'Brien[9] have recognised that (*a*) is due to the presence of a large excess of zirconium, and suggest the slow, drop by drop, addition of the zirconyl solution as being effective in producing a filterable precipitate. Their method, although an improvement, still does not obviate (*d*) and (*e*).

Experimental—

Tannin was again employed in the investigation of these objections, using the zirconium-tannin complex (described by Schoeller[10] for the quantitative separation of zirconium from aluminium, iron, manganese, etc.). The reagent was very effective in producing a filterable precipitate; removal of excess of tannin was effected by addition of a few drops of zirconium solution to the filtrate, but the resulting solution contained some zirconium. Attempts to precipitate this with saturated potassium sulphate and with potassium ferrocyanide were made, but they were unsuccessful for the small quantities of zirconium present, since they merely formed a cloudiness in the solution on standing for some time and this could not be removed by filtration. Rendering the zirconium non-interfering as fluozirconate was also tried, equally without success. The method was no improvement on that of Reilly and O'Brien.

In order to remove the excess of tannin, resort was made to the tin-tannin complex again, by addition of ammonium stannichloride, with subsequent removal of residual tin with hydrogen sulphide. This was found to be very satisfactory: the successive steps prevent any interference that may result from the presence of zirconium, tannin, or tin.

Procedure—

The following details apply to solutions containing up to 0·7 g. of phosphate ion. Transfer the filtrate from Group II to a 100-ml. beaker, boil to remove hydrogen sulphide, and test a

small portion for the presence of phosphate with ammonium molybdate. If positive, add 2 g. of ammonium chloride, boil, and add slowly 10 ml. of *either* an 8 per cent. aqueous solution of zirconyl chloride *or* a 7 per cent. aqueous solution of zirconyl nitrate, and boil again. Add diluted ammonia solution (1 + 1) until the solution is just alkaline to litmus, followed by *N* hydrochloric acid until it is just acid and boil. Add 10 ml. of 1 per cent. tannin solution, boil and add 10 ml. of a 1 per cent. solution of ammonium stannichloride in 0·5 *N* hydrochloric acid. Boil, filter, and discard the precipitate. Test a portion of the filtrate for the presence of phosphate ions.

If the test is negative, pass H_2S into the remainder of the filtrate, filter (rejecting the precipitate), boil off hydrogen sulphide from the filtrate, oxidise any ferrous iron with concentrated nitric acid and precipitate the iron group by addition of ammonia in the usual way.

In order to obtain some estimate of the loss of ions occasioned by the separation, figures were obtained for the recovery of a fixed amount of cation from solutions containing varying amounts of phosphate. It was thought that iron, aluminium, zinc, and barium would be representative of the metals of Groups IIIa, IIIb, and IV, and they were investigated as follows:

Iron—A soln. of ferric alum was made up, and standardised by precipitation as hydroxide and ignition to Fe_2O_3. Seventy-five-ml. portions were withdrawn, A.R. diammonium hydrogen phosphate was added, the separation as described above effected, and the iron determined as Fe_2O_3.

Aluminium—Three 50-ml. portions of a stock soln. of ammonium alum were treated with differing amounts of A.R. diammonium hydrogen phosphate, the phosphate was removed as described and the aluminium recovered as Al_2O_3.

Zinc—Separations were made on three solns. of zinc sulphate, each containing a known amount of zinc and A.R. diammonium hydrogen phosphate, after which the zinc was determined by titration against a standard potassium ferrocyanide solution.

Barium—The barium content of 50 ml. of a solution of barium chloride was determined as $BaSO_4$. To three other equal volumes of the same solution were added different weights of A.R. diammonium hydrogen phosphate; removal of the PO_4''' ion was carried out as above and the barium again determined as $BaSO_4$.

No.	Phosphate added g. $(NH_4)_2HPO_4$	Cation taken g.	Cation found g.	Recovery %
1	0·05	0·284 Fe_2O_3	0·277 Fe_2O_3	97·5
2	0·20	0·284 "	0·268 "	94·0
3	0·60	0·284 "	0·255 "	92·0
4	1·00	0·284 "	0·236 "	83·0
5	0·05	0·193 Al_2O_3	0·182 Al_2O_3	94·5
6	0·50	0·193 "	0·169 "	87·5
7	1·00	0·193 "	0·154 "	80·0
8	0·05	0·072 Zn	0·071 Zn	98·0
9	0·50	0·072 "	0·068 "	93·5
10	1·00	0·072 "	0·060 "	83·0
11	0·05	0·252 $BaSO_4$	0·246 $BaSO_4$	98·0
12	0·50	0·252 "	0·244 "	97·0
13	1·00	0·252 "	0·241 "	96·0

DISCUSSION—

The original procedure as first proposed was undoubtedly unsatisfactory, but it has been substantially improved in various ways by later workers. The most significant advance has been that of Reilly and O'Brien,[9] but their method of removing the surplus zirconium by boiling in alkaline solution leaves much to be desired. In the revised procedure given above, the system is such that each successive reagent removes excess of the former, and interference is prevented. Thus the phosphate is removed by a zirconium solution, excess zirconium is removed by tannin (which also serves as a flocculating agent), and excess of tannin is removed by tin. Excess tin is removed by H_2S. The average time for the complete sequence of operations is 15 minutes.

A further advantage of this scheme is that different amounts of phosphate are removed with equal ease, since the method is a "sliding scale" one. A small amount of phosphate leaves a large excess of zirconium, which is removed as described, leaving a comparatively large excess of tin, which is conveniently precipitated as sulphide. Similarly, a large amount

of phosphate leaves a small quantity of tin, which is also dealt with easily by the H_2S. The recovery figures quoted show that for qualitative purposes, the loss in cations is not great.

One apparent objection would seem to be the expense of the zirconium salts. Actually, however, the quantities used are such as to make the method cheaper than that using stannic chloride: 5 g. of $SnCl_4.5H_2O$ are used for every operation by the stannic chloride method, whereas only 0·8 g. of $ZrOCl_2.8H_2O$ and 0·1 g. of $(NH_4)_2SnCl_6$ are used in the revised method described.

The modified procedure has become standard practice in these laboratories, and has been in satisfactory use for many months.

SUMMARY

Two methods for the removal of the phosphate ion in qualitative analysis have been examined and discussed, *viz.*, the stannic chloride and the zirconium phosphate methods. It is seen that, of the two, the latter has the wider application. A modified procedure has been outlined whereby the rapid and complete removal of quantities up to 0·7 g. of phosphate may be effected whether it be present as the major or the minor constituent of the sample

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The Quantitative Analysis of Crude and Refined Carbazole

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THE growing demand for carbazole as an intermediate for the preparation of a number of technically useful derivatives has made a re-examination of the various extant or proposed methods for estimating the carbazole content of materials of coal tar origin desirable. Coal tar contains a relatively high proportion (about 2 per cent.) of carbazole and is not likely to be displaced as its source. The closest congeners of carbazole in tar are anthracene and phenanthrene and they are therefore the chief contaminants of crude and refined specimens of carbazole submitted to analysis. Besides these, a number of other substances, chiefly aromatic hydrocarbons but not excluding heterocyclic nitrogen compounds, may be present to a lesser extent.

Of the older analytical methods[1] only one based on an estimation of the nitrogen content (which is assumed to be wholly due to carbazole) by Kjeldahl's process has been used extensively. This has been said[2] to yield results unduly high by 2 to 4 per cent., but our experience with material known to be high in carbazole was quite the reverse. If the method is employed on very crude materials, containing nitrogenous compounds other than carbazole, errors of unknown magnitude and direction must arise. However, the main drawback to the method is that carbazole is extremely resistant to decomposition by the Kjeldahl process and specimens require 20 to 30 hours digestion before clear and colourless solutions suitable for ammonia estimation are secured. This prolonged heating is not a direct cause of loss of ammonium salts and the consequent low analytical results.

Two other methods[1] depended upon (*a*) the quantitative separation of carbazole as the N-potassium derivative, which was decomposed and the recovered carbazole weighed, and (*b*) a quantitative bromination. The first has no feature to recommend it, and although Ardashev[2] preferred the bromination method to the Kjeldahl nitrogen estimation, it does require a supplementary independent estimation of the anthracene content (anthracene, if present, is

also brominated), and a very close control of experimental conditions is needed, as he showed, to define exactly the number of molecules of bromine (2 or 3) that react with the carbazole. Neither method was thought sufficiently attractive to warrant any further examination.

Cohn[3] suggested that a method of analysis could be based on the acetylation of carbazole, excess of acetic acid being titrated. Some preliminary experiments on these lines were not promising. The presence of N-acetyl carbazole in the mixture to be titrated is very inconvenient and the method was not persevered with.

Recently, Chmelevsky and Postovsky[4] have proposed a method based on the quantitative nitrosation of the carbazole in specimens derived from coal tar. This has the merit of being rapid and is not interfered with by any anthracene or phenanthrene present. The specimen is treated with nitrous acid, generated from sodium nitrite and acetic acid, and the excess of nitrous acid is decomposed to produce nitric oxide (NO), which is swept out in a stream of carbon dioxide into strong caustic potash solution and its volume measured. The technique of this method has now been closely studied and it has been found that when adequate precautions are taken, as detailed below, the results with purified and crude carbazole specimens and with mixtures of purified carbazole, anthracene and phenanthrene are reproducible.

1. The carbon dioxide used for sweeping out air initially, and nitric oxide finally, from the reaction flask, was generated from marble chips, broken small and kept beneath boiling water for 2 or 3 hours before use, and recently boiled aqueous hydrochloric acid (equal volumes of concentrated acid and distilled water). It was dried by bubbling through concentrated sulphuric acid before passage into the reaction flask.

2. The specimen for analysis was placed in the reaction flask and dissolved in a fixed amount of chlorobenzene and acetic acid. The acetic acid, previously distilled from chromic acid, was freed from peroxides before use by boiling for 30 minutes under reflux with cupric acetate and then distilling. The chlorobenzene – acetic acid solution was heated just to boiling to expel dissolved air.

3. The purity of the sodium nitrite was checked by titration with standard potassium permanganate solution and a correction was determined, from blank experiments, for the residual amount of nitric oxide dissolved in the reaction mixture.

4. The reaction mixture was warmed to 40° C. towards the end of the evolution of nitric oxide.

5. The nitric oxide collected was not transferred to a graduated tube and measured over water but measured in the nitrometer after allowing time for it to reach room temperature. When nitric oxide is transferred over water an appreciable but indeterminate amount is lost by solution. For example, in an experiment in which 22·2 ml. at N.T.P. were measured over 50 per cent. caustic potash solution the amount after transference diminished to 21·3 ml. at N.T.P. Nitric oxide is about three times as soluble in water at room temperatures as nitrogen.

If the precautions detailed above are neglected considerable errors arise in both directions and the reproducibility of the analytical results is much reduced. Thus if air is not eliminated from the system and if the acetic acid contains peroxide some of the nitric oxide is oxidised to nitrogen peroxide (NO_2) and lost, but the volume of gas collected will be contaminated with nitrogen. This generally leads to low results for the carbazole content of a specimen. On the other hand, if the nitric oxide is not fully swept out, and the residual amount dissolved is not allowed for, results will be high. A further source of variability is incomplete nitrosation. Our results with highly purified specimens of carbazole showed that under the conditions laid down about 4 per cent. escaped nitrosation. This was allowed for in computing the carbazole content of specimens submitted to analysis.

The technique recommended reduces the uncertainty in a single estimation to ± 0·6 per cent. with a carbazole specimen upwards of 90 per cent. purity, and to ± 0·2 per cent. if the carbazole content is about 30 per cent.

Experimental

Crude and purified carbazole specimens—

Samples of carbazole from three sources were used in this work.

Specimen A—A specimen of "pure carbazole" separated from coal tar and of German origin had m.p. 243° to 245° C.

Specimen B—Specimen A was recrystallised three times from chlorobenzene to constant m.p. 244·5° to 245·5° C. (corrected) and taken as being then sufficiently pure to use as a standard, *i.e.*, its purity was taken as nominally 100 per cent.

Specimen C—A sample of synthetic carbazole was prepared from the phenylhydrazone of *cyclo*hexanone through tetrahydrocarbazole,[5] the latter being dehydrogenated by boiling a solution in xylene with chloranil.[6] The crude synthetic carbazole was recrystallised from chlorobenzene to m.p. 244° to 245° C. (corrected).

Specimen D—A crude crystalline fraction containing about 30 per cent. of carbazole was obtained from a firm of tar distillers. This was fully representative of material derived from coal tar at an early stage in the separation of carbazole. It was somewhat oily and the analytical results reported herein seem to show that it contained nitrogenous substances additional to carbazole. Specimens taken for analysis were from a portion that had been thoroughly ground and mixed to render it homogeneous.

ESTIMATION OF NITROGEN CONTENT IN CARBAZOLE-CONTAINING MATERIALS BY KJELDAHL'S METHOD—

The method adopted followed orthodox lines.[7] The specimen, containing about 0·8 g. of carbazole, was digested with 23·5 ml. of concentrated sulphuric acid containing 0·5 g. of copper sulphate crystals, 0·1 g. of selenium and 16 g. of potassium hydrogen sulphate. A further 10 ml. of concentrated sulphuric acid were added during the digestion. Generally from 15 to 30 hours were allowed, this being 2 or 3 hours additional to the time required for the disappearance of organic coloration.

Duplicate results on Specimen A were 7·63 and 7·70 per cent. of nitrogen, from which the carbazole content, assuming all nitrogen found arises from carbazole, is 91·0, 91·9 per cent. In a mixture of this specimen with equal weights of pure phenanthrene and anthracene, the nitrogen content was found to be equivalent to a weight of carbazole equal to 91·7 per cent. of the weight of Specimen A taken.

Similar duplicate results on Specimen B (the standard specimen) corresponded to 91·9 and 92·6 per cent. for the carbazole content.

Specimen D gave the following figures for carbazole content:—31·6, 31·1, and 31·2 per cent.

Additionally, three further experiments were carried out. The first, a blank estimation (*i.e.*, without introduction of carbazole but in which the digestion was continued for 4 hours) showed nitrogen, 0·0 per cent. The other two were designed to estimate any loss of ammonium salts due to the lengthy digestion period. First, a weighed amount of ammonium sulphate was added to the Kjeldahl reagents and immediately estimated in the usual way: 99·3 per cent. of the weight taken was found, which may be taken as an estimation of the purity of the ammonium salt. Then the experiment was repeated, but 16 hours digestion was allowed before estimation of the ammonium salt. The amount found was now 98·9 per cent. of that taken. The difference in the two results (less than 0·5 per cent.) is small enough to show that the larger loss of nitrogen occurring in actual carbazole estimations does not take place after conversion to ammonium sulphate.

QUANTITATIVE NITROSATION OF CARBAZOLE-CONTAINING MATERIALS

OUTLINE OF RECOMMENDED METHOD—

The apparatus employed is shown diagrammatically in Fig. 1. A weighed amount of the specimen for analysis (containing about 0·5 g. of carbazole) is placed in the reaction flask (F) and dissolved in a mixture of 20 ml. of chlorobenzene and 20 ml. of acetic acid by heating to boiling. After cooling, the flask is attached to the rest of the apparatus: the sodium nitrite (about 0·33 g.), weighed accurately in its container (D), is previously introduced into the inlet tube. A vigorous stream of carbon dioxide is passed for about 20 minutes and the rate then reduced to 2 to 3 bubbles per second, when it will be found, by the usual test of filling the nitrometer with potash, that the volume of undissolved gas collected is negligible (*i.e.*, less than 0·1 ml. in 10 mins.). The sodium nitrite container is allowed to fall and its contents are mixed, with occasional shaking, into the contents of the flask. After about 30 minutes, when evolution of nitric oxide has almost ceased, the reaction flask is immersed in a water bath maintained at 40° C. for about 15 minutes and shaken from time to time, after which no appreciable further increase in the volume of nitric oxide in the nitrometer (G) will occur. The potash reservoir is raised so that the liquid levels are equal, and between 30 minutes and 1 hour allowed for the gas to assume room temperature before final measurement of its volume and temperature and the barometric pressure.

Calculation of carbazole content—The calculation is based upon the following equations:

$$C_{12}H_9N + NaNO_2 + CH_3COOH = C_{12}H_8N_2O + CH_3COONa + H_2O$$

$$3NaNO_2 + 2CH_3COOH = 2NO + NaNO_3 + 2CH_3COONa + H_2O.$$

In one estimation, 0·5042 g. of pure carbazole reacted with 0·3290 g. of sodium nitrite (purity estimated by titration 99 per cent.) and the volume of nitric oxide collected was 27·8 ml. at

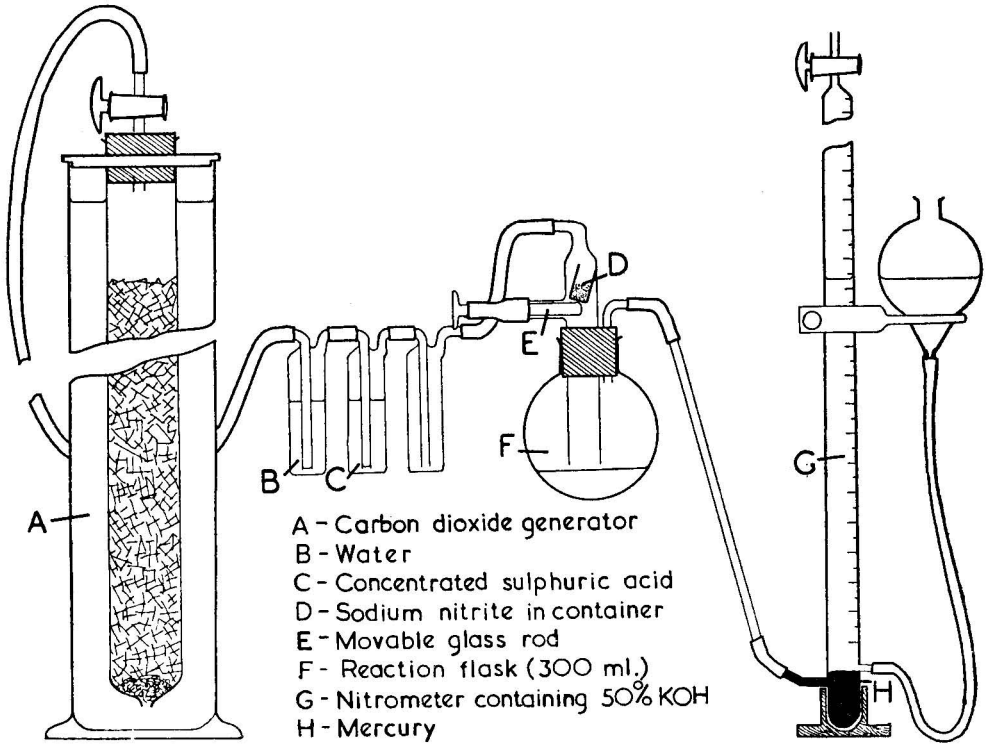

Fig. 1

20·5° C. and 766·8 mm., corresponding to 26·09 ml. at N.T.P. A standard correction of 1·0 ml. to allow for dissolved nitric oxide (see below) is added, making 27·09 ml. Hence, weight of carbazole nitrosated =

$$\frac{167}{69}\left(0{\cdot}3290 \times \frac{99}{100} - 27{\cdot}09 \times \frac{3}{2} \times \frac{69}{22400}\right) = \frac{167}{69} \times 0{\cdot}2005,$$

which is increased by a factor 1·04 to allow for incomplete nitrosation (see later, p. 50), so that the carbazole content is in

$$\frac{167}{69} \times \frac{0{\cdot}2005}{0{\cdot}5042} \times 1{\cdot}04 \times 100 = 100{\cdot}1 \text{ per cent.}$$

Establishment of the recommended method

Correction for solubility of nitric oxide—

Six identical experiments were made in which sodium nitrite in quantity sufficient to furnish about 26 ml. of nitric oxide was decomposed in the apparatus in absence of carbazole. The sodium nitrite was 99 per cent. pure by titration: from the amounts of nitric oxide collected, the purity was calculated to be 95·0, 94·5, 94·8, 94·6, 95·6 and 95·5 per cent.; mean 95·0 per cent., standard deviation for a single estimation 0·46 per cent. When the standard correction 1·0 ml. was added to the gas volumes after reduction to N.T.P., the mean of the calculated values for the purity of the sodium nitrite became 99·1 per cent. These experiments indicate the preferability of estimating the nitrite by titration rather than by a single blank experiment, and they justify the correction of the volume collected by the standard addition of 1·0 ml. for the solubility of nitric oxide in the reaction medium.

Estimations on carbazole-containing material—Correction for incomplete nitrosation—

Six identical experiments on carbazole Specimen B, using sodium nitrite in excess sufficient to provide the same volume of nitric oxide as in the blank experiments, gave the following

results after applying the standard correction to the volume of gas collected: 96·7, 95·6, 96·8, 95·5, 96·2 and 96·2 per cent. (mean 96·2 per cent. standard deviation 0·54 per cent.).

If the carbazole percentages as found are multiplied by 1·04, the mean is now 100·0 per cent. and standard deviation 0·6 per cent., and it therefore appears legitimate to allow for incompleteness in nitrosation in this way.

The carbazole Specimen C was found in three estimations to have the purity 99·8, 99·8 and 99·6 per cent. (mean 99·7 per cent.) in terms of Specimen B = 100 per cent.

Two estimations on the cruder carbazole (Specimen D) gave carbazole content 28·4, 28·0 per cent., mean 28·2 per cent. Compared with the results of the Kjeldahl estimation (mean of three, 31·3 per cent.), they appear low, and as the Kjeldahl method applied to purer carbazole specimens (A and B) gave markedly lower results than the nitrosation method, it must be supposed that the cruder material contains nitrogen compounds other than carbazole that are not nitrosated.

Experiments were next made with mixtures containing only carbazole, anthracene, and phenanthrene. In a mixture of commercial "pure" anthracene and phenanthrene, containing roughly equal amounts by weight, the carbazole content was found to be 0·24 per cent. When to this mixture approximately half its weight of carbazole (Specimen B) was added, the carbazole found was 32·7 per cent., against 32·5 per cent. calculated.

In order to make more certain that the figures obtained for the carbazole content of crude material (Specimen D) by the nitrosation method are more reliable than those obtained through the Kjeldahl nitrogen estimation, duplicate experiments were made in which a mixture of Specimens D and B was analysed. The results were—Found in the mixture 0·5141 and 0·5063 g. of carbazole; Calculated from prior analyses 0·5135 and 0·5058 g., *i.e.*, weight found = 100·1 per cent. of weight calculated in each instance. These experiments show clearly that in the nitrosation method it is the actual carbazole content that is determined and impurities in crude specimens, whether nitrogen-containing or not, do not interfere with the method.

Additional experiments—

The use of a mixture of chlorobenzene and acetic acid as the reaction medium has some advantage. If acetic acid is used alone, the carbazole is largely undissolved at the beginning of the reaction with nitrous acid, and nitrosation tends to be less complete, giving lower results. When a mixture of acetic acid with a small proportion of acetic anhydride was used in an effort to make the nitrosation more complete, by removal of water produced in the reaction itself, the opposite result was found and was evidently due to preferential acetylation of the carbazole. An increase in the proportion of sodium nitrite with a corresponding diminution in the weight of specimen analysed effected no improvement.

In two blank experiments sodium nitrite was decomposed first in acetic acid and then in the same volume of a mixture of chlorobenzene and acetic acid (in equal volumes—the proportions finally adopted). It was found that the nitric oxide collected in the latter case corresponded stoichiometrically more closely to the sodium nitrite taken (97·0 and 98·2 per cent). and it is supposed that this is due to a lessened solubility of nitric oxide in the mixture. In these estimations the reaction mixture was heated finally to 50° C. Choice of 40° C. as the temperature to be adopted was made because in an actual carbazole estimation on Specimen B in which the temperature was raised to 85° C., the nitrosate visibly decomposed with evolution of gas. The correction for dissolved and unswept nitric oxide is not large if the mixture is heated to 40° C., which is a convenient temperature to standardise.

When the amount of acetic acid was reduced to 3 ml. in 70 ml. of chlorobenzene (an amount of acid which is still 10 mols. per mol. of sodium nitrite), reaction between it and the sodium nitrite was impracticably slow.

Summary

It has been shown that crude and refined carbazole specimens can be analysed by quantitative nitrosation with convenience and accuracy. The uncertainty can be reduced to ± 0·6 per cent. on an individual estimation with a purified carbazole specimen, and less, ± 0·2 per cent. (calculated on the sample), with a crude specimen containing about 30 per cent., if a number of detailed precautions are taken.

Thanks are due to Mr. D. W. Milner of Yorkshire Tar Distillers Ltd. for the sample of crude carbazole.

The work described above has been carried out as part of the research programme of the Chemical Research Laboratory, and this paper is published by permission of the Director of the Laboratory.

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June, 1948

Notes

CALCIUM TARTRATE CRYSTALS IN PROCESSED CHEESE

To investigate a complaint that a certain type of cheese was being sold containing "bits of glass," a specimen was obtained from a "5-lb." block of cellophane-wrapped imported processed cheese; and on making a cross-cut with a knife, at least one of the particles in question was usually visible on the newly-exposed surface. The particles were immediately recognisable as crystals and were remarkably perfect as to the facets; but all had milky opaque spots at or near the centre due to inclusions. The crystals were fairly uniform in size, and their over-all dimensions were about 0·9 mm. × 0·9 mm. × (1·5 to 2 mm.). Fig. 1 gives the side and end view of a well developed crystal. The almost square end-view was typical of every crystal seen, but the development of the pyramidal facets varied, so that the edge corresponding to *ab* could be long or short. The small triangular facets *w*, *x*, *y*, *z*, placed alternately at the two ends of the crystal, were always seen and were relatively larger in the smaller crystals. The inclusions, Fig. 2, were well shown by immersing a small crystal in clove oil in a hollow-ground slide. The crystals were found to contain tartrate and calcium.

The 11th World's Dairy Congress, 1937, recommended that no more than 3 per cent. of emulsifying salts should be permitted in processed cheese.[1] A standard of "not more than 3 per cent. of harmless emulsifying agents" is in force in New South Wales.[2] For the purposes of the Processed Cheese-Order, 1942, processed cheese was defined as a cheese which after manufacture has been heated and to which an emulsifying agent has been added. Under the Labelling of Food Orders 1944 and 1946, processed cheese is exempted from the requirements as regards declaration of ingredients. J. R. Nicholls[3] and H. E. Cox[4] mention disodium phosphate, sodium citrate and sodium potassium tartrate as being the commonest emulsifiers.

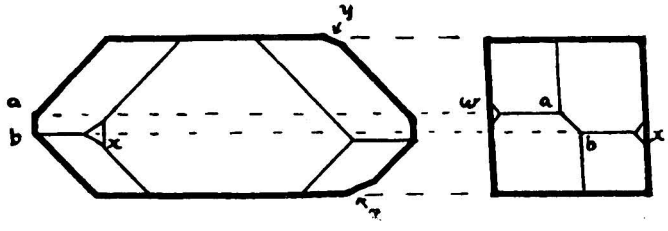

Fig. 1

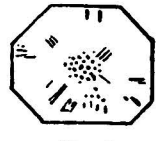

Fig. 2

In the present instance it appears likely that a tartrate has been added as an emulsifier during manufacture and that it has reacted with the calcium present in the residual whey incorporated in the cheese. Calcium tartrate crystallises well on the microscope slide in prismatic crystals, which have long been known as a micro-test for calcium or tartrates.

The inclusions lend some support to the view that the crystals may have grown *in situ* in the cheese, and the perfection of the facets would indicate slow growth. The presence of these crystals is to be regarded in my view as a blemish or defect in quality, but would not render the cheese injurious to health.

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January, 1947

A DIAPHRAGM PUMP FOR AIR AND OTHER GASES

In microchemical operations a stream of low-pressure air finds numerous applications, *e.g.*, to assist evaporation,[1] to free solutions from dissolved gases,[2] and as a stirring agent in micro-titrations.[3] For the small rates of air flow required for such purposes, the electrically-operated diaphragm pump is well suited. Such devices have long been used in the aeration of aquaria, and the like; the modification here described is inexpensive and of simple construction.

As shown in Fig. 1, the device is actuated by an electric bell movement. By unscrewing the pump mechanism retaining screw A and then replacing the gong, the bell may be restored to its original form. The pump assembly, B, is constructed mainly from a thistle funnel and is held by its stem in clamp C. The latter is of sheet metal and lined with sheet rubber or cork. Stretched over the mouth of B and secured

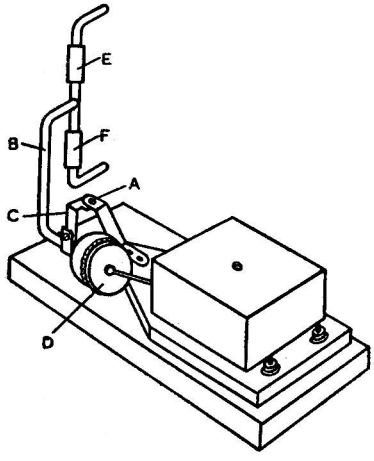

Fig. 1

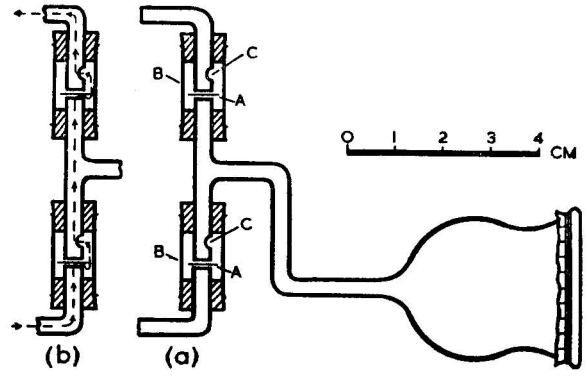

Fig. 2

by a few turns of thread is a diaphragm, D, of sheet rubber cut from an old cycle inner tube. The position of B in the clamp is adjusted so that the centre of the diaphragm is sharply depressed when the hammer of the bell moves inwards. Air is then forced out through upper valve E, and the elasticity of the diaphragm causes it to follow the hammer on the return stroke, thus drawing in air through lower valve F. The cycle of operations is repeated as long as the current flows, so that a virtually continuous stream of air passes through the apparatus.

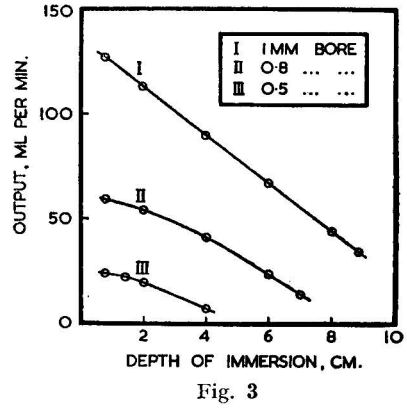

Fig. 3

Details of the pump mechanism are shown at (*a*) in Fig. 2. Valves A A are discs of thin sheet rubber cut by means of a wetted corkborer and are about 1 mm. smaller in diameter than the bore of valve chambers B B. Owing to the high impulse frequency, the valve-lift should be small; about 1 mm. is suitable. A manometer attached to the pump outlet is useful in determining the most suitable lift. Unidirectional flow of air, as shown at (*b*) by broken lines, is secured by blowing small holes C C in the valve-stops.

Performance naturally depends on the power of the bell mechanism and on various other factors. Results shown in Fig. 3 are merely typical and were obtained by measuring the rates of delivery of free air from various capillaries immersed in water. The delivery may be controlled by means of a screw clip or a fine-control stopcock.[4]

Another use for the pump is for the circulation of gases in a closed system; no metal comes into contact with the gases and leakage, other than by diffusion through the rubber, cannot occur. Provided the resistance is not too great, the device also functions effectively as an aspirator. We have used it in this way for the generation of hydrogen sulphide for micro-qualitative analysis.[5]

To produce a pressure higher than that obtainable with the diaphragm pump the complete valve assembly (*b*, Fig. 2) may be attached by its side tube to vent tube H of the micro-stirrer unit previously described.[6] In this manner pressures up to about 50 cm. of water are obtainable.

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September, 1947

THE COLORIMETRIC DETERMINATION OF TRACES OF BISMUTH IN LEAD

COLORIMETRIC methods for the determination of traces of bismuth in lead have been described by Robinson[1] and by Zischkau.[2] Robinson showed that his method, which involves removal of most of the lead as chloride,

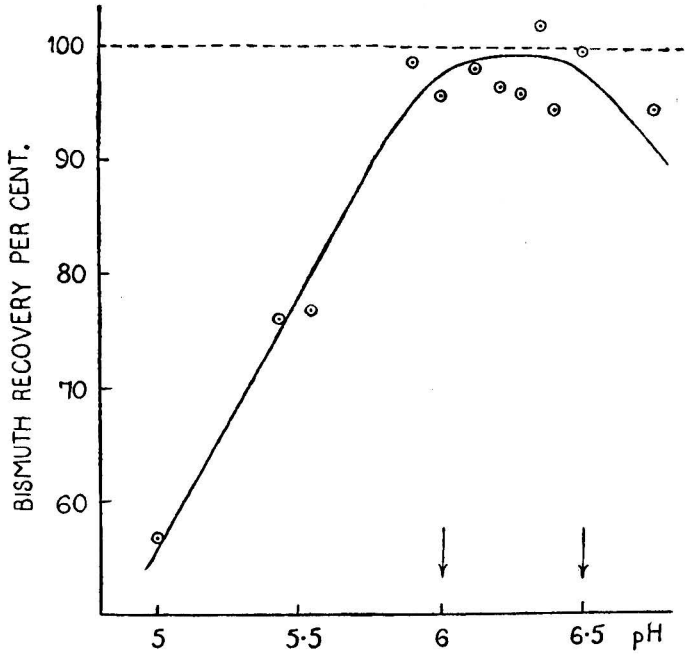

Fig. 1. **Effect of *p*H of solution before filtering on the recovery of bismuth, showing optimum *p*H range.**

separation of the bismuth by co-precipitation with ferric hydroxide and final colorimetric determination as iodide, gave satisfactory recovery of bismuth added to pure lead in amounts from 0·0005 per cent. to 0·002 per cent. Zischkau has described two methods: one, which is applicable to lead containing from about 0·003 per cent. to 0·01 per cent. of bismuth, is open to serious criticism because it involves separation of lead as sulphate, and was found by the present authors to be unsatisfactory; the other, for quantities of bismuth from about 0·005 to 0·1 per cent., uses thiourea for final colorimetric determination and is simple and direct, the lead not being removed.

When Robinson's method and Zischkau's second method were applied, using the Spekker photoelectric absorptiometer, to samples of lead used in chemical plant discordant results were obtained (Table I). In Robinson's method difficulty was experienced in adjusting the reaction of the solution, prior to filtering off the ferric hydroxide, owing to a blue colour obscuring the precipitated lead hydroxide acting as indicator. In establishing conditions for the separation of bismuth with ferric hydroxide, Robinson used the equivalent of only 0·0015 per cent. of bismuth but by determinations on lead with known amounts of bismuth added it

was found by the present authors that Robinson's method is not entirely reliable for amounts of bismuth higher than about 0·002 per cent., low recoveries often being obtained.

The effect of *p*H at this stage was studied by making determinations on lead with added bismuth equivalent to 0·0045 per cent., the ferric hydroxide being filtered off from solutions of various *p*H values determined by glass electrode. Fig. 1 shows that the optimum *p*H range is 6 to 6·5. Following Robinson's original method it was found that the *p*H could be as low as 5 and in such cases the filtrate was brown and on standing several hours deposited a flocculent precipitate containing more bismuth. However, even at *p*H 5 satisfactory recovery of 0·0045 per cent. of bismuth was obtained if the precipitated ferric hydroxide was coagulated by boiling before filtration, the filtrate then being colourless and yielding no precipitate on standing.

TABLE I

Sample	Zischkau's method Bi %	Robinson's method	
		Original Bi %	Modified as suggested Bi %
1	0·003	0·0025	0·0035
2	0·0033	0·0015	0·003
3	0·0025	0·002	0·0023

It appears therefore that for more than about 0·002 per cent. of bismuth the solution should be boiled before filtration or in cases where coloured solutions are encountered the *p*H should be previously adjusted to from 6 to 6·5, and that in any event by adjusting to this *p*H range correct results may be obtained on material containing up to 0·0045 per cent. of bismuth without repeating the determination on a smaller sample.

From Table I it is seen that results by the modified method agreed satisfactorily with those by Zischkau's method.

Thanks are due to the directors of May and Baker, Ltd., for permission to publish these results.

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E. J. BALLARD
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TITRIMETRIC ESTIMATION OF FLUORINE

A NEW method for the titrimetric estimation of micro-quantities of fluorine was suggested by Milton, Liddell, and Chivers in 1942.[1] It involved the use of the sodium salt of sulpho-dichlorohydroxy dimethylfuchsin dicarboxylic acid (Solochrome Brilliant Blue B.S., Colour Index 723) in place of the alizarinsulphonate indicator normally used.

It was claimed that the method proposed allowed of a much sharper endpoint and gave a higher degree of precision than the alizarin technique.

Owing to the fact that the stocks of the new indicator soon became exhausted and it was withdrawn from the I.C.I. dyestuff catalogue, many workers anxious to utilise the method were unable to obtain supplies.

A dyestuff of similar composition is now made available by the Geigy Company under the name of Chrome Azurol S.[2] This indicator has been extensively tried out in the writer's laboratory for the past year and has been found to be quite as satisfactory as the original Solochrome Blue in micro-fluorine determinations. The method has been applied to the estimation of fluorine in atmospheres, soils, waters, bones and teeth, foodstuffs, vegetation including grass, blood, and urine, and it is confirmed that exceedingly precise results are obtainable.

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R. F. MILTON
October, 1948

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

Food and Drugs

South African Fish Products. Part XXVI. Application of the Fitelson Method of Squalene Determination to some Marine Oils. M. L. Karnovsky and W. S. Rapson (*J. Soc. Chem. Ind.*, 1947, **66**, 124–125)—The method described by Fitelson (*J. Assoc. Off. Agric. Chem.*, 1943, **26**, 499; 1945, **28**, 282: *cf.* Drummond and Thorbjarnarson, *Analyst*, 1935, **60**, 23) for determining unsaturated hydrocarbons in the unsaponifiable matter of fats has been applied to several marine oils.

Method—The unsaponifiable matter was separated by the S.P.A. method, four extractions being made. For the estimation of squalene, the directions of Fitelson were followed exactly, except that 2 g. of oil usually provided sufficient unsaponifiable matter for at least one determination. The following quantities gave satisfactory results in an alumina column 10 × 0·8 cm.:

Unsaponifiable fraction high in content of	Weight of material used per chromatogram	Volume of reagent used
Fatty alcohols	Not more than 120 mg.	10 ml. probably sufficient
α-Glyceryl ethers	" " " 200 "	10 " " "
Sterols	" " " 200 "	10 " " "
Vitamin-A	About 50 to 60 mg.	10 " " "
Unsaturated hydrocarbons	" 25 to 35 "	15 to 20 ml.

With oils containing considerable amounts of vitamin A some adjustment of the amount of material used was necessary in order to retain a rapidly moving yellow band. The error involved in allowing the yellow band to pass into the filtrate is small where the method is used to estimate more than traces of unsaturated hydrocarbon.

A selection of the results obtained is given below.

Specific name	Common name	Oil from	Unsaponifiable matter, per cent.	Per cent. unsaturated hydrocarbon calculated as squalene in oil
Physeter macrocephalus	Sperm whale	head	47·28	0·12
Congermuraena australis	Cape eel	liver	11·80	0·18
Lophius piscatorius	Angler fish	"	2·62	0·12
Galeorhinus canis	Soup-fin shark	"	2·68	0·05
Carcharodon carcharias	Man-eater	"	36·13	33·55
Cetorhinus maximus (1)	Basking shark	"	47·73	39·31
	" "	kidney	45·90	1·86
(2)	" "	liver	32·75	24·52
(3)	" "	"	35·32	28·82
Chimaera monstrosa	Common chimaera	"	74·80	62·83
Arctocephalus pusillus	Seal	"	29·41	0·50

H. K. DEAN

Component Acids and Glycerides of Neat's Foot Oil. T. P. Hilditch and R. K. Shrivastava (*J. Soc. Chem. Ind.*, 1948, **67**, 139–142)—The mixed acids of a sample of neat's foot oil were subdivided into three groups by low temperature crystallisation, as follows—15·2 per cent. (iodine value 2·7) were obtained by crystallisation from 10 per cent. solution in ether at −40° C.; 57·1 per cent. (iodine value 85·2) were obtained from the remainder by crystallisation from 10 per cent. solution in acetone at −60° C.; the balance, 27·7 per cent., had iodine value 101·0. Each group was examined by the ester-fractionation method and the weight-percentages of the component acids were calculated as: myristic 0·7, palmitic 16·9, stearic 2·7, arachidic 0·1, tetradecenoic 1·2, hexadecenoic 9·4, oleic 64·4, octadecadienoic 2·3, octadecatrienoic 0·7, and unsaturated C_{20-22} acids 1·6. Constants of the oil were: saponification equiv. 286·3, iodine value 73·3, free fatty acids (as oleic) 0·7 per cent., unsaponifiable matter 0·3 per cent., and n_D^{40} 1·4610. The component glycerides were studied after partial separation by low-temperature crystallisation from acetone. The percentages of their constituents were approximately: palmitodiolein 35, hexadecenodiolein 23, polyethenoid-diolein 8, oleopalmitostearin 7, and probably not much more than 10 of triolein, with minor proportions of stearodiolein, palmito-polyethenoid-oleins, and hexadeceno-polyethenoid-oleins. The lubricant properties of the oil are probably not due to the small amount of triolein present, but may be connected with the specific *mixed* unsaturated glycerides present, *i.e.*, to hexadecenodiolein and perhaps also to the di-oleoglycerides in which the third acyl group is a polyethenoid member of the C_{18}, C_{20} (or C_{22}) series. The presence of fairly large proportions of hexadecenoic acid in neat's foot oil had not been previously noted.

E. B. DAW

Titration of Hyoscine, Hyoscyamine, and Atropine with Picric Acid in Chloroform: Separation and Fractionation of the Picrates. E. M. Trautner, O. E. Neufeld, and C. N. Rodwell (*Aust. Chem. Inst. J. Proc.*, 1948, **15**, 55–61)—Hyoscine, hyoscyamine, and atropine together with the related alkaloids and free bases associated with them in solanaceous plants can be titrated in chloroformic solution with picric acid in chloroform, dimethyl-amino-azo-benzene (butter-yellow) being used as an indicator. The colour

change is from yellow to orange; it is sharp and similar to that of methyl orange in aqueous solution. During the titration, or shortly afterwards, hyoscine picrate is precipitated, and if trichloroethylene is added to the chloroform solution, the solubility of hyoscine picrate is sufficiently reduced for it to be quantitatively precipitated while hyoscyamine and atropine picrates remain in solution. The optimum mixture of chloroform and trichloroethylene is two to one and working with such a solvent, mixtures of hyoscine and hyoscyamine (or atropine) varying in proportions from 9 : 1 to 1 : 9 have been assayed with a maximum error of 10 per cent. of the amount of the constituent present in smaller amount.

Procedure—Shake 60 g. of finely-powdered leaf with 1000 ml. of industrial methylated spirit for several hours, filter the extract, and evaporate an aliquot to the consistency of a thick syrup. To the cold residue add 30 ml. of 3 per cent. phosphoric acid, allow the syrup to disintegrate to a well-defined precipitate, and filter. Shake the filtrate with successive small quantities of chloroform, wash the bulked chloroform extracts with a small amount of the 3 per cent. phosphoric acid solution, reject the chloroform, and add the acid washings to the bulk of the acid solution. Make the acid solution alkaline to phenolphthalein test paper by adding aqueous ammonia solution and extract with successive portions of chloroform. Mix the chloroformic extracts, wash with a few ml. of water, and evaporate to dryness in a current of air. At this stage make sure that the residue is entirely free from traces of chloroform or other solvents. Add 20 ml. of light petroleum (b.p. 90° to 110° C.), heat nearly to boiling, decant the supernatant liquid, and repeat with two successive portions of 10 ml. of light petroleum.

Mix the nearly water-white light petroleum fractions and extract quantitatively with successive portions of 3 per cent. phosphoric acid. Add aqueous ammonia solution to the bulked acid extracts until the mixture is alkaline to phenolphthalein test-paper and again extract with succesive quantities of chloroform. Adjust the volume of the final chloroform solution, either by dilution or by evaporation, to obtain an approximately 0·05 *N* solution of total alkaloids.

Titrate slowly with 0·05 *N* picric acid in dry chloroform to an orange-yellow colour, using dimethyl-amino-azo-benzene as indicator, and from the volume of 0·05 *N* picric acid used calculate the total alkaloids in the aliquot.

Add a volume of pure trichloroethylene equal to half the volume of the combined chloroformic solutions, allow to stand overnight, and filter. Wash the residue in the filter with chloroform, dry at 100° C., weigh, and calculate the proportion of hyoscine in the aliquot.

If the amount of total alkaloids in the sample is not even approximately known, extract the bulked light petroleum fractions with a known amount of standard acid, and titrate back with aqueous standard alkali to determine this concentration. Make the solution alkaline and extract the alkaloids as previously described. A. H. A. Abbott

Colorimetric Assay of Digitoxin. A. T. Warren, F. O. Howland, and L. W. Green (*J. Amer. Pharm. Assoc.*, 1948, **37**, 186–188)—The colorimetric method devised for the determination of digitoxin can be applied to tablets containing the drug. The procedure depends on the formation of a fugitive purple colour when digitoxin reacts with sodium β-naphthaquinone-4-sulphonate in alkaline solution, and addition of acetic acid at the time of maximum colour development to change the colour to yellow and to stabilise it.

Procedure—Prepare a chloroformic solution of the sample such that 1 ml. may be expected to contain approximately 20 μg. of digitoxin. Evaporate 10 ml. of the solution to dryness in a small flask under a gentle stream of air. Add 0·1 ml. of chloroform, swirl the flask to wet the residue, and then add 4 ml. of aldehyde-free ethyl alcohol and 0·5 ml. of 0·05 *N* sodium hydroxide. Place the flask in boiling water and exactly 1 min. later add, as rapidly as possible, 1 ml. of a reagent containing 0·024 per cent. of sodium β-naphthaquinone-4-sulphonate and 0·024 per cent. of sodium sulphite in water. Mix well and, exactly 1·5 min. after the addition of the reagent, add 0·5 ml. of 13 per cent. acetic acid in aldehyde-free alcohol. Remove the flask from the bath immediately, cool, and dilute to 25 ml. with alcohol. Measure the transmission of the solution within 2 hr. in a photoelectric colorimeter and make a blank determination on the reagents. Calculate the proportion of digitoxin in the sample by reference to a curve prepared by submitting aliquots of a standard solution of pure digitoxin in chloroform to the test procedure.

Procedure for tablets—Grind 20 tablets to fine powder and weigh a sample approximately equal to 10 tablets. Treat the powder with 20 ml. of boiling chloroform, filter the mixture, and wash the residue several times with warm chloroform. Combine the filtrate and washings, dilute to 50 ml. with chloroform, transfer 10 ml. of the solution to a small flask, and continue the assay as previously described. The above dilution is for tablets containing 0·1 mg. of digitoxin.

A. H. A. Abbott

Biochemical

Iodimetric Method of Estimation of Penicillin. A. M. Wild (*J. Soc. Chem. Ind.*, 1948, **67**, 90–92)—The method of Alicino (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 619; *cf. Analyst*, 1947, **72**, 68) has been studied and the following conclusions have been drawn.

(1) The degree of iodination is a linear function of temperature.
(2) The concentration of iodine has only a slight effect on absorption.
(3) The concentration of potassium iodide in the iodine solution used exerts a noticeable effect. That used by the author was 0·2 per cent.
(4) The *p*H should be between 6 and 7 and adjusted again to within that range before the final thiosulphate titration.

(5) The time of standing of each unhydrolysed sample in the presence of iodine should be carefully controlled according to its impurity content.

(6) Within the limits 0·1 to 0·33 *N*, neither the concentration nor the quantity of alkali has any effect on the subsequent titration. The reaction is complete within 15 min. and setting aside for more than 30 min. has no effect on the final result.

(7) Because of the different iodine absorption by impurities before and after alkali treatment, it is doubtful whether the method could be adapted to low-grade penicillin.

METHOD—*Procedure*—These conclusions lead to the following method of analysis being recommended.

Stock solutions—(1) 0·1 *N* Sodium thiosulphate containing also 0·2 g. of $Na_2CO_3.10H_2O$ per litre, and accurately standardised. (2) 0·1 *N* Iodine containing 2 per cent. of potassium iodide. (3) Aqueous phosphate buffer (I), *p*H 7·0; 2·835 g. of $NaH_2PO_4.2H_2O$; and 9·76 g. of $Na_2HPO_4.12H_2O$ per litre. (4) Aqueous phosphate buffer (II), *p*H 6·24; a mixture of 4 volumes of *M*/15 KH_2PO_4 and 1 volume of *M*/15 Na_2HPO_4. (5) 1 Per cent. starch solution in water.

Prepare 250 ml. of 0·01 *N* sodium thiosulphate by diluting the 0·1 *N* sodium thiosulphate with distilled water, free from carbon dioxide. Prepare 0·01 *N* iodine by pipetting 25 ml. of 0·1 *N* iodine into a 250-ml. graduated flask containing approximately 125 ml. of phosphate buffer (II), and diluting to volume with distilled water.

Procedure—Dissolve not less than 50 mg. of the sample in phosphate buffer (I) in a dry 250-ml. conical flask to give not more than 900 to 1000 units per ml. (Volume of solvent = *V*.) Pipette 5 ml. of the penicillin solution into each of two 4-oz. stoppered bottles *A* and *B*. Into *A* pipette 10 ml. of 0·01 *N* iodine, set aside in the dark for *exactly* 5 min., and titrate immediately with 0·01 *N* sodium thiosulphate, adding 2 ml. of starch solution.

Into *B* pipette 1 ml. of *N* sodium hydroxide and set aside for 30 min. Neutralise with 1 ml. of *N* hydrochloric acid. Add 10 ml. of 0·01 *N* iodine and set aside in the dark with another bottle (*C*) containing water and a thermometer. After 30 min., take the temperature (t°) in the bottle *C*, and immediately titrate the contents of *B* with 0·01 *N* sodium thiosulphate, adding 2 ml. of starch solution near the end-point.

Calculation—

$$\text{Percentage of total penicillin} = \frac{(\text{Titre difference in } A \text{ and } B) \times V \times 100 \times \text{thiosulphate factor}}{\{2{\cdot}31 + 0{\cdot}015\ (t - 20)\}\ (\text{weight of sample}) \times 5}$$

For samples in which the relative proportion of penicillin G is high, a fair estimate of the potency is given by:—

$$\frac{(\text{Titre difference in } A \text{ and } B) \times V \times 1660 \times \text{thiosulphate factor})}{\{2{\cdot}31 + 0{\cdot}015\ (t - 20)\}\ (\text{weight of sample}) \times 5}$$

For precise results, the relative proportions of the penicillins must be known.

For maximum accuracy, the following points must be observed.

(1) Starch solution and 0·01 *N* sodium thiosulphate must be freshly prepared each day.

(2) A 10-ml. burette graduated in 0·02 ml. should be used.

(3) During the standing periods the temperatures of *A* and *B* should be within a few degrees of one another.

(4) Titrations of blank determinations should be done rapidly, especially when the penicillin is impure.

(5) The temperature in *B* while it is set aside should not vary more than ± 1° C. If fluctuations of more than ± 2° C. are encountered, thermostatic control at 25° C. is advised.

(6) The 5-ml. aliquot for assay must contain 4500 ± 500 units.

The reproducibility of the results obtained by this method is about ±1·5 per cent.

H. B. JOHNSON

New Colour Reactions and a New Method for the Photometric Determination of Vitamin B_1. C. S. Runti (*Intern. Z. Vitaminforschung*, 1948, 19, 282)—This work originated as a study of the possibility of diazotising vitamin B_1. By reaction of a diazotised solution of aneurine hydrochloride with various amino compounds in presence of alkali, typical azo colours were obtained. To ascertain if these colours were due to a coupling of the diazo derivative of the vitamin with the amines or to the coupling of the diazo derivatives of the amines with the vitamin, the experiments were repeated after blocking the amino group of the vitamin by treatment with acetyl chloride. The colours were obtained as before and, similarly, the same colours were produced if the amino compounds were first diazotised, the excess of nitrous acid was removed by urea, the solution of vitamin added and the liquid made alkaline by adding potash. It concluded therefore that the vitamin is not itself diazotised under these conditions but reacts with the diazo derivatives of the amines employed. These included sulphonamides, *p*-aminobenzoic acid, and *p*-aminophenylarsonic acid. The reaction with 2(*p*-aminobenzene sulphonamido)pyridine was found to be particularly suitable for the photometric estimation of the vitamin. The concentration of the sodium nitrite solution employed has an important influence on the stability of the colours obtained. With the use of dilute (0·5 per cent.) solution these remain unchanged for a considerable time.

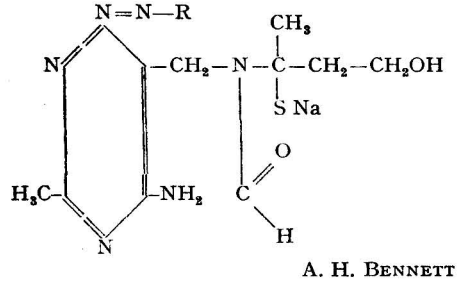
Estimation of vitamin B_1. Preparation of a standard curve—For this purpose use a Pulfrich step photometer and make a series of measurements with successive dilutions of a standard solution of aneurine hydrochloride containing, in 3 ml., 375 μg. of the vitamin. Measure 3 ml. of this solution or of its dilutions into a tared 25-ml. flask. Add 1 ml. of diluted hydrochloric acid (1 : 4) and 1 ml. of

0·5 per cent. sodium nitrite solution. Add 3 ml. of a saturated aqueous solution of 2(*p*-aminobenzene sulphonamido)pyridine, shake for 1 to 2 min. at room temperature and make alkaline by adding 1 ml. of a 33 per cent. solution of caustic potash. Allow to stand for 5 min., in which time the colour, at first orange, changes to pink. Dilute to the mark with water and after allowing to stand for 20 min., take readings in the photometer, 20 mm. cuvettes and the filter S.53 being used.

This procedure is specially adapted for the determination of the vitamin in pharmaceutical preparations. For biological material, a different standardisation would be required.

Of the other water-soluble vitamins, only ascorbic acid interferes with the results and this can be eliminated by oxidation with iodine.

From experiments with other substances containing a thiazole or pyrimidine nucleus the author concludes that the latter and not the former takes part in the reaction with the diazo compounds, and proposes the following general formula for the azo colours.



A. H. BENNETT

Colorimetric Estimation of Pregnandiol in Urine. H. S. Guterman and M. S. Schroeder (*J. Lab. Clin. Med.*, 1948, **33**, 356–365)—METHOD —*Preparation of special reagent*—Dissolve 8 g. of sodium hydroxide pellets in 100 ml. of absolute methyl alcohol, allow to stand for a short time, and remove the precipitated carbonate by filtration. Determine the sodium hydroxide concentration of the solution by titration with 0·1 *N* sulphuric acid and adjust to a concentration of 2 per cent. with absolute methyl alcohol. Prepare the solution afresh each week.

Procedure—Boil 100 ml. of urine, 50 ml. of toluene, and 10 ml. of concentrated hydrochloric acid under a reflux condenser for 15 min., cool to room temperature, and transfer the mixture to a separating funnel. Allow to separate and, if an emulsion is formed, break it by addition of 1 or 2 drops of a detergent solution. Discard the lower (urine) layer and wash the toluene and any emulsion twice with 15 ml. of 0·1 *N* sodium hydroxide and then twice with 15 ml. of water. Transfer the washed toluene to a flask and boil off any dissolved water. When the toluene is boiling steadily add 10 ml. of 2 per cent. sodium hydroxide solution in absolute methyl alcohol and continue boiling until approximately half the original volume remains and a granular precipitate has formed. Filter hot, and if the filtrate is orange, pink or brown in colour, again add 10 ml. of 2 per cent. sodium hydroxide solution in absolute methyl alcohol and heat with more toluene until the volume has again been reduced by one half. When the filtrate is yellow, or yellowish-green, wash the residue in the filter with 15 ml. of hot toluene and evaporate the combined filtrate and washings to dryness. Remove the last traces of toluene by a stream of air.

Add 5 ml. of acetone to the residue, warm until dissolution is complete, slowly add 20 ml. of 0·1 *N* sodium hydroxide, and boil the mixture for 3 min. Cool, allow to stand at 5° C. for 1 hr., filter, and wash the residue with 15 ml. of water. Dissolve the residue in 10 ml. of hot absolute ethyl alcohol, evaporate the solution to dryness, and dissolve the residue in 10 ml. of concentrated sulphuric acid. Allow the solution to stand for 1 hr., dilute an aliquot to 5 ml. with concentrated sulphuric acid, and read the colour in a spectrophotometer at 430 mμ., concentrated sulphuric acid being used as a blank.

Prepare a standard solution of pregnandiol in absolute ethyl alcohol (100 μg. per ml.), evaporate aliquots containing 10 to 100 μg. of pregnandiol to dryness, dissolve the residues in 5-ml. quantities of concentrated sulphuric acid, allow to stand for 1 hr., and read the colours in a spectrophotometer. Plot the concentrations against the logarithms of the percentage absorptions and use the curve to determine the concentration of pregnandiol in the sample.

Recovery experiments indicate that the method has an average accuracy of 95 per cent., and androsterone was found not to interfere with the colour reaction. A. H. A. ABBOTT

Delta-Tocopherol. I. Its Isolation from Soyabean Oil and its Properties. M. H. Stern, G. D. Robeson, L. Weisler, and J. G. Baxter (*J. Amer. Chem. Soc.*, 1947, **69**, 869–874) —α-, β-, and γ-tocopherols give substantially complete colour formation in 2 min. in a modified Emmerie and Engel assay method (see the next abstract) with only a small increase in colour intensity when the time is increased to 10 min. The mixture of tocopherols in the distillate from soyabean oil, however, showed an increase of about 10 per cent. The substance responsible for this has been separated and is a tocopherol, *viz.*, 8-methyltocol, differing from α-, β-, and γ-tocopherols in having only one methyl group in the aromatic ring of the chroman nucleus. It is the first monomethyltocol to be isolated from natural sources. It constitutes about 30 per cent. of the mixed tocopherols of soyabean oil (Weisler, Robeson, and Baxter, *infra*) and 5 per cent. of those in wheat germ oil (Kascher, unpubl.) and occurs in cottonseed and peanut oils (Weisler *et al.*, *infra*).

Properties—δ-Tocopherol is unique among the known tocopherols in giving with the ferric chloride and dipyridyl reagent a slow steady increase in colour intensity after the initial rapid oxidation is completed (see the next abstract). Of the tocopherols so far isolated it is the most active antioxidant for vitamin A and carotene *in vitro*, the order of decreasing activity being δ-, γ-, β-, α-tocopherol.

δ-Tocopherol reacts with diazo reagents and the

reaction provides a method whereby it and also γ-tocopherol may be estimated in mixtures (see the next abstract).

Oxidation with 1·7 molecular proportions of gold chloride in ethanol for 45 min. according to the procedure of Karrer and Geiger (*Helv. Chim. Acta*, 1940, **23**, 455) yielded a reddish oil having the properties of a *p*-quinone ($E^{1\%}_{1\,cm.}$ at 257 mμ = 222) that contained 12 per cent. of unchanged δ-tocopherol. When δ-tocopherol (0·055 g.) and silver nitrate (0·15 g.) were heated with 1 ml. of ethanol at 50° C. for 1 hr. and the product was extracted with ether, a reddish oil having the ultra-violet light absorption properties of an *o*-quinone ($E^{1\%}_{1\,cm.}$ at 435 mμ = 15·2) and containing 6·5 per cent. of unchanged tocopherol was obtained. When δ-tocopherol (0·03 g.) in 10 ml. of ethanol is treated slowly with 22 ml. of nitric acid and, after shaking, is allowed to stand for 2·5 hr. at room temperature the orange-yellow pigment formed has an extinction coefficient at 373 mμ. of 59. Oxidation of α-, β-, and γ-tocopherols with nitric acid yields *o*-quinones. The different behaviour of δ-tocopherol with nitric acid may possibly provide a method for its determination in mixtures with other tocopherols. Possibly the mixture might be oxidised with nitric acid and the δ-tocopherol determined spectrophotometrically by measuring the intensity of the absorption at 373 mμ.

The experimental determination of the structure of δ-tocopherol is described. A. O. JONES

Delta-Tocopherol. Assay of Total Tocopherols in Mixtures Containing Delta-Tocopherol. M. H. Stern and J. G. Baxter (*Anal. Chem.*, 1947, **19**, 902–905)—Delta-tocopherol is distinguished from α-, β-, and γ-tocopherols by the fact that it gives more colour on a molecular basis with the ferric chloride and α,α′-dipyridyl reagent of Emmerie and Engel (*Rec. Trav. Chim.*, 1938, **57**, 1351; *Analyst*, 1939, **64**, 216), so that its presence in a mixture of tocopherols results in assay values that are too high.

The reagent of Emmerie and Engel has been used in acetic acid solution and in ethanol solution. The assay of mixtures of tocopherols by means of the reagent in acetic acid is unsatisfactory because the intensity of colour produced with the individual tocopherols is not a linear function of the concentration, and lack of linearity makes it difficult to standardise a method which is to be applied to mixtures in which the proportion of the various tocopherols is unknown. Also the rate of oxidation of the individual tocopherols by the acetic acid reagent varied, so that a time could not be selected in which all the tocopherols gave about the same colour intensity, and it would thus be difficult to use the reagent for assaying mixtures of tocopherols except where the composition of the constituents was known and it was possible to calibrate the method with pure tocopherols having the same composition.

When the reagent is used in ethanol solution the rate of oxidation of α-, β-, and γ-tocopherols is rapid and the intensity of the colour produced is substantially the same when allowance is made for differences in the molecular weights. Colour formation with α-, β-, and γ-tocopherols is complete in 2 min. with only a slight increase when the reaction time is extended to 10 min. With δ-tocopherol, however, extension of the time of reaction causes an increase of 30 per cent. in the colour, and thus time becomes a critical factor in mixtures containing this tocopherol. For mixtures of unknown composition the best reaction time is 2·5 min., since colour formation for three of the tocopherols is then complete and the colour intensities for all four are most nearly the same. If this time is taken as the basis of an assay procedure for mixtures containing δ-tocopherol, the assay may be standardised either with pure natural α-tocopherol or preferably with a 50 to 50 mixture of α- and γ-tocopherols.

Procedure—Separate solutions of ferric chloride (0·1 g. of $FeCl_3.6H_2O$ per 100 ml.) and α,α′-dipyridyl (0·25 g. per 100 ml.) in purified absolute ethanol are prepared and stored in amber glass bottles, preferably painted black, fresh solutions being made every 2 weeks. To a 1-ml. aliquot of the sample containing 50 to 150 μg. of tocopherol in purified ethanol (for concentrates) or light petroleum (for low potency preparations containing glycerides) in a 2-oz. glass-stoppered bottle painted black add, in order, 1 ml. of the dipyridyl reagent, 1 ml. of ferric chloride reagent, and 22 ml. of purified ethanol, the last from a 50-ml. burette. When 17 ml. have been added, start a clock with a seconds hand while the remainder is being run in, insert the stopper, swirl the mixture gently and set it aside for 2·5 min. A blank liquid previously prepared (1 ml. of ethanol, 1 ml. of each reagent, then 22 ml. of ethanol) is used to adjust the galvanometer of an Evelyn colorimeter to 100 with a No. 520 filter. About 10 sec. before the completion of the 2 5 min. period pour the test solution into the tube in the colorimeter, cover it with a black cap, and take the galvanometer reading at 2·5 min. Determine the tocopherol content by means of a curve relating the L value ($2 - \log G$) to the concentration, which has been prepared either with pure natural α-tocopherol or with a 50 to 50 mixture of natural α- and γ-tocopherols. With galvanometer readings of 25 to 70, L is a linear function of the concentration. The absolute ethanol must be purified by distillation with potassium permanganate and potassium hydroxide through a Vigreux column, and the light petroleum (Skellysolve B, b.p. 64° to 68° C.) by washing with concentrated sulphuric acid, and then with dilute sodium hydroxide and distilling. Excessive exposure of either the sample solution or the blank solution to light must be avoided.

Improved procedure for assay of soyabean oil—A sample of crude soyabean oil was "degummed" by stirring at 40° C. with 2 per cent. by weight of water. The sludge was allowed to separate overnight and the oil was filtered through a bed of sodium sulphate to remove traces of water. To a 10-ml. aliquot of purified light petroleum containing about 0·1 g. of the oil in a conical glass-stoppered centrifuge tube, 2 ml. of 85 per cent. (w/w) sulphuric acid were added and the tube was inverted five times and centrifuged. The supernatant liquid was

decanted into a clean tube, shaken with 5 ml. of 1 per cent. potassium hydroxide solution, and again centrifuged. A 3-ml. aliquot of the liquid, containing about 60 μg. of tocopherol, was placed in a reaction bottle and the solvent was removed in a stream of nitrogen. To the residue were added light petroleum (1 ml.), dipyridyl reagent (1 ml.), ferric chloride reagent (1 ml.) and ethanol (22 ml.). The value of L was determined by the procedure of Baxter *et al.* (*Biol. Symposia*, 1947, **12**, 484), which is substantially that already described but with a 2-min. reaction period. Reference to a calibration curve prepared with a 50 to 50 mixture of α- and γ-tocopherols gave the partly corrected value of 0·176 per cent. for the total tocopherol content of the water-refined oil. A correction was made for the inhibition of colour formation that occurs with vegetable oils and with some mineral oils by the method of Kaunitz and Beaver (*J. Biol. Chem.*, 1944, **156**, 653). A 3-ml. aliquot containing about 60 μg. of pure γ-tocopherol was treated with the dipyridyl reagent, the ferric chloride reagent and ethanol in the usual way and the L value was determined after a reaction time of 2 min. Another aliquot was evaporated to dryness in a stream of nitrogen and a 3-ml. aliquot of the light petroleum sample solution (after purification with sulphuric acid and potassium hydroxide) containing about 60 μg. of tocopherols was added to it and the L value determined. If Lt, Ls, and Lc represent the values of L for the pure tocopherol, the sample solution and the combination of both respectively, the percentage recovery is 100 $Lt/Lc - Ls$. The value of 0·176 per cent. was thus corrected for the inhibition and the value 0·190 per cent. was found for the soyabean oil. Further work suggests that this assay procedure would be improved by the use of the 2·5-min. reaction period in the assay and by the use of a 70 to 30 mixture of γ- and δ-tocopherols in the inhibition determination.

The cause of the inhibition of colour formation occurring with many vegetable oils is not known with certainty. In assaying preparations of low potency a correction for inhibition should be applied and in its determination a mixture of pure tocopherols having approximately the same composition as those in the sample should be used.

A. O. JONES

Delta-Tocopherol. Assay of Individual Tocopherols in Mixtures Containing Delta-Tocopherol. L. Weisler, C. D. Robeson, and J. G. Baxter (*Anal. Chem.*, 1947, **19**, 906–909)—When γ- and α-tocopherols were believed to be the only commonly occurring members of the vitamin E complex, their determination in mixtures was relatively simple and proeedures based on the determination of γ-tocopherol by selective oxidation with silver nitrate or nitric acid or by coupling with diazotised *p*-nitraniline were developed, α-tocopherol being then found by difference from the total tocopherol content as measured by the Emmerie and Engel method (*Rec. Trav. Chim.*, 1938, **57**, 1351; ANALYST, 1939, **64**, 216). Another method was the direct determination of α-tocopherol by selective oxidation with ferric chloride (Hove and Hove, *J. Biol. Chem.*, 1944, **156**, 601). The presence of δ-tocopherol causes considerable error in these methods, since it behaves like γ-tocopherol, giving a colour with silver nitrate and with nitric acid.

The method now presented for determining the individual tocopherols in mixtures of α-, γ-, and δ-tocopherols depends upon coupling duplicate samples with diazotised *o*-dianisidine in alkaline solution. Both γ- and δ-tocopherols have unsubstituted positions in the aromatic ring of the chroman nucleus and couple with the reagent to give red pigments absorbing at 510 mμ. The relative intensity of colour formation for the two tocopherols at different degrees of alkalinity, however, is different, and this difference provides a basis for determining each in mixtures. α-Tocopherol has no unsubstituted position and hence does not couple, but it can be measured by difference from the total tocopherol content determined by the modified Emmerie and Engel procedure of Stern and Baxter (see the preceding abstract).

To prepare *o*-dianisidine dihydrochloride add to 100 g. of crude powdered dianisidine (m.p. 134° to 136° C.) 140 ml. of water and 6 ml. of concentrated hydrochloric acid, stir well, boil gently until dissolution is complete, add 0·5 g. of stannous chloride, boil for 5 min., add 2 g. of decolorising carbon, boil gently for a further 5 min., and filter rapidly by suction through Celite. To the hot filtrate add 50 ml. of concentrated hydrochloric acid, cool in an ice-bath and collect the crystals by suction, washing three times with ethanol and once with ether, and dry the crystals at 40° C. for 1 hr. (m.p. 283° C. with decomposition).

To prepare the diazotised reagent add 6 ml. of concentrated hydrochloric acid to 0·5 g. of *o*-dianisidine dihydrochloride dissolved in 60 ml. of water and then add 12 ml. of 5 per cent. aqueous sodium nitrite solution. Mix thoroughly and, after 5 min., add 12 ml. of 5 per cent. aqueous urea solution. Set the thoroughly mixed reagent aside for 24 hr. at room temperature before using. It is stable for about 10 days.

Procedure—Two coupling reactions are necessary on duplicate samples of the preparation. Dilute a sample containing 50 to 100 μg. of γ- and δ-tocopherols to 3 ml. with absolute ethanol in a 50-ml. glass-stoppered cylinder. Add 7·5 ml. of a 2 per cent. aqueous solution of sodium carbonate monohydrate and 1 ml. of diazotised *o*-anisidine solution, shake the mixture, allow it to stand at room temperature for 5 min., then add 0·5 g. of sodium sulphate and 12 ml. of light petroleum (Skellysolve H). Shake the mixture vigorously three times, allowing the phases to separate completely after each shaking. Prepare a blank liquid by the same procedure, omitting the tocopherols. The small interfacial precipitate that forms does not affect the determination. Transfer the mixture to a separating funnel and discard the lower phase. Dry the light petroleum layer with sodium sulphate and determine its L value (2 — log G) in an Evelyn colorimeter with a No. 250 filter and with the instrument set at 100 for the blank liquid.

For the second coupling, dilute a sample containing 50 to 200 μg. of γ- and δ-tocopherols to

4 ml. with absolute ethanol in a 50-ml. glass-stoppered cylinder, add 2 ml. of a 2 per cent. solution of potassium hydroxide in absolute ethanol and 0·3 ml. of diazotised *o*-dianisidine solution. Shake the mixture, allow it to stand for 2 min., and then add 8 ml. of water, 12 ml. of light petroleum, and 0·5 g. of sodium sulphate. Extract the dye in the manner described for the coupling reaction in sodium carbonate solution, preparing a blank solution in the same manner.

To prepare calibration curves couple the two pure tocopherols at the two alkalinities and plot the L values against the concentrations. The high intensity produced by coupling δ-tocopherol in sodium carbonate solution contrasts with the lower intensity produced by coupling in potassium hydroxide solution. The curves for the dye from γ-tocopherol are intermediate. Finally determine the total tocopherols by the method described in the preceding abstract.

The use of these differences in colour formation to assay γ- and δ-tocopherols is based on two equations relating the intensity of colour formed in the two alkaline solutions to the percentage of individual tocopherols present.

$$L_C^S = (\%\ \gamma)L_C^\gamma/100 + (\%\ \delta)L_C^\delta/100$$

$$L_H^S = (\%\ \gamma)L_H^\gamma/100 + (\%\ \delta)L_H^\delta/100$$

where L_C^γ, L_C^δ, L_H^γ, and L_H^δ are constants representing the L values of pure tocopherols coupled in sodium carbonate and potassium hydroxide solutions and L_C^S and L_H^S represent the observed L values for the total tocopherols in the sample being assayed. The L value is $(2 - \log G)/1{\cdot}9\ C$, where G is the galvanometer reading and C is the number of grams of total tocopherols per 100 ml. in the standard volume used (12 ml.). By substituting the experimentally determined L values and solving the equations, the percentages of γ- and δ-tocopherols are calculated.

The accuracy of the diazo method can often be increased by a preliminary adsorption to separate α- and γ-tocopherols from δ-tocopherol. Only a single calibration curve, preferably prepared by coupling γ-tocopherol in potassium hydroxide solution is then needed. The firmness of adsorption of these three tocopherols on zinc carbonate increases in the order α, γ, δ. By passing a light petroleum solution of mixed tocopherols through a column of zinc carbonate and Celite under controlled conditions, it is possible to collect the α-tocopherol and a portion of the γ-tocopherol in the filtrate, leaving the δ-tocopherol and certain pigments on the column. Application of the diazo reaction to the tocopherols recovered from the filtrate permits calculation of the percentage of the α-compound in the tocopherols of the original sample, since the total tocopherol content of the latter can be determined and the weight of sample adsorbed is known.

A satisfactory column (2·5 × 60 cm.) can be prepared by inserting 50 g. of a 70 : 30 mixture of zinc carbonate and Celite in four portions in the tube, suction being applied from a water pump through a filter flask after each addition. Since the adsorption method depends upon the separation of the α-tocopherol from such interfering reducing compounds as δ-tocopherol, the ratio of tocopherol concentrate to adsorbent which will allow all the α-tocopherol and a portion of the γ-tocopherol but no δ-tocopherol to pass through the column must first be determined. The diazo method is used to establish absence of δ-tocopherol from the filtrate. A 2-g. sample is satisfactory for concentrates having a potency of approximately 30 per cent. of mixed tocopherols. For higher concentrates (60 to 80 per cent.) a 1-g. sample is preferable. First wash the column with 125 ml. of light petroleum, add the sample in 25 ml. of light petroleum and rinse it into the column with another 25-ml. portion of solvent. Draw the solution through the column by gentle suction and wash with 300 ml. of light petroleum. The top of the column must remain covered with solution or solvent at all times after the initial wetting. Follow the course of the washing operation by means of ultra-violet light.

Clean, sharp bands are observed if the packing is satisfactory, and the α-tocopherol is associated with three blue fluorescent bands that pass down the column first and into the filtrate. Evaporate the filtrate on the steam-bath under a stream of nitrogen and weigh the residue. Then assay it for total tocopherols as described in the preceding abstract and for γ-tocopherol by the diazo reaction. The percentage of α-tocopherol in the original sample is then readily calculated. A. O. JONES

Determination of Individual Tocopherols. M. Kofler (*Helv. Chim. Acta*, 1947, **30**, 1053–1070)—δ-Tocopherol (8-methyltocol), recently isolated from soyabean oil, resembles α-tocopherol (5 : 7 : 8-trimethyltocol) in biological activity, whereas β-tocopherol (5 : 8-dimethyltocol) and especially γ-tocopherol (7 : 8-dimethyltocol) exhibit less activity. Synthetic 5 : 7-dimethyltocol (not yet found in nature) and synthetic 8-monomethyltocol (possibly a stereo-isomer of the natural product) are biologically inactive. Methods for the determination of individual tocopherols are therefore required.

OXIDATION METHODS—In methods depending upon oxidation to tocopheryl *p*-quinones the consumption of oxidising agent is directly or indirectly measured. *Ceric sulphate method*—Dissolve about 10 mg. of the tocopherol in 10 ml. of alcohol, add 2 drops of diphenylamine indicator solution, and titrate with 0·01 *N* ceric sulphate until the colour changes to violet. If the colour change is indistinct use leuco-malachite green as external indicator. Each millilitre of the ceric sulphate solution ≡ 2·15 mg. of α-tocopherol, 2·08 mg. of dimethyltocol, and 2·01 mg. of δ-tocopherol. In the *ferric chloride - dipyridyl method* of Emmerie and Engel (*Rec. Trav. Chim.*, 1938, **57**, 1351; *Analyst*, **1939**, **64**, 216, 446) remove the solvent from the tocopherol extract and treat the residue with 15 ml. of the reagent. After 10 min. with α- or β-tocopherol and 30 min. with γ- or δ-tocopherol, measure the extinction in a Pulfrich photometer with filter S.50 and a cell thickness of 5 cm. against the reagent in a similar cell. To prepare the reagent add 100 parts of alcohol to 1 part of an aqueous 20 per

cent. solution of ferric chloride hexahydrate, mix this solution with an equal volume of a 0·05 per cent. solution of α,α′-dipyridyl in alcohol and store in the dark. Determine the concentration of the α- or β-tocopherol by means of a standard curve. In the determination of β-tocopherol the α-curve may be used if the final result, in accordance with the difference in the molecular weights, is reduced by 3 per cent. For the determination of tocopherol in blood plasma shake 5 to 10 ml. with light petroleum and adsorb the tocopherol chromatographically on weakly active aluminium oxide, subsequently eluting the tocopherol with benzene. This purification is particularly necessary with bovine plasma to separate carotenoids. With human plasma, in which only the α-compound occurs in measurable amount, and with rat plasma, the results from purified and unpurified samples differ by only 5 to 20 per cent. Treat the benzene eluate after removal of the solvent as already described, and determine the concentration from the extinction curve.

The Furter - Meyer reaction—(*Helv. Chim. Acta*, 1939, **32**, 240; *Analyst*, 1939, **64**, 217). All four tocopherols form reddish-brown oxidation products when treated with nitric acid in alcohol, but the optimum reaction period is different for the individual compounds. To 10 ml. of the alcohol solution add 2 ml. of concentrated nitric acid, heat to the boiling point, place the mixture in boiling water for 5 min., cool, and dilute with alcohol to 20 ml. Determine the extinction in a Pulfrich photometer against alcohol. α-Tocopherol does not give this reaction in chloroform solution. For the determination of β-, γ-, or δ-tocopherol (also in presence of α-tocopherol) by oxidation in chloroform solution, treat 10 ml. of the solution in a 25-ml. stoppered cylinder with 1 ml. of concentrated nitric acid and shake for 15 sec. After a few more sec., and before nitrogen peroxide vapour is evolved, withdraw the chloroform layer, filter it into a thin-walled test-tube, place this for a few seconds in warm water to dispel turbidity during the measurement and measure the colour in a Pulfrich photometer with filter S.50 and a cell thickness of 2 cm. against chloroform as blank liquid.

FLUORIMETRIC METHOD—According to Smith *et al.* (*J. Amer. Chem. Soc.*, 1939, **61**, 2424) *o*-quinones exist in the red compound formed by oxidation of the tocopherols with nitric acid. The evidence for this is that these compounds condense with *o*-phenylenediamine to form phenazine derivatives, and, on the basis of this reaction, a fluorimetric method has been developed. With α-tocopherol, the oxidation must be made in alcohol; with β- and γ-tocopherols, it may be made in alcohol or chloroform. For the determination in alcohol the procedure is as previously described (*Helv. Chim. Acta*, 1943, **26**, 2166; *Analyst*, 1944, **69**, 156). The determination is best made in a fluorimeter, otherwise comparison solutions are necessary. If equal amounts of α- and γ-tocopherol are converted into their phenazine derivatives, the product from γ-tocopherol exhibits only about half the fluorescence of the derivative of the α-compound and also the fluorescence is greener. The method is not so suitable for β- and δ-tocopherol nor for 5 : 7-dimethyltocol, but it may be used for these compounds if the phenazine derivative is purified on a weakly active aluminium oxide column. γ-Tocopherol can be determined fluorimetrically in presence of α-tocopherol if the oxidation is made in chloroform solution. Treat 10 ml. in a stoppered graduated cylinder with 1 ml. of concentrated nitric acid, shake for 2 min., add 20 ml. of low-boiling petroleum spirit and about 20 ml. of water, shake, remove the aqueous layer, wash with water, and then with strong brine, and remove the solvent. Condense the residue with *o*-phenylenediamine as usual in glacial acetic acid. This method also is less suitable for β- and δ-tocopherol. The glacial acetic used must be purified with animal charcoal since even the best commercial grades contain an inhibitor of the condensation. The brown colour that the diamine solution acquires in air does not interfere.

THE DIAZOTISATION METHOD—This method is applicable only to γ- and δ-tocopherols. To prepare the diazonium salt solution dissolve 0·7 g. of *p*-nitraniline in 20 ml. of diluted hydrochloric acid (1 + 1), place the solution in ice and, immediately before use, mix 1 part of this solution with 1 part of a cooled 1 per cent. sodium nitrite solution. Treat 15 ml. of an alcoholic solution of γ- or δ-tocopherol and, at the same time, 15 ml. of alcohol, each with 1 ml. of the diazonium salt solution. After 60 min. measure the extinction in a Pulfrich photometer with filter S.53 with a cell-thickness of 5 cm., keeping the solutions in the dark until the measurement is made. If the coloured solution is turbid, add, after the 60-min. period, 1 ml. of water, extract with about 15 ml. of light petroleum (b.p. 80° to 100° C.), wash the extract with water, filter, and adjust the volume to 20 ml. Measure the colour as before against the solvent as blank liquid. If the petroleum extract contains other coloured substances, chromatographic separation may be tried with a short column of weakly active aluminium oxide, eluting with a mixture of light petroleum and benzene. The composition of the eluate depends upon the activity of the oxide (*infra*).

MIXTURES OF TOCOPHEROLS—To a mixture of another tocopherol with α-tocopherol apply the nitric acid method in chloroform solution. At the same time the colour will indicate which tocopherol is present, a red-violet indicating β-tocopherol and a red-orange, γ- or δ-tocopherol. To estimate γ- and δ-tocopherols in presence of α- and β-tocopherols use the diazo method. To determine β-tocopherol in presence of α-tocopherol use the nitric acid method. If γ- or δ-tocopherol is present, the β-compound must be determined by difference, the γ- and δ-compounds being determined by the diazo reaction and the β-, γ- and δ-compounds by the nitric acid reaction in chloroform. α-Tocopherol can be separated chromatographically from γ- and δ-tocopherols. In presence of β-tocopherol it can either be determined fluorimetrically (when practically only the α-compound is measured) or the β-tocopherol can be determined by the nitric acid

reaction in chloroform and then the α- and β-compounds by the ferric chloride reaction or the nitric acid reaction in alcohol. If, at the same time, γ- or δ-tocopherol is present these are separated from α-tocopherol chromatographically, the α-compound with a portion of the β-compound being found in the benzene eluate.

To prepare the aluminium oxide for the chromatographic separations treat 4 kg. of the oxide with 2 litres of concentrated hydrochloric acid in which 200 g. of stannous chloride have been dissolved. Add 2 litres of water and shake the mixture from time to time. After 3 days wash the treated oxide with distilled water by decantation and finally on a Buchner funnel until the washings are no longer acid to methyl red. Finally wash the oxide with acetone and dry it in air. Ignite a portion of the oxide and re-mix it with the un-ignited portion. Determine the correct degree of activity for the oxide with α- and γ-tocopherol, adding so much ignited to un-ignited oxide that α-tocopherol but not γ-tocopherol is quantitatively eluted with methanol.

The theoretical bases of the methods described are discussed and examples are given of their application to the determination of individual tocopherols in wheat germ oil and cottonseed oil.

A. O. JONES

Bacteriological

A Strain of *Lactobacillus plantarum* suitable for use in the Estimation of Antiseptic Power [of Hops, Hopped Worts, and Beers]. D. Kulka and T. K. Walker (*J. Inst. Brew.*, 1948, **54**, 130–132)—This lactic acid bacterium was substituted for Massol's bacterium, a strain of *Lactobacillus bulgaricus* isolated from St. Ivel cheese, which had lost its acid-forming capacity.

It was isolated by Walker and Hastings [also from St. Ivel cheese] and proved by them to be very trustworthy in the study of hop antiseptic by the log-phase methods during the period 1933–40. In 1946, cultures that had been grown in liquid wort, neutralised with calcium carbonate and sealed, were found to behave exactly like the original cultures 14 years before in their resistance to phenol and humulone. Detailed examination of the organism was not then made, but has been carried out by the present authors, who now give a full description and show that it is not a strain of *Lactobacillus bulgaricus* but corresponds very closely to the *Lactobacillus plantarum* strain: Reading 4125 of the collection of the National Institute for Research in Dairying.

It is a straight-sided, Gram-positive bacterium of variable length, non-motile and without spores, and a facultative anaerobe. It gives no catalase reaction, gives a positive Voges-Proskauer reaction, and acid production is given with maltose, lactose, sucrose, L-arabinose, D-xylose, glucose, fructose, raffinose, and salicin. It does not, like *L. casei*, peptonise sour milk at 14 days at 25° C.

D. R. WOOD

Agricultural

Quantitative Estimation of Hemicelluloses by Direct Isolation. H. D. Weihe and M. Phillips (*J. Agric. Res.*, 1947, **74**, 77–85)—In the method of Preece (*Biochem. J.*, 1931, **25**, 1304) the sample (20 to 25 g.) is extracted first with a hot 0·5 per cent. ammonium oxalate solution, then with a 1 per cent. boiling sodium hydroxide solution in 50 per cent. ethanol, and finally with 50 per cent. ethanol. The residue is then repeatedly extracted at room temperature with a 4 per cent. aqueous sodium hydroxide solution until a portion of the extract no longer gives a precipitate when neutralised with acetic acid and diluted with an equal volume of acetone. The combined alkaline extracts are first acidified with glacial acetic acid and then treated with an equal volume of acetone. The precipitate is collected and dried *in vacuo*, and the weight of the moisture-free and ash-free product is determined and reported as free hemicellulose. The material remaining from the extraction with the alkali solution is then extracted with 4 per cent. boiling sodium hydroxide solution, the product is isolated in the manner described, and the result is reported as combined hemicellulose.

In this procedure the free and combined hemicellulose fractions are both contaminated with lignin and considerable degradation of the combined hemicellulose is likely to occur when it is extracted with boiling sodium hydroxide solution.

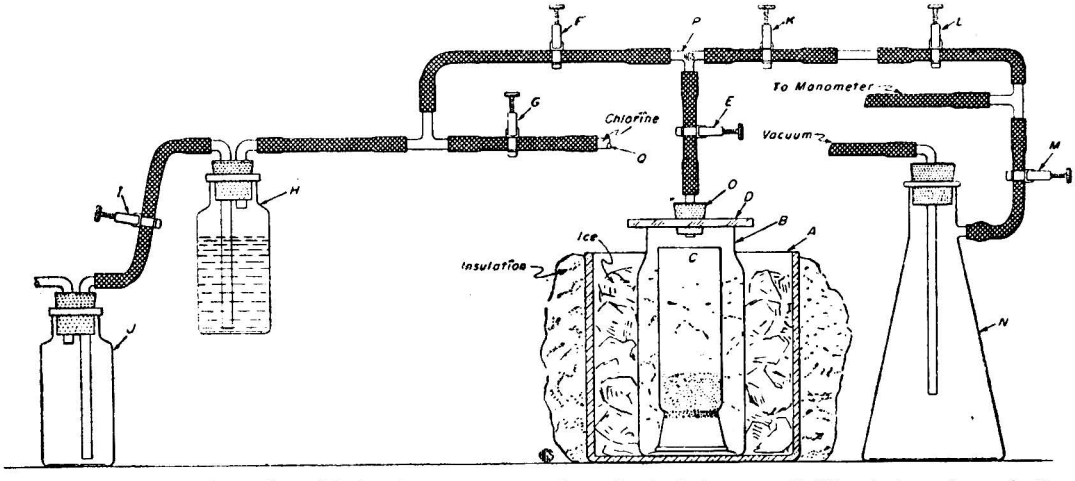
The method now presented consists essentially of the isolation of the holocellulose from a sample that has previously been freed from alcohol-benzene soluble extractives and pectic substances, and isolation of hemicellulose from the holocellulose. The residual material is weighed to determine the total amount of material extractable with dilute sodium hydroxide solution.

For determining pectic substances and holocellulose the apparatus recommended consists of a sintered-glass crucible ($5\frac{1}{2}$ in. × $1\frac{3}{4}$ in.) with a disc of 80- to 100-mesh. The end below the disc is rimmed to take a rubber stopper carrying a glass tube bent upwards parallel to the length of the crucible and connected with suction apparatus for removing solvent from the crucible.

To determine the alcohol - benzene (1 + 2) extractives place a 5-g. sample of the plant material in the crucible and extract it in a Soxhlet extractor for 30 hr. Remove as much as possible of the solvent by suction, dry the crucible *in vacuo* at 60° C., and weigh. For the extraction of pectic and other substances soluble in 0·5 per cent. aqueous ammonium oxalate solution, attach the rubber stopper and suction tube, nearly fill the crucible with 0·5 per cent. ammonium oxalate solution at 85° C., and place the assembly in a beaker of water maintained at that temperature for 1 to 1·5 hr., stirring the contents occasionally. Remove the solvent by suction and continue the digestion repeatedly with fresh solvent until a portion of the extract, when treated with four times its volume of ethanol previously acidified with hydrochloric acid, gives no precipitate. Finally, wash the residue

with water until free from ammonium oxalate, then with 96 per cent. ethanol, dry *in vacuo* at 60° C., and weigh.

The procedure for determining the holocellulose in the residue is a modification of that of Ritter *et al.* (*Paper Trade J.*, 1941, **113**, 83, Tappi sect., 143; 1937, **105**, 127, Tappi sect., 277). The apparatus for chlorination, shown in the figure, consists of a glass jar *B* with a tightly fitting, ground-glass cover *D* carrying a rubber stopper *O*. The glass tube passing through *O* is connected by means of rubber tubing provided with a Hofmann clamp *E* to the T-tube *P*, which, in turn, is connected in the manner shown to the bottles *H* and *J* and to the litre suction flask *N* containing diluted sodium hydroxide solution for the absorption of chlorine. Clamps are provided at *F*, *G*, *I*, *K*, *L*, and *M*. *H* is almost filled with saturated sodium chloride solution. Chlorine from a cylinder enters the system at *Q*. By closing the clamp *F* and opening clamps *G* and *I*, chlorine from *Q* can be led into *H* and the salt solution driven over into *J*. *B* is placed in an insulated jar *A* containing crushed ice. The sample to be chlorinated is placed in the sintered-glass crucible *C*.



Moisten the sample in *C*, which has been successively extracted with alcohol - benzene mixture and with 0·5 per cent. ammonium oxalate solution (*supra*) with about 12 ml. of water and place the crucible in the chlorination chamber *B*. Place *B* in *A* and fill the latter with crushed ice. Place the cover on *B* and replace *O* with a thermometer in a suitable stopper and with its bulb within an inch of the sample in *C*. When the temperature in *C* is 15° C. or less, remove the thermometer and insert *O*. With *F* closed and *E*, *K*, and *L* open, evacuate the system by opening *M*. By means of a manometer ensure that the system is gas-tight. and, with *K*, *L*, and *M* closed and *G* and *I* open, pass chlorine from a cylinder through a water wash-bottle into the system at *Q* until about one-half of the salt solution in *H* is forced into *J*. Open *F* slowly and increase and adjust the flow of chlorine so that the level of the liquid in *H* remains almost constant. When the pressure in *B* is approximately atmospheric, allow *H* to fill with chlorine, then close the clamp *G* and simultaneously stop the chlorine supply. The level of the liquid in *H* will rise as chlorine is absorbed by the sample. When absorption has ceased, close *F*, remove *C* from the chlorination chamber, and thoroughly mix the contents with 95 per cent. ethanol. After 2 min., draw off the solution in *C* with the aid of a suction flask and repeat the washing. Add to *C* a 3 per cent solution of ethanolamine in 95 per cent. ethanol and stir. Draw off the liquid and repeat the washing. Wash again twice with ethanol and once with ether and, finally, free the sample from solvent by means of the suction pump. Moisten the sample with 12 ml. of water and repeat the process of alternate chlorination and extraction, as described, until the chlorinated sample no longer gives a colour with ethanolamine solution. Dry the crucible and its contents *in vacuo* at 60° C., weigh, and calculate the amount of holocellulose in the sample.

To isolate the hemicelluloses from holocellulose add to 1 g. of holocellulose in a 200-ml. Erlenmeyer flask 100 ml. of a 4 per cent. aqueous sodium hydroxide solution, stopper the flask and digest the mixture at room temperature (25° to 30° C.) for 2 hr. with occasional stirring. Filter the mixture through cloth in a small Hirsch funnel, wash the residue with 15 ml. of water, and add the washings to the main filtrate. Repeat the digestion in the same manner three more times. To the combined filtrates add 3 volumes of ethanol, make the liquid slightly acid with acetic acid, and allow it to stand overnight at room temperature. Draw off the supernatant liquid, collect the hemicellulose material in a sintered-glass crucible of fine porosity, washing with 100 ml. of a mixture of 25 ml. of water, 1 ml. of glacial acetic acid, and 75 ml. of 95 per cent. ethanol. Wash successively with 95 per cent. ethanol, absolute ethanol, and ether. Finally, dry *in vacuo* and weigh the hemicelluloses.

In this work hemicelluloses are defined as carbohydrate complexes that are extracted from holocellulose by diluted aqueous sodium hydroxide solution at room temperature and that are precipitated from the alkaline extract by excess of ethanol. This definition is no more arbitrary than any other that might be proposed. A. O. JONES

Organic

Automatic Micro-determination of Carbon and Hydrogen. R. O. Clark and G. H. Stillson (*Anal. Chem.*, 1947, **19**, 423–426)—Combustion cycles for the analysis of a wide range of samples were determined, a semi-automatic combustion unit being used.

METHOD—*Apparatus*—The vaporisation furnace is 6·35 cm. long and contains six heating elements, each consisting of a length of Alundum thermocouple tubing (4·25 mm. outside diameter) wound with No. 30 platinum wire to give a total resistance of 5·5 ohms with the windings connected in series. The windings are spaced so that the end of the furnace nearer the combustion furnace operates at a temperature 50° C. higher than the other. The temperatures of the vaporisation and combustion furnaces, the pre-burner and heating mortar are all read, through a selector switch, on a single millivoltmeter calibrated in degrees centigrade. The vaporisation furnace is advanced by means of a threaded brass half-bearing and Monel-metal screw, both having ⅜-in. thread, 12 threads to the inch. This permits the furnace to be moved manually to any position by lifting the bearing from the screw drive, which is driven by a 100 r.p.m. uni-directional motor. Power is transmitted by a 30 to 1 worm and gear combination so that the driving screw rotates at 3·3 r.p.m. when the motor is operating at maximum speed. By means of a rheostat, rates of advance of 0·5 to 7 mm. per min. can be obtained. When the vaporisation furnace reaches the combustion furnace it is automatically stopped as the bearing runs on to a smooth section of driving screw.

Procedure—Place the vaporisation furnace 5 cm. from the combustion furnace, and place the sample so that half the boat is outside the movable furnace, on the side nearer the combustion furnace. Open the absorption tubes, turn on the heat to the vaporisation furnace, with the current adjusted to give a maximum temperature of 500° C. Allow the furnace to advance at 0·75 mm. per minute, for 4 min. then increase the rate to the maximum of 7 mm. per min. and maintain this rate until the sample has completely vaporised, after which decrease the rate to 0·75 mm. per min. After the sample has vaporised into the combustion tube again operate the motor at its maximum speed and raise the furnace temperature to 650° C. Three minutes after the bearing disengages, turn off the current.

Five slightly different cycles, covering a range of compounds boiling from 100° to 220° C., are illustrated. The accuracy of the semi-automatic unit is at least equal to that of the manual analysis, a conclusion borne out by many analyses performed over a number of years. The precision attainable is also satisfactory.

COMPARISON OF DEVIATION ON MANY ANALYSES

	No. of samples	Standard deviation parts per 1000	
		Carbon	Hydrogen
Semi-automatic ..	54	1·4	10
Power	218	2·5	18
Manual	76	1·4	9·1

Comparison of the combustion cycles employed by Hallett (*Ind. Eng. Chem., Anal. Ed.*, **1938, 10**, 101) and Royer *et al.* (*Ibid.*, 1940, **12**, 688) shows that (i) the Royer cycle, using a constant rate of advance throughout the combustion period, imposes a definite limitation as regards its application to compounds that must be vaporised under carefully controlled conditions, and (ii) Hallett's cycle should have a wider application, but it assumes that all compounds will have been vaporised into the combustion tube by the time the speed of the furnace is increased to a maximum; many cases are observed in which this is not so. Data indicate that with compounds that are volatilised with difficulty only the semi-automatic procedure gives accurate and consistent results. The semi-automatic unit was converted to fully automatic operation in which the five cycles could be selected by means of a five-gang, ten-circuit selector switch. The conclusions drawn from the semi-automatic unit are applicable to the fully automatic units.

H. K. DEAN

Analysis of Chloroacetic Acids. Determination of Dichloroacetic and Acetic Acid. G. A. Dalin and J. N. Haimsohn (*Anal. Chem.*, 1948, **20**, 470–472)—Most specifications issued by purchasers of chloroacetic acid include analytical methods for determining dichloroacetic acid based on the work of Pool (*Pharm. Weekblad.*, 1905, **42**, 165). The present authors have shown that the accuracy of this method is dependent on the quantity of reagent used; by standardising the quantities of reagents and by adding manganous sulphate the accuracy has been brought within the range required by the specifications' normal limit of approximately 2 per cent. of dichloroacetic acid. Determination of acetic acid by difference has also been shown to be unsatisfactory, and a direct method involving titration after distillation with an inert, intermediate-boiling solvent is proposed.

Determination of dichloroacetic acid—Dissolve 2·5 g. of the sample in 40 ml. of water, add 14 g. of sodium hydroxide pellets, and boil the solution under a reflux condenser for 1 hr. Cool, acidify with concentrated hydrochloric acid, neutralise with concentrated aqueous ammonia solution, and add 3 ml. of aqueous ammonia solution in excess. Concentrate the solution by boiling until crystals begin to form and, while hot, add 11 ml. of freshly prepared, 5 per cent. calcium acetate solution and 5 ml. of concentrated aqueous ammonia solution. Allow the mixture to stand, with occasional stirring, for 1 hr. and filter through a No. 40 Whatman filter paper. Wash the flask and the precipitate successively with 20 ml. of 10 per cent. aqueous ammonia solution, 20 ml. of 1 per cent. calcium acetate solution, and 20 ml. of water. Return the filter paper to the flask, add 200 ml. of 10 per cent. sulphuric acid, heat to 70° C., and add 3 ml. of 0·2 per cent. manganous sulphate solution. Titrate immediately with 0·1 *N* potassium permanganate from a micro-burette, and carry out a blank determination on the reagents. One ml. of 0·1 *N* potassium permanganate ≡ 0·12895 g. of dichloroacetic acid.

There must be no delay between the addition of the sulphuric acid and the titration: any delay increases the attack on the filter paper and results in an inaccurate blank.

Determination of acetic acid—Mix 50 ml. of the sample with about twice its volume of methyl *n*-amyl ketone and distil the mixture through a column about 60 cm. long and 10 mm. inside diameter, packed with 1/16-in. helices. Collect the distillate in small fractions, titrate each fraction with standard alkali, and calculate the acid as acetic acid. Continue until the ratio of acid determined to size of fraction is constant and plot the cumulative acid values against the cumulative distillate. Extrapolate the straight portion of the curve back to zero distillate to eliminate the effect of the acetic acid evolved in the distillation, and hence read the quantity of acetic acid present in the sample.

Other solvents proposed include xylene and di-*n*-butyl ether. The boiling point of all three solvents is intermediate between that of acetic acid (118° C.) and chloroacetic acid (189° C.) but, since methyl-*n*-amyl ketone is soluble in water, the use of this solvent avoids the necessity of extracting the acetic acid by shaking with water.

The method should be applicable to any series of homologous compounds for which a series of inert intermediate solvents can be found, and the accuracy can be improved by increasing the size of the sample and the amount of solvent.

A. H. A. ABBOTT

Absorptiometric Micro-determination of Chloralose in Alcoholic Solution. Preliminary Note. J. Delvenne and G. Barac (*Rec. Trav. Chim.*, 1947, **66**, 443–444)—An absorptiometric method, suitable for the determination of 50 to 200 μg. of chloralose, and based on a colour reaction with resorcinol in concentrated sulphuric acid, is proposed.

Procedure—To 2 ml. of a solution of chloralose in 94 per cent. alcohol add 4 ml. of resorcinol reagent (0·25 g. of resorcinol in 100 ml. of sulphuric acid of *d* 1·84) and mix well. Set aside for 5 min. and add 2 ml. of water. Allow to stand for 25 min. more and measure the absorption of the solution at 500 mμ. in a 0·5-cm. cell, using as a reference solution 2 ml. of 94 per cent. alcohol treated in the same manner as the test solution.

Read off the chloralose content of the test solution from a calibration graph prepared by using standard chloralose solutions.

H. J. CLULEY

Electrometric Ferrocyanide Method for Determining Sugars. E. T. Podlubnaya and P. S. Bukharov (*J. Anal. Chem. Russ.*, 1948, **3**, 131–136)—In the variations of the ferrocyanide method for determining sugars, difficulties arise in the observation of the end-point in dark-coloured solutions and in the different behaviour of certain indicators in hot and cold solutions. The electrometric method described avoids these difficulties.

METHOD—*Apparatus*—The electrodes are platinum wires fused into glass tubes. One of them, the reference electrode, is immersed in an alkaline 1 per cent. potassium ferrocyanide solution or 1 per cent. potassium ferricyanide solution that has been titrated with a sugar solution. It is contained in a tube with a tap at the bottom, and the whole passes through a hole in the rubber bung of a conical flask used as titrating vessel. Two further holes carry the indicator electrode and a connection to a burette, from which the sugar solution is delivered. The electrodes are connected through a tapping key to a galvanometer. A known volume of the standard potassium ferricyanide solution is placed in the conical flask.

Standardisation—Dissolve in water 4·75 g. of sucrose, dried over calcium chloride, heat the solution at 100° C. for 10 to 15 min. with 50 ml. of 2 per cent. hydrochloric acid solution to produce inversion, cool, neutralise with sodium carbonate, and dilute to 1 litre. For use, dilute the solution until it is exactly 0·2 per cent., and transfer a portion to the burette. In the conical flask place 40 ml. of 1 per cent. potassium ferricyanide solution and 10 ml. of 2·5 *N* sodium hydroxide. Insert the electrodes, heat the contents of the flask to the boiling point, and add the sugar solution dropwise with constant shaking, until the galvanometer needle swings to the other side of zero. When this reading has been obtained, further titrations may be carried out by adding most of the sugar solution required for a titration at the start. The titre of the solution is established on the figure obtained after adding at the start 85 to 95 per cent. of the required amount of sugar solution.

Procedure—Take an amount of the test solution corresponding to about 4·5 g. of sugar, add 50 ml. of 2 per cent. hydrochloric acid, boil, cool, neutralise, and titrate with it as described in the standardisation.

The method was compared with that of Bertrand on samples of pure sugars, raspberry and blackcurrant liquors, chartreuse, brandy, etc. Results agreed to about 1 part in 300. The optimum concentration of sugar in the solution used for titration is 0·2 per cent.

G. S. SMITH

Kjeldahl Determination of Nitrogen without Distillation. Application to Samples containing Phosphorus. K. Marcali and W. Rieman, 3rd (*Anal. Chem.*, 1948, **20**, 381–382)—The disadvantages of the formaldehyde method recommended by the authors (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 709; *Analyst*, 1947, **72**, 163) are the conversion of any phosphorus present from primary to secondary phosphate between the methyl red and phenolphthalein end-points, and the precipitation of the sulphates of barium and calcium and the hydroxides of iron and aluminium, which make the end-point less sharp. In the modification now described phosphorus is precipitated as zirconyl phosphate with the hydroxides of zirconium, calcium, barium, iron, and aluminium.

Method—Weigh a sample containing 10 mg.-equivalents of nitrogen and digest and dilute as in the general procedure (*loc. cit.*). Transfer quantitatively to a 250-ml. volumetric flask and add 15 ml. of sodium bromide solution, 5 ml. of a zirconyl chloride solution (for 150 mg. of phos-

phorus) prepared by dissolving 322 g. of the octohydrate in 600 ml. of *N* hydrochloric acid and diluting to 1 litre with *N* acid and 3 drops of methyl red indicator. Add 10 *N* sodium hydroxide until the colour is yellow and *N* sulphuric acid until it is just pink. Cool and dilute the solution. Filter through a rapid, fluted 15-cm. paper, discarding the first 5 ml., and pipette 100 ml. into a 250-ml. Erlenmeyer flask. Add 1 drop of methyl red solution and adjust with 0·1 *N* sodium hydroxide to the end-point. Read the burette and continue the titration as previously described.

Results—The method is accurate to within 1 in 175 on pure organic compounds containing 2 per cent. of phosphorus added as sodium phosphate, and to within 0·05 per cent. on blood samples containing between 3·5 and 7·5 per cent. of nitrogen as determined by the standard Kjeldahl method.

The modification has not been extended to the micro- and semimicro-scales. M. E. DALZIEL

Factors affecting the Determination of Furfural. G. A. Adams and A. E. Castagne (*Canad. J. Res.*, 1948, **26**, 314–324)—The known gravimetric and volumetric methods for determining furfural also measure the methyl-furfural and hydroxymethylfurfural derived from the cellulose, starch, hexose sugars, and methyl pentoses often associated with furfural-yielding substances in plant materials. The colorimetric method, based on the reaction between furfural and aniline in acetic acid solution, developed by Stillings and Browning (*Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 499–502; *Analyst*, 1940, **65**, 663–664), is specific for furfural, and the present authors have confirmed that methylfurfural and hydroxymethylfurfural do not interfere with the determination. They have also stabilised the colour by adding oxalic acid and sodium chloride. The modified procedure has been used to determine the furfural yield in such plant materials as wheat straw, wheat bran, corn cobs, oat hulls, soya bean hulls, peat moss, and sphagnum moss. The results were compared with those obtained by the use of the bromine oxidation method (Hughes and Acree, *Ind. Eng. Chem., Anal. Ed.*, 1934, **6**, 123–124; *Analyst*, 1934, **59**, 430) and this procedure gave results consistently higher than those obtained by the colorimetric method. Since both methods yield identical results with pure furfural, it is contended that the titration procedure measures other substances, including methylfurfural and hydroxymethylfurfural, whilst the proposed colorimetric method gives an accurate determination of furfural from plant materials.

Procedure—Reduce the air-dried material to No. 40 powder and transfer a quantity of material expected to contain between 10 and 100 mg. of furfural to a two-necked, round-bottomed, 500-ml. flask. Add 100 ml. of 12 per cent. hydrochloric acid and 20 g. of sodium chloride. Fit a dropping funnel containing 12 per cent. hydrochloric acid to one neck of the flask, and connect the other neck, by all-glass joints, to a condenser leading to a flask fitted with a Kjeldahl-like trap partially filled with 12 per cent. hydrochloric acid. Boil the liquid in the flask, and heat so that the level in the flask is maintained at 100 to 125 ml. by the continual addition of 12 per cent. hydrochloric acid from the dropping funnel, and the distillate collects at the rate of 400 ml. per hour. To test for complete removal of furfural, after about 1·5 hr. with pure pentose and 2·5 hr. for plant materials, neutralise 1 ml. of distillate with sodium hydroxide solution and add 1 ml. of a reagent prepared by mixing 1 ml. of freshly distilled aniline, 10 ml. of water, and 9 ml. of glacial acetic acid. If no colour develops in 10 to 15 min., the test sample contains no furfural.

Dilute the distillate to a known volume, usually 200 ml., neutralise a 10-ml. aliquot with sodium hydroxide solution, and dilute to 100 ml. with water. Mix 5 ml. of freshly distilled aniline with 50 ml. of glacial acetic acid and 10 ml. of 20 per cent. sodium chloride solution. Add 5 ml. of 5 per cent. oxalic acid solution, 5 ml. of 10 per cent. disodium phosphate solution, and place the flask containing the aniline reagent in a water-bath at 20° ± 0·5° C. Cool the diluted furfural distillate to 20° C. and transfer 5 ml. to the whole of the aniline reagent and dilute the mixture to 100 ml. with water. Maintain the solution at 20° C. for 1 hr. in the dark and then read the transmittance in a suitable colorimeter. Simultaneously, prepare a blank of all reagents and read its transmittance as 100 per cent. Calculate the quantity of furfural in the sample by reference to a standard curve prepared by applying the above procedure to known amounts of pure furfural.

The accompanying table, besides illustrating the higher results obtained with the bromine-titration process, compares the potential value of some plant materials as sources of furfural.

Material	Furfural, per cent.	
	Colorimetric method	Titration method
"Brewing" bran* ..	31·2	33·0
Corn cobs	24·9	27·1
Oat hulls	22·1	25·5
Wheat straw (crop I) ..	17·6	19·6
Wheat bran	17·2	18·9
Sunflower seed hulls ..	16·8	19·0
Wheat straw (crop II) ..	16·0	17·6
Flax shives	14·0	16·4
Milkweed pod hulls ..	9·8	13·3
Soya bean hulls	6·7	8·1
Peat (source, New Brunswick)	6·7	13·7
Sphagnum moss	5·3	9·4
Peat (source, British Columbia)	4·3	9·1

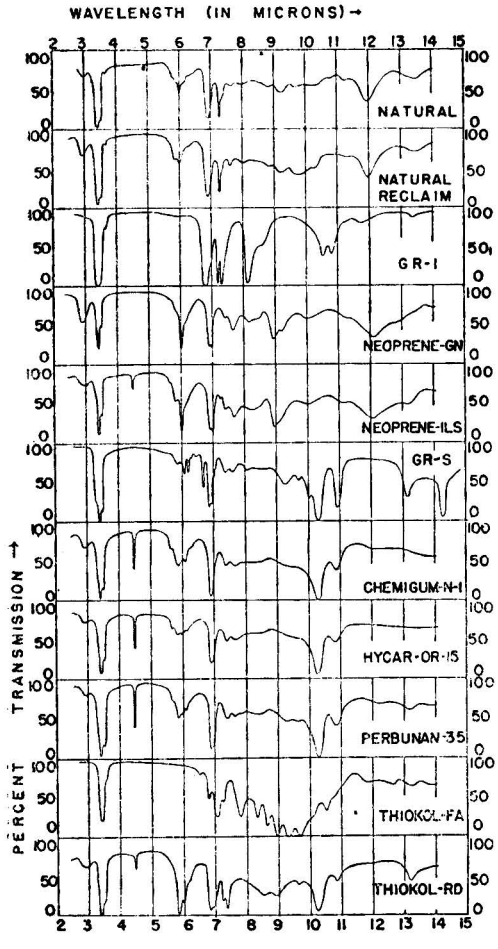
* A wheat bran product made by the patented Earle process and supplied for this work by the Continental Baking Co., Kansas City, Mo.

A. H. A. ABBOTT

Analysis of Natural and Synthetic Rubber by Infra-red Spectroscopy. H. L. Dinsmore and D. C. Smith (*Anal. Chem.*, 1948, **20**, 11–24)—The preparation of samples of raw and vulcanised rubbers for examination by infra-red spectroscopy is described. The identification of unknown rubbers is also described and directions are given

for the quantitative determination of natural rubber and GR-S (the general purpose butadiene-styrene co-polymer) in admixture with each other, and the determination of the acrylo-nitrile content of oil-resisting rubbers. The relation between polymer structure and absorption spectrum is discussed.

METHOD—*Preparation of samples*—Extract unvulcanised rubber with a suitable solvent and then dissolve, either by 6 to 8 hr. shaking, or by mixing rubber and solvent in a mixing mill, in ethylene



Spectra of Cured Elastomers

dichloride. Comminute vulcanised rubbers on a standard roll mill prior to extraction, and then dissolve by boiling under reflux in a suitable solvent having a boiling point above 100° C. Remove any fillers by centrifuging or filtration, concentrate the solution to a viscous mucilage, and spread between parallel metal spacing bars on a sodium chloride or potassium bromide plate. Allow the solvent to evaporate, leaving a dried film of the rubber. An appropriate film thickness must be found by trial, and should be such as to give 80 to 90 per cent. absorption at the 6·9-μ. band.

Spectroscopic measurements—The measurements reported were made on a large recording prism spectrometer of high resolving power. Two traces were recorded, one through the sample and the other through a blank plate. The ratios of corresponding ordinates were found. The percentage transmittances so obtained were reproducible to within ± 1·0 per cent. and the wavelength measurements to within ± 0·01 μ. from 2 to 15 μ.

Identification—Individual polymers can be identified from the reference spectra shown in the diagram.

Quantitative analysis—For mixtures of natural rubber and GR-S, measure the transmittance (T) as the ratio of the energy transmitted by sample and blank respectively, at 7·60, 7·25, and 6·70 μ. Duplicate measurements should be made, and transmittance values outside the range 20 to 65 per cent. should not be used. The extinction $E = \log (1/T) = KCt$, where K is a constant, C the concentration, and t the thickness. To eliminate the effect of the thickness, use extinction ratios and plot $E_{6\cdot70}/E_{7\cdot60}$ against GR-S content, and $E_{7\cdot25}/E_{7\cdot60}$ against natural rubber content for a series of standard samples, where the subscripts refer to the wavelengths used. Determine the composition of unknown samples from the calibration thus obtained. The presence of reclaimed natural rubber is found to interfere with the accuracy of the results, the error increasing with increasing amounts of reclaimed rubber.

The acrylonitrile content of the Buna N type rubbers is obtained by a similar method, the ratio $E_{4\cdot47}/E_{3\cdot50}$ being used. W. C. WAKE

Inorganic

Potentiometric Determination of Small Amounts of Carbon Dioxide and Hydrochloric Acid in presence of each other in Distilled Water. Analytical Contribution for the Existence of Barium Bicarbonate. F. Cůta and R. Kohn (*Coll. Czech. Chem. Comm.*, 1947, **12**, 384–398)—Carbon dioxide can be determined in distilled water by potentiometric titration with barium hydroxide, using the antimony electrode. The method is suitable for concentrations of 0·01 to 5 mg.-mol. of carbon dioxide per litre.

In the presence of hydrochloric acid the sum of the hydrochloric acid and carbon dioxide is determined, necessitating the separate determination of the hydrochloric acid by another method. The simultaneous determination of hydrochloric acid and carbon dioxide is possible only within a narrow range of concentrations.

For the potentiometric titration of carbon dioxide alone the inflection point corresponds approximately to the formation of barium bicarbonate, and this fact is advanced as proof of the existence of barium bicarbonate in solution. However, if the inflection point is taken as the equivalence point, results for carbon dioxide are about 2 per cent. low; this effect is attributed to slight decomposition of the barium bicarbonate. It is necessary to determine the potential at the equivalence point by measuring the potential of solutions containing equivalent amounts

of potassium bicarbonate and barium chloride, covering a range of concentrations. The equivalence potential thus determined varies with the concentration, and the potential to be taken for the end-point therefore depends upon the carbon dioxide content of the sample. By this method of titrating to a pre-determined potential carbon dioxide can be determined with an average error of 0·3 per cent.

Determination of carbon dioxide - barium hydroxide equivalence potentials—Introduce 500 ml. of water free from carbon dioxide into a flask in a current of pure air and add barium chloride and potassium bicarbonate in amounts equivalent to the formation of barium bicarbonate. Insert the antimony and saturated calomel electrodes and record the potential when it remains stable for 2 min. Repeat the procedure with barium bicarbonate solutions similarly produced covering a range of concentrations, and plot a curve of equivalence point potentials against corresponding carbon dioxide concentrations.

Determination of carbon dioxide in absence of hydrochloric acid—Weigh a flask containing sufficient paraffin oil to form a layer 1 to 2 cm. thick. Syphon in the sample below the oil and re-weigh to determine the quantity of sample taken. Introduce into the aqueous layer a stirrer, the tip of the burette, and the antimony and saturated calomel electrodes. Add 0·1 ml. of saturated potassium chloride solution per 100 ml. of sample to increase the conductivity.

Begin stirring, taking care that no oil is dragged down into the aqueous layer, and titrate with 0·01 *N* barium hydroxide solution. Calculate the approximate carbon dioxide content from the inflection point of the curve obtained and use this value to establish the equivalence potential from the equivalence potential curve. The amount of barium hydroxide added to give this potential is equivalent to the carbon dioxide in the sample.

The titration should be completed in 15 min., as in longer periods loss of carbon dioxide into the paraffin layer becomes significant.

Determination of carbon dioxide in presence of hydrochloric acid—Carry out the titration by the above method; two inflection points will be obtained. At the second inflection point the volume of barium hydroxide solution added will be equivalent to the sum of the hydrochloric acid and carbon dioxide present, subject to correction as above from the equivalence potential curve. To obtain the carbon dioxide content, determine hydrochloric acid alone, *e.g.*, by titration, in presence of methyl orange as indicator.

The first inflection point corresponds accurately to the hydrochloric acid content only under limiting conditions, when the hydrochloric acid and carbon dioxide contents are both in the region of 3 to 4 mg.-mol. per litre. H. J. CLULEY

Determination of Magnesium in Dolomites by Photo-turbidimetric Titration. B. E. Reznik and G. P. Fedorova (*J. Anal. Chem. Russ.*, 1948, **3**, 92–95)—Most of the difficulties of nephelometry connected with the effects of various factors on the properties and physical structure of the precipitates obtained disappear when the method of photo-turbidimetric titration is used. This method has been applied to the determination of magnesium in the form of magnesium ammonium phosphate.

Development of the method—Preliminary experiments on the possibility of nephelometric determination of magnesium by means of di-sodium hydrogen phosphate in ammoniacal medium showed that although after 5 min. the optical density of the suspension became constant and the absorption strictly followed the Lambert-Beer law, yet the effect of temperature was too critical for the practical application of normal nephelometry. With photo-turbidimetric titration the equivalence point appeared at the same place at temperatures as far apart as 13° and 26° C., and it was unaffected by varying the waiting time after addition of each portion of the reagent solution over the range of 2 to 15 min. Calcium interfered but it could be removed as the molybdate. The molybdate is preferred to the oxalate method of precipitating calcium since the compound formed does not adsorb or occlude magnesium, thus rendering a double precipitation unnecessary, and the precipitate may be filtered off after a shorter time.

Procedure—Dissolve 0·5 g. of dolomite in 15 to 20 ml. of concentrated hydrochloric acid, previously treated with 0·5 to 1 ml. of concentrated nitric acid, evaporate to dryness, extract the residue with hydrochloric acid, boil, and filter. Add aqueous ammonia solution to precipitate the sesquioxides, dissolve and reprecipitate them, boil the combined filtrates, add 15 ml. of slightly ammoniacal 10 per cent. ammonium molybdate solution at the rate of 1 drop per sec., and then boil for 15 to 20 min. to coagulate the calcium molybdate. Cool, filter by suction, wash the precipitate six to eight times with hot water, and dilute the filtrate and washings to 250 ml. in a graduated flask. Transfer 5 to 10 ml. to a photo-turbidimetric cell, add 5 ml. of diluted aqueous ammonia solution (1 + 9) and half the calculated amount of disodium hydrogen phosphate solution. Then add during constant stirring 0·1-ml. portions of this solution at 2-min. intervals until the absorption is constant, as shown by the galvanometer needle. Determine the titre of the phosphate solution by similar means, using a solution of known magnesium content. One ml. should correspond to approximately 0·0016 g. of magnesium oxide.

With dolomites containing 10 to 20 per cent. of magnesium oxide, results agreed with those obtained by the pyrophosphate method to within 0·2 to 0·3 per cent. of the sample weight. G. S. SMITH

Inorganic Drop Reaction for Copper. F. Goldschmidt and B. R. Dishon (*Anal. Chem.*, 1948, **20**, 373–374)—With concentrated hydrobromic acid cupric ions give a violet colour; the limit of concentration is 1 in 5×10^5.

Procedure—Place 1 drop of the test solution on copper-free filter paper (Green No. 803), and dry it in a warm air-current. Add 1 drop of a solution containing 5 g. of ammonium bromide and 4 ml. of syrupy phosphoric acid in 100 ml.; the appearance of a violet colour indicates the presence of copper. On adding a drop of water and drying in a warm

air-current, a violet ring forms. In moist air the colour fades, but drying in warm air regenerates it.

In 0·05 ml., 0·2 μg. of copper can be detected in the presence of 12·5 parts of gold, or 25 parts of tervalent chromium, or 25 parts of ferric iron, or 125 parts of nickel, or 5 parts of cobalt, without special precautions; also 1 μg. of copper in 50 parts of bismuth.

Interfering substances—Mercurous mercury, stannous tin, and ferrous iron must be oxidised by boiling 1 drop of the test solution with 1 drop of saturated bromine water before applying the test.

Gold, bismuth, and ferric bromides colour the test solution when present in excess of the quantities stated above. If gold is present, boil 1 drop of the solution with 1 drop of the reagent and a few grains of silver and apply the test to the resulting solution; thus 0·2 μg. may be detected in presence of 500 μg. of gold. For bismuth, add 1 drop of 1 per cent. sodium acetate solution to 1 drop of the test solution and 1 drop of the reagent, and apply the test to the resulting suspension of bismuth phosphate: 0·2 μg. in presence of 500 μg. of bismuth can then be detected. For large amounts of iron, boil 1 drop of the solution with 1 drop of saturated bromine water, and add 1 drop of the reagent and 1 drop of 4 per cent. sodium fluoride solution. Apply the test to the cooled suspension of ferric phosphate; then 1 μg. can be detected in presence of 200 μg. of iron.

The photochemical colour change of silver compounds interferes, therefore boil 1 drop of the solution with 1 drop of the reagent to precipitate silver bromide, and to 1 drop of the supernatant liquid add a further drop of the reagent for the test; this method detects 0·2 μg. in presence of 2500 μg. of silver.

Chromium, cobalt, and nickel must be removed by ordinary analytical methods.

Oxidising acids give a yellow stain due to the liberation of bromine but, on drying, only the violet ring remains, except with iodates or periodates, which give a persistent stain. In this case, treat 1 drop of the solution with 3 drops of the reagent and dry in warm air; after some time a violet coloration develops, indicating copper. In this way 0·1 μg. of copper is detectable in presence of 1550 μg. of nitrate, and 0·5 μg. in presence of 2090 of chlorate, 3200 of bromate, 4380 of iodate, or 4800 of periodate. M. E. DALZIEL

Rapid Colorimetric Determination of Copper in Tin-base Alloys. G. Norwitz (*Anal. Chem.*, **1948, 20,** 469–470)—The sample is attacked by *aqua regia*, and the solution heated after addition of phosphoric acid to prevent precipitation of the tin on neutralising the solution. Water and ammonia are added and the resultant blue colour is measured in a Klett-Summerson photo-electric colorimeter with a 4-cm. glass absorption cell and a Klett-Summerson filter No. 59 (maximum transmittance at 580 mμ.)

Method—Treat 1·000 g. of the sample in a 500-ml. Erlenmeyer flask, marked to indicate the 500-ml. level, with 10 ml. of concentrated hydrochloric acid and then with 10 ml. of concentrated nitric acid; heat on a hot-plate to dissolve the sample. Add 30 ml. of 85 per cent. phosphoric acid solution and heat strongly until the solution is clear green in colour (12 to 15 min.). Allow to cool somewhat and add 200 ml. of cold water, followed cautiously by 120 ml. of concentrated aqueous ammonia while swirling. Cool the solution to room temperature, dilute it to the mark, stopper the flask, and shake it well. Read the blue cuprammonium colour in a colorimeter set to zero with distilled water. Deduce the copper content from a calibration curve prepared by using tin-base samples of known copper content.

Results—The method is suitable for copper contents between 1 and 10 per cent. and on 2 samples containing between 3 and 4 per cent. the values obtained were within ± 0·05 per cent. of the correct values.

The colour is stable for 24 hr. in a tightly-stoppered flask. Nickel, chromium, and cobalt interfere, but are usually absent in tin-base alloys; up to 5 per cent. of iron does not interfere. Tartaric acid does not keep tin dissolved in ammoniacal solution without the presence of phosphate. Tin and antimony are apparently not lost in driving off hydrochloric and nitric acids. M. E. DALZIEL

Concentration Methods in the Determination of Traces of Nickel. I. V. T. Chuyko (*J. Anal. Chem. Russ.*, 1947, **2**, 328–333)—Traces of nickel in aqueous solutions and in solutions of aluminium and zinc salts can be concentrated, prior to colorimetric determination, by co-precipitation with magnesium hydroxide. Traces in chromium and iron salts can be co-precipitated with zinc ferricyanide in acid solution, and traces in cobalt salts by addition of potassium cyanide and precipitation with sodium hydroxide and bromine water in presence of paper pulp.

Co-precipitation with magnesium hydroxide and colorimetric determination of nickel—To samples of nickel-free distilled water varying amounts of nickel equivalent to 0·001 to 0·1 mg. per litre were added, and the solutions treated with 40 ml. of 0·1 *M* magnesium sulphate and 20 ml. of 2 *N* sodium hydroxide per litre. After several hours, the clear supernatant liquid was siphoned off, the precipitate was dissolved in hydrochloric acid, its solution diluted to 0·1 *M* concentration in magnesium, and part of the magnesium precipitated with sodium hydroxide solution, and the solution filtered. For colorimetric determination of the nickel, the magnesium hydroxide was dissolved in a few millilitres of hot, 10 per cent. hydrochloric acid, the solution carefully neutralised with alkali, and then treated with 0·5 ml. of 0·1 *N* iodine in potassium iodide solution, followed by a saturated solution of sodium pyrophosphate with dimethylglyoxime, added until the precipitate first formed re-dissolved. In 5 to 10 min. the iodine colour disappeared and was replaced by the colour of the nickel complex. Sometimes it was necessary to add a small additional amount of solid sodium pyrophosphate to give a definite colour change. In further experiments the addition of magnesium salt and decantation by siphoning were replaced by filtration after addition of paper pulp.

Calcium, sodium, and potassium salts in concentrations of 1 g.mol. per litre had no effect on the co-precipitation, but ammonium salts required the addition of 2·5 g.-equivalents of sodium hydroxide for each gram-equivalent of ammonium salts for quantitative results. Colour matching was carried out against standards prepared in a similar way. The maximum error in the indicated range was 6 parts in 100.

Aluminium and zinc salts—Solutions containing known amounts of nickel were treated with 40 per cent. sodium hydroxide solution until the precipitated aluminium and zinc hydroxides were dissolved. After suitable dilution, paper pulp from a 7-cm. ashless filter paper was added, the solution well shaken and then filtered, and the paper with adsorbed nickel hydroxide washed two or three times with 2 *N* sodium hydroxide, and then with distilled water. Subsequent treatment was as before.

Apparently paper pulp was as good a collector of nickel hydroxide as magnesium hydroxide.

The error in determining 0·01 mg. of nickel in presence of 0·1 to 1 g. of aluminium or zinc did not exceed 5 parts per 100. In presence of chromium salts the magnesium precipitate did not settle properly and the cloudy solution passed through a filter. With paper pulp in place of magnesium hydroxide, no nickel appeared on the paper.

Co-precipitation with zinc ferricyanide—Solutions of chromium salts containing small amounts of nickel were acidified with a few drops of hydrochloric acid and heated to 40° C. For every 50 to 70 ml. of solution 2 ml. of 0·5 *M* potassium ferricyanide were added and then 10 ml. of 0·5 *M* zinc sulphate in drops. The precipitate was filtered off, washed with water to remove chromium salts, and then dissolved on the filter in 2 *N* sodium hydroxide. The filtrate was mixed with paper pulp and poured through the same filter which was then washed with alkali to remove ferricyanide. Further treatment was as described above. Quantitative precipitation of nickel occurred only when the zinc ferricyanide formed in presence of excess of ferricyanide.

Solutions of ferrous sulphate were freed from about 0·002 per cent. of nickel that was present, by oxidation with nitric acid, successive addition of 4 ml. of 0·5 *M* potassium ferricyanide and 4 ml. of 0·5 *M* zinc sulphate for each 70 to 100 ml. of solution, and filtration from the nickel-containing precipitate. To the weakly acid filtrates known amounts of nickel were added, and the solutions treated at 40° to 50° C. exactly as with chromium solutions. Some ferric hydroxide usually appeared during the alkali treatment. This could act as an efficient collector of the nickel unless the amount formed were very small, in which case the addition of paper pulp to the filtrate and re-filtration as described above were necessary. With 0·005 to 0·1 mg. of nickel in presence of 1 to 2 g. of iron, the results were normally slightly low but by not more than 6 to 8 parts in 100.

Determination in presence of cobalt—Solutions, 50 to 100 ml., of cobalt salts, containing small amounts of nickel, were treated with potassium cyanide solution until the precipitate first formed disappeared except for a slight cloudiness. They were cleared by heating and filtering. The filtrates were mixed with 10 ml. of 2 *N* sodium hydroxide, 75 ml. of bromine water, a further 25 ml. of alkali, and about 0·1 g. of paper pulp. After shaking for 15 to 20 min., they were filtered and the filters were washed with alkaline water. The precipitates were dissolved in hot, 20 per cent. hydrochloric acid solution and nickel was determined in the solutions. With 0·01 to 0·05 mg. of nickel in presence of 0·5 to 1·0 g. of cobalt the maximum error was 6 parts in 100. G. S. SMITH

Colorimetric Determination of Small Quantities of Iron in Water by means of Dimethylglyoxime. P. Lieffrig and X. Buron (*Chimie Analyt.*, 1948, **30**, 36)—*Method*—To 100 ml. of the sample, in a Nessler tube with a ground-glass stopper, add 2 drops of concentrated hydrochloric acid and then a few crystals (10 to 20 mg.) of sodium dithionite ($Na_2S_2O_4$). Mix by inverting the tube a few times, add 8 to 10 drops of a 1·5 per cent. solution of dimethylglyoxime in alcohol, and mix again. Add 2 ml. of aqueous ammonia solution and mix once more. Compare the red colour produced (which is stable for several days) with standards of known iron content. The method works well if the iron content of the water is not above 1 mg. per litre. If the iron content is higher, a suitable volume of the sample should be diluted to 100 ml. with distilled water. A set of permanent standards can be made by dissolving amounts of cobalt chloride as given in the table in 100-ml. portions of 60 per cent. phosphoric acid.

mg. of $CoCl_2$ in 100 ml. of 60% phosphoric acid	Iron, mg. per litre
0·10	0·10
0·16	0·20
0·25	0·30
0·30	0·40
0·40	0·50

These standards should be checked with known iron solutions and adjusted if necessary: they may then be used for several years. L. A. DAUNCEY

Critical Examination of a Perchloric Acid Colorimetric Method of Analysis for Chromium in Carbon and Low Alloy Steel. Report of the Methods of Analysis Committee of the Metallurgy Division of The British Iron and Steel Research Association (*Metallurgia*, 1948, **37**, 211–216)—The method makes use of the optical absorption of dichromate, which is increased in presence of iron perchlorate. The effect of various factors on the results is given, and it is concluded that the method is not suitable as a standard one, but should be valuable for routine analysis, provided that the limitations are appreciated. The following findings have been taken into account in deciding the experimental conditions, which should be closely controlled.

(1) The absorption is substantially independent of perchloric acid concentration within the range 34 to 44 per cent.

(2) The colour system behaves as if it had two components, one due to the chromium alone and

the second being an intensification proportional to the amount of iron in the solution.

(3) Of the common alloying elements, only molybdenum and vanadium in amounts greater than 1 per cent. interfere. The use of sulphuric or phosphoric acid should be avoided.

(4) The optical absorption is influenced by the temperature of the solution, which should be 20° ± 2° C. during the measurement.

(5) The use of more ferrous sulphate than the suggested amount causes low results.

(6) Filtration of the solution is not necessary when the silicon content is low (0·1 to 0·2 per cent.). When filtration is necessary a sintered-glass and not a paper filter should be used.

Procedure—Dissolve 1 g. of sample in 10 ml. of diluted nitric acid (1 + 1) and 23 ml. of perchloric acid (sp.gr. 1·54) in a covered, 200-ml. conical beaker). (A 0·5 g. sample may be used, and the volume of perchloric acid is then reduced to 18 ml.). Evaporate the solution to perchloric acid fuming and boil gently for 5 min. Cool rapidly, add 20 ml. of distilled water to dissolve the salts, and cool again. Dilute exactly to 50 ml. and measure the absorption in a Spekker absorptiometer, using a water : water setting of 1·20. With 1·0-g. samples and chromium contents less than 0·15 per cent. use 2-cm. cells, and for chromium contents between 0·15 and 0·35 per cent., use 1-cm. cells; with 0·5 g. samples and chromium contents less than 0·1 per cent., use 4-cm. cells and for chromium contents between 0·1 and 0·4 per cent., use 2-cm. cells. With a mercury vapour lamp use either Chance No. 6 and Wratten No. 50 filters or Ilford 601 and H.503 filters. With a tungsten lamp use Ilford 601 and H.503 filters. Reduce the solution (either that in the cell or a further portion) with 0·02 to 0·04 g. of ferrous sulphate and measure the absorption immediately. The slope of the line connecting the Spekker difference readings and the chromium content depends on the iron content of the solution.

L. A. DAUNCEY

New Method for Determining Tungsten in Steels by means of β-Naphthaquinoline. R. B. Golubtsova (*J. Anal. Chem. Russ.*, 1948, **3**, 118–122)—β-Naphthoquinoline can be used instead of cinchonine for the determination of tungsten in steels, ferro-tungsten, and pure tungsten. Results of the same order of accuracy are obtainable.

Properties of β-naphthaquinoline—Preliminary experiments were carried out by adding 5 to 10 ml. of a 2 per cent. aqueous solution of the reagent acidified with sulphuric acid to solutions containing various metallic ions in acidic and alkaline media. No reaction occurred with ferrous iron, nickel, aluminium, titanium, zinc, lead, niobium, nitrate, or chromate. Molybdenum gave a dense white precipitate in neutral or weakly acidic solution, but none in more acidic solutions. The reagent is practically insoluble in water. On being heated with very dilute hydrochloric acid, it dissolves and is re-precipitated in the cold as yellow needles. These after filtration and drying can be dissolved in water to give a 2 per cent. solution. Both alcoholic and acetic acid solutions of the reagent give only colloidal suspensions with soluble tungstates. The reagent dissolves readily in water acidified with sulphuric acid; a 2 per cent. solution can be kept unchanged for a long time in an unsealed vessel. This solution gives precipitates with tungstates in both hot and cold solutions and, although heating may be used to coagulate the precipitate, this is not essential. The precipitate that has been formed in the cold may be filtered through a close filter after 10 to 15 min. standing. Results expressed in the form of a Gibbs triangular diagram showed that 1 ml. of a 2 per cent. solution of the reagent precipitates 29·6 mg. of tungsten in the presence of 1 ml. of 10 per cent. sulphuric acid solution.

Determination of tungsten in steels—Dissolve 1 to 2 g. of steel in a covered beaker by heating with 80 ml. of diluted hydrochloric acid (1 + 4), followed by the addition of a few drops of nitric acid. Heat until the tungstic acid that separates is bright yellow, then add 60 ml. of water, and heat again for 20 to 30 min. Cool, add 10 ml. of 2 per cent. β-naphthoquinoline in water containing sulphuric acid, filter after 15 min., wash first with 10 per cent. hydrochloric acid solution until a test shows that all iron has been removed, and then with 2 per cent. ammonium nitrate solution. Ignite the precipitate at first at a low temperature and then at 800° C. to tungstic oxide (factor 0·7931).

If the precipitate is contaminated, fuse it with 3 g. of potassium-sodium carbonate mixture, extract with water, acidify the solution with diluted hydrochloric acid solution (1 + 1), and re-precipitate etc. as before. [Treatment with hydrofluoric acid may be advisable. G.S.S.]

Determination in ferro-tungsten and metallic tungsten—Treat 0·5 g. in a platinum dish with 10 ml. of hydrofluoric acid and 10 ml. of concentrated nitric acid added in drops. After dissolution, add 2 to 3 ml. of concentrated sulphuric acid and heat for 20 min., avoiding the deposition of salts. Transfer the clear solution to a beaker, add 10 ml. of the reagent solution, and evaporate on asbestos to a volume of 50 to 60 ml. Cool, filter, and proceed as described above for steel.

[ABSTRACTOR'S NOTE—Tables in the paper intended to show accuracy give, for steels, two columns, "W% taken" and "W% found," *e.g.*, taken 1·24%, found 1·25, 1·20, 1·21. The "taken" figure might be that obtained by the cinchonine method, but there is nothing to show this. The tables are not referred to in the text, but there is a statement that the accuracy is as good as that of the cinchonine method.] G. S. SMITH

Electrometric Determination of Vanadium in Steels. A. Claassen and J. Corbey (*Rec. Trav. Chim.*, 1948, **67**, 5–10)—In the volumetric method of Thanheiser and Dickens (*Arch. Eisenhüttew.*, 1931, **5**, 105) steel is dissolved in a mixture of sulphuric and phosphoric acids, and carbides are oxidised by adding potassium permanganate. Chromium and vanadium are reduced by means of ferrous sulphate and sufficient permanganate is added to the cold solution to give a lasting faint colour. The small excess of permanganate is destroyed by adding oxalic acid and the vanadium

is titrated electrometrically with ferrous sulphate solution. This method gives low results, probably because the oxalic acid reduces some vanadium, and other reducing agents have been used without success. The method of the A.S.T.M. (1946) in which the steel solution is reduced with ferrous sulphate, excess of which is destroyed by means of ammonium persulphate, and the vanadium is titrated with potassium permanganate solution, also gives low results.

The method now described is claimed to be satisfactory. Excess of oxidising or reducing agent is removed by means of the titrant and the amount of vanadium is determined by the amount of reagent consumed between two electrometric end-points. By the determination of formal redox potentials and other experimental work, conditions have been found that make both end-points sharp.

Methods—Vanadium in tungsten-free steel—Dissolve up to 1 g. of steel in 6 ml. of phosphoric acid (sp.gr. 1·7), 8 ml. of diluted sulphuric acid (1 + 1), and 20 ml. of water. When the vanadium content is small, use up to 4 g. of steel and 3 ml. more of diluted sulphuric acid (1 + 1) for each extra gram of steel. Dilute to 70 ml., boil, add 2·5 per cent. potassium permanganate solution until the solution is strongly coloured and boil for a few min. Add solid ferrous sulphate in small portions until no further colour change occurs. Dilute to 200 ml., cool to below 30° C. and add, with constant stirring, potassium permanganate solution until a small excess is present. Add 2 to 4 drops in excess and leave for 2 min. to make certain that the colour persists. Titrate electrometrically with 0·02 to 0·1 *N* ferrous sulphate solution. When the first equivalence point is reached (0·90 to 0·95 v. with respect to the saturated calomel electrode) add 40 ml. of diluted sulphuric acid (1 + 1) and continue the titration to the second end-point (0·62 to 0·67 v. *versus* S.C.E.).

Vanadium in tungsten steels—With moderate amounts of vanadium in the presence of tungsten the above method can be used if the amount of phosphoric acid is increased to 30 ml., but the first end-point is not sharp. The following method is preferred. Dissolve the steel in 16 ml. of diluted sulphuric acid (1 + 1), 20 to 40 ml. of water, and 6 ml. of phosphoric acid for up to 1 g. of sample, 14 ml. for 2 g. and 22 ml. for 3 g. These quantities are adequate for steels with up to 25 per cent. of tungsten. Heat until the evolution of hydrogen ceases and then add 2·5 per cent. potassium permanganate solution in excess and boil until the evolution of gas ceases. Dilute to 75 ml., and add solid ferrous sulphate to the hot solution until the colour of the solution shows no further change. Boil for 5 min. to effect quantitative reduction of the vanadium from the vanadophosphotungstate complex, cool, dilute to 175 to 200 ml., and titrate electrometrically with 0·02 to 0·1 *N* potassium permanganate to the first end-point (0·58 to 0·63 v. *versus* S.C.E.). Dilute the solution to 350 to 400 ml. (600 ml. for a 3-g. sample), heat to 60 to 70° C., and continue the titration to a second end-point at 0·84 to 0·92 v. *versus* S.C.E.

L. A. DAUNCEY

Composition of the Phospho-vanado-molybdate Complex Obtained in Colorimetric Determination of Phosphorus by Misson's Method. N. V. Maksimova and M. T. Kozlovsky (*J. Anal. Chem. Russ.*, 1947, **2**, 353—358) —Misson (*Chem.-Ztg.*, 1908, **32**, 633) used the yellow coloration given by phosphates in acid solution with ammonium metavanadate and molybdate for the colorimetric determination of phosphorus. He ascribed to the complex formed the formula $(NH_4)_3PO_4,NH_4VO_3,16MoO_3$ largely on the basis of determinations of phosphorus and vanadium in precipitates from more concentrated solutions. This formula, which has been taken as correct by other workers, is not, however, in accordance with the theory of the structure of complex compounds of phosphorus and molybdenum.

It is now shown that the formula is wrong. The complex contains no ammonium, and the ratio $P_2O_5 : V_2O_5 : MoO_3$ is 1 : 1 : 22. Attempts at separating the complex from the medium in which colorimetric determinations of phosphorus are carried out involved a search for precipitants and a method of extraction with a solvent. Barium chloride, silver nitrate, mercurous nitrate, and magnesium chloride gave no precipitate, but 8-hydroxyquinoline gave an orange precipitate of the composition $6C_9H_7ON.P_2O_5.V_2O_5.22MoO_3.nH_2O$, in which *n* varied between 12 and 35. Analyses for molybdenum, vanadium, phosphorus, and 8-hydroxyquinoline were made, and it was established incidentally that weighing of the precipitate might be used for gravimetric determination of phosphorus, and titration of the 8-hydroxyquinoline present for volumetric determination of phosphorus. Extractions were attempted with *iso*amyl alcohol, ether, and chloroform. Chloroform had no effect on the complex or its components, but the other solvents removed the complex entirely in one extraction. They also extracted much of the excess of molybdate present, but only traces of vanadate and these could be removed from the extract by two or three washings with *N* nitric acid saturated with *iso*amyl alcohol. Phosphate was not extracted. Thus, the extraction method could be used to give the ratio $P_2O_5 : V_2O_5$, but no further information. Analysis of the residue after evaporation of the *iso*amyl alcohol extract gave this ratio as 1 : 1.

An indication that no components other than P_2O_5, V_2O_5, MoO_3, H_2O, and possibly hydrogen were present, was obtained by using only phosphoric acid, vanadium pentoxide, soluble molybdic acid, and nitric acid. The coloration was exactly the same as that obtained under normal conditions. The complex is represented as $[P_2(V_2O_6)(Mo_2O_7)_{11}]$.

Formation of the 8-hydroxyquinoline compound and its analysis—To separate 1-, 2-, and 3-mg. quantities of phosphorus in the form of potassium dihydrogen phosphate, there were added 10 ml. of 0·02 *M* ammonium metavanadate in 0·125 *N* nitric acid, 5 ml. of 10 per cent. ammonium molybdate solution, 10 ml. of diluted nitric acid (sp.gr. 1·2), and water to make 50 ml. Then 20 ml. of 1 per cent. 8-hydroxyquinoline solution in alcohol were added

in the cold with stirring. Precipitation was immediate. After a test for complete precipitation the solution was filtered through a porcelain filtering crucible, and the precipitate washed with 0·2 per cent. 8-hydroxyquinoline solution in *N* nitric acid. Washing with water would have caused the precipitate to run through the filter. The crucible was dried to constant weight at 100° C. An aliquot part of the dried precipitate was further ignited to constant weight at 450° C.

The ignited precipitate was dissolved by heating in concentrated hydrochloric acid, followed by addition of water to give an acid concentration of 15 per cent. Then 3 g. of tartaric acid were added to combine with the vanadium, the solution was boiled, a rapid stream of hydrogen sulphide was passed through it for 15 min., an equal volume of water was added, the passage of gas continued for 10 min. more, the solution was boiled for 3 min., and hydrogen sulphide passed for another 10 min., whereupon the solution was left to stand in the cold for 1 hr. The precipitated molybdenum sulphide was filtered off and washed with a 2 per cent. solution of tartaric acid in 1 per cent. hydrochloric acid saturated with hydrogen sulphide. It was then dissolved in ammonia solution and transformed to lead molybdate. The filtrate was boiled, and oxidised with ammonium persulphate, and the remaining traces of molybdenum were precipitated by hydrogen sulphide. After filtration the solution was evaporated to dryness with nitric acid, and the residue ignited and dissolved in sodium hydroxide solution. The solution was neutralised with nitric acid and 5 per cent. in excess was added, and the vanadium was determined colorimetrically with hydrogen peroxide. The solution was then boiled to remove hydrogen peroxide, vanadium was reduced with sulphur dioxide solution, and phosphorus determined as magnesium pyrophosphate.

For the determination of 8-hydroxyquinoline the complex dried at 100° C. was dissolved by heating in a mixture of alcohol and hydrochloric acid, the solution was mixed with an equal volume of water, cooled, treated with an excess of standard potassium bromate-bromide solution, and then with potassium iodide, and the liberated iodine titrated with thiosulphate solution in presence of starch. [No further details are given.]

Results showed that the weight of the ignited residues and the amounts of 8-hydroxyquinoline found were proportional to the weights of phosphorus taken. Hence the methods described above up to the weighing of the ignited complex and the titration of 8-hydroxyquinoline in the dried complex may be used for gravimetric and volumetric determinations of phosphorus. The gravimetric factor is 0·0174 and the volumetric factor is 0·000259 for 1 ml. of 0·1 *N* potassium bromate. The actual results were: 1·0 mg. of phosphorus gave 60·0 and 58·4 mg. of ignited precipitate and 14·08 and 14·04 mg. of 8-hydroxyquinoline, 2·0 mg. gave 115·0 and 114·8 mg. and 28·02 and 28·04 mg and 3·0 mg. gave 174·0 and 168·0 mg. and 42·05 and 42·09 mg., respectively. G. S. SMITH

Determination of Molybdenum in Ferro-Molybdenum by a Potentiometric Method. H. Wirtz (*Z. anal. Chem.*, 1939, **116**, 240–243)—Previous methods use the precipitation of molybdenum as its sulphide or as lead molybdate after separation of the iron by sodium peroxide fusion. Molybdenum is so easily reduced by impurities that quantitative precipitation of the sulphide is difficult and the precipitate obtained is not easy to handle; in the fusion some molybdenum is lost by co-precipitation with iron and chromium hydroxides. The potentiometric method described entails reduction of hexa- to penta-valent molybdenum by tervalent titanium. The apparatus consists of a saturated calomel reference electrode, a platinum gauze indicator electrode, connected through a galvanometer, and a motor-driven stirrer led through the gauze. The potential of the standard electrode is such that the end-point is indicated by zero deflection of the galvanometer needle. The titanous chloride solution must be added from a burette with 0·05-ml. graduations. Chromium, at the concentration present in ferro-molybdenum, does not interfere.

Titanous chloride solution—Treat 80 ml. of a 10 to 15 per cent. solution of iron-free titanous chloride with 300 ml. of concentrated hydrochloric acid and then dilute to 5 litres.

Standardise the solution by dissolving copper oxide equivalent to 1 g. of copper, checked electrolytically, in 30 ml. of hydrochloric acid and diluting the solution to 1 litre. Dilute 50 ml. of the solution to 200 ml. in a 600-ml. beaker, and add 10 ml. of 15 per cent. potassium thiocyanate solution, and 10 ml. of diluted hydrochloric acid (1 + 1). Titrate with titanous chloride as described below. The concentration towards molybdenum is obtained by multiplying the concentration towards copper by 1·51.

Procedure—Fuse 1 g. of the sample with 20 g. of sodium peroxide in an iron crucible and extract the cooled melt with water in a beaker. Boil the solution with sodium peroxide for 3 to 5 min., then cool, and dilute to 500 ml. in a graduated flask. Evaporate the solution to perchloric acid fuming Pipette 50 ml. of the clear liquid into a 600-ml. beaker and dilute to 200 ml. with water. Add 10 ml. of 15 per cent. potassium thiocyanate solution, neutralise to litmus with diluted hydrochloric acid (1 + 1), and add 10 ml. in excess. Place the beaker in the apparatus and start the titration. Add 3 to 5 ml. of the standard titanous chloride solution dropwise and then continue the addition in a stream until disturbance of the galvanometer needle shows equivalence is approached. Continue adding the reagent dropwise until the galvanometer reads zero.

To clean the apparatus stir with aqueous ammonia solution for 1 min., and then rinse the electrodes thoroughly with water to remove the titanium hydroxide.

Results—Compared with 7 results obtained by the sulphide method, agreement is to within 1 in 380; and with 9 results by the molybdate method, to within 1 in 680. M. E. DALZIEL

Quantitative Determination of Lead in Presence of Cations of the Second Analytical Group [Barium, Strontium, Calcium, and Magnesium]. V. P. Shvedov, E. O. Goldshteyn, and N. I. Seletkova (*J. Anal. Chem. Russ.*, 1948, **3**, 109–112)—Existing methods of separating lead from the alkaline earths and magnesium are unsatisfactory. It is now shown that quantitative separation occurs when lead is precipitated as the hydroxybromide or hydroxyiodide. Determination of lead can then be made by the chromate method. Completeness of precipitation of lead was proved radiometrically and also by tests with hydrogen sulphide. In the former case the radioactive isotope ThB was added to the original lead solution as an indicator. Precipitation must be carried out at room temperature, and the precipitate filtered after not less than 1 hr. The ratio of bromide or iodide ions to hydroxyl ions in the solution should be 2 : 1.

Procedure—(Tests were carried out on solutions containing lead acetate and the nitrates of the other metals). To a solution containing barium, strontium, calcium, magnesium, and lead, add a mixture of equal volumes of 2 *N* ammonium bromide or iodide and *N* aqueous ammonia solution at room temperature. After 1 hr. filter off the precipitate and wash it five times with the precipitating solution diluted ten times. Treat the precipitate on the filter three times with boiling ammonium acetate solution, and then wash with hot water, collecting the filtrate in the original beaker. Dilute the filtrate with water, boil for 5 min. with 10 ml. of concentrated potassium dichromate solution, cool, filter through a filtering crucible, wash with water acidified with acetic acid, dry the lead chromate at 100° to 110° C., and weigh.

Tests carried out with 0·1 g. of lead in the presence of equal and five- and ten-fold quantities of the individual alkaline earths and in the presence of equal quantities of all the elements together gave results correct to 0·5 parts or less in 100.

G. S. SMITH

Polarographic Analysis of Refined Lead. M. Zotta (*Gazz. Chim. Ital.*, 1948, **78**, III, 143)—The principal metals that it is necessary to determine in lead used for accumulators are antimony, bismuth, arsenic, iron, copper, silver, tin, and zinc. For silver, arsenic, and tin, the spectrographic method is the most suitable. The remaining metals and cadmium and nickel can be rapidly and accurately determined polarographically.

Procedure—Dissolve 10 g. of lead shavings freed from grease, in a graduated 100-ml. flask, in 50 ml. of pure nitric acid (sp.gr. 1·3) diluted with three parts of water. If traces of tin or more than 0·03 per cent. of antimony are present, the solution will not be perfectly clear.

Add to the liquid 5 ml. of concentrated sulphuric acid, dilute to the mark with water, mix, and allow to stand for about 30 min.

(*a*) To determine copper, zinc, cadmium, and nickel, pipette 20 ml. of the clear liquid from above the lead sulphate precipitate into a small dish of Jena glass and evaporate to dryness, heating cautiously with the naked flame. Dissolve the residue in 3 ml. of a solution having the composition –$(NH_4)_2SO_4$, 1·5 *N*, $(NH_4)_2CO_3$, 0·1 *N*, NH_4OH, 0·5 *N*, and gelatin 0·005 per cent. After eliminating the dissolved oxygen from the solution, obtain the polarogram. The first step obtained is due to copper, the second to the small amount of lead remaining in solution, and the following steps to cadmium, nickel, and zinc, in this order.

(*b*) To determine iron and bismuth, take 40 ml. of the clear liquid, evaporate first on the water bath and then by cautious heating with the naked flame until sulphuric fumes are evolved. Pour the liquid into a centrifuge tube and wash the dish with 2 ml. of water. Precipitate the hydrates by addition of concentrated aqueous ammonia solution drop by drop, heat for 20 min. on the water-bath, cool, and centrifuge. Wash the residue twice with a small amount of dilute aqueous ammonia solution. Dissolve in 1·5 ml. of 4 *N* sulphuric acid. Add 3 ml. of *M* sodium citrate, a drop of methyl red, and aqueous ammonia solution drop by drop until the colour change begins. Obtain the polarogram. The step due to iron is followed by that of bismuth and then by that due to the sum of lead and antimony present.

(*c*) To determine antimony, take 5 ml. of the clear liquid and obtain the polarogram directly. If the amount of antimony exceeds 0·02 per cent. the results will be low, since part of the antimony remains undissolved by the nitric acid.

Prepare standards by adding the various metals to lead whose impurities are known, uniformity of the alloy being secured by prolonged heating at 550° C. in vacuum in a sealed glass tube with continuous oscillation.

The maximum sensitivity is 0·0005 per cent. for cadmium, bismuth, and iron; 0·0004 per cent. for copper; 0·0002 per cent. for zinc; and 0·001 per cent. for antimony. The average errors are ±8 per cent. for antimony, ± 6 per cent. for copper, and ± 3 per cent. for all the others. A. H. BENNETT

Precious Metal Sulphide Series. G. A. Medvedeva (*J. Anal. Chem. Russ.*, 1948, **3**, 103–108)—The order of decreasing solubility of the precious-metal sulphides in solutions of salts of these metals is Ir_2S_3, Rh_2S_3, PtS_2, Ru_2S_3, OsS_4, PdS, and Au_2S_3. Osmium, palladium, and gold can be completely precipitated as sulphides by 10 min. boiling of solutions of these metals with sulphides of iridium, rhodium, platinum, or ruthenium. A platinum solution can thus be freed from impurities of osmium, palladium, and gold; it is only necessary to treat the solution with platinum sulphide.

G. S. SMITH

Physical Methods, Apparatus, etc.

Measurement of Water in Gases by Electrical Conduction in a Film of Hygroscopic Material and the Use of Pressure Changes in Calibration. E. R. Weaver and R. Riley *J. Res. Nat. Bur. Stand.*, 1948, **40**, 169–214)—The electrical conductivity of a thin film of phosphoric acid alters considerably with alteration in concentration of water vapour. For example, the resistance of such a film changes from 2870 × 10^3 ohms for a

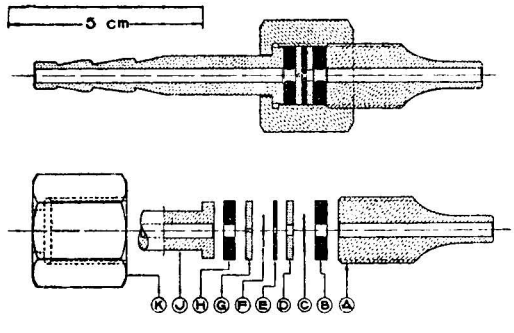
partial pressure of water vapour of 0·011 mm. of mercury to 8×10^3 ohms for a partial pressure of water vapour of 20·2 mm. If two samples of gas, one containing a known concentration of water vapour, S, and one unknown, W, are adjusted to give the same electrical resistance across a hygroscopic film by varying the pressure of the gases to P_S for the standard gas and to P_X for the unknown gas, then the concentration of water vapour in the unknown gas can be obtained from the simple relationship $W = S/P_S P_X$, assuming no deviations from Boyle's and Dalton's laws. To allow for such deviations, another and more complicated equation has been deduced, but circular slide rules have been developed to simplify calculations.

The apparatus is described in detail, especially the film detectors of various forms, electrical indicators, saturators, and valves. A discussion is given of the effectiveness of different electrolytic films, made from phosphoric and sulphuric acids and from lithium chloride in glycerol and other solvents.

The apparatus appears to have been extensively used for determining moisture in oxygen used by aviators, in dichlorodifluoromethane, in air, in powdered solids, and in organic liquids, and for testing the capacity of drying agents. The pressures used have mostly been in excess of atmospheric pressure but the authors think that the method might be used at low pressures also. They further suggest the method should be particularly useful for detecting oxygen in a combustible gas or *vice versa* by means of the water produced on combustion.

W. J. GOODERHAM

Membrane Valve for Constant Gas Flow. G. W. Harmsen (*Chem. Weekblad*, 1947, **43**, 205–206)—Needle valves and similar means for regulating the flow of gas do not allow of a small rate of flow being kept constant over a long period of time without adjustment. The author therefore used a "membrane valve," the construction of which is evident from the figure. B and H are thick rubber rings with large openings, whilst E is a thinner ring with a smaller opening, larger,



however, than that in the metal plate G. This ring, E, presses the membrane F against the plate G, which is bored with a hole of about 0·3 mm. diameter. E should not be too thick or soft. Before the gas passes through the flow-controlling membrane, F, it passes through a similar membrane, C, on the plate D, which has a much larger opening. This serves as a filter. The membranes are cut out of paper—thick filter paper for rapid gas flow and sized writing paper for lower rates.

G. MIDDLETON

The Critical Air-blast Test. F. G. Kreulen, van Selms, and D. J. W. Kreulen (*Chem. Weekblad*, 1948, **44**, 208–211)—The "critical air-blast test" (Methods of Test of Transport Gas Producer Fuel, B.S. 1264, 1945) determines the suitability of a coke for various purposes, including its use as household fuel. The test is defined as the minimum blast rate, measured in cubic feet per minute, at 60° F. and 30 in. mercury pressure (dry gas), that will maintain combustion in a fully ignited bed of dry fuel 40 mm. in diameter, and not less than 25 mm. deep, for a period of not less than 20 min. Combustion is judged to be maintained if, after the blast rate is increased to 0·150 cu.ft. per min., a visible glow appears within 20 min.

For household coal, the value should be not more than 0·055. Samples of coke made from coals of different origins all showed values between 0·06 and 0·08, but there was no clear relation between the degree of coking of a sample of coal and the critical air blast. A number of abnormal cokes gave the following values:—

Sample	Ash per cent.	Volatile matter per cent.	Critical air blast
Petroleum coke ..	0·1	8·1	0·034
Retort graphite ..	0·6	0·0	0·118
Peat coke (war-time production) ..	6·2	35·5	0·004
Coke from high-volatile non-coking coal	4·3	5·1	0·031
Coke from wood ..	1·0	1·9	0·026
Coke from peat ..	3·5	3·8	0·024
Coke from Dutch brown coal	13·1	6·7	0·020
Coke from anthracite	5·8	2·8	0·086
Coke from Cannel coal	11·6	4·2	0·033

With peat coke, the first sample was made at low temperature, but present methods of production use a high temperature of 1050° to 1100° C. With anthracite and similar coals, the critical air blast shows a direct relation to the amount of volatile matter; anthracites with a low content of volatile matter give a high critical air blast value; this figure thus should be useful for evaluating such samples. There appears to be a straight-line relationship between the critical air blast and the temperature at which the coke is made. This dependence on temperature is greater with anthracites than with high-volatile, non-coking, coals. When cokes with different values for the critical air blast are mixed, the curve obtained is not quite rectilinear but somewhat S-shaped. This indicates that household cokes are improved by the addition of anthracite; it appears probable that petroleum coke would also be a useful addition.

G. MIDDLETON

Polarographic Behaviour of Ions with Sodium Fluoride as Supporting Electrolyte. P. W. West, J. Dean, and E. J. Breda (*Coll.*

Czech. Chem. Comm., 1948, **13**, 1–10)—The polarographic behaviour of a number of inorganic ions, using sodium fluoride as supporting electrolyte, has been investigated to find whether the complex-forming properties of sodium fluoride have an important effect on the reduction waves of these ions. Of the twenty ions reported upon, only three, cobalt, tin and antimony, give steps whose half-wave potentials are significantly different from the corresponding potentials obtained when hydrochloric acid or potassium chloride is used as supporting electrolyte. J. G. WALLER

Determinations with Radioactive Isotopes by Measuring Specific Activity. P. Süe (*Bull. Soc. Chim.*, 1947, 405–406M)—In a solution containing a mixture of ions, an ion can be determined by adding a known weight of its radioactive isotope, converting the active and inactive ions into an insoluble salt and measuring the specific activity (number of particles emitted per minute per unit weight) of the precipitate. If the specific activity of the added isotope in the form of the insoluble salt is known, the observed reduction in specific activity due to its dilution with the inactive salt can be used to calculate the concentration of the ion originally present. The advantage of the method is that the precipitation need not be quantitative, as the proportion of active to inactive ion will remain the same, and the method should be of special value in the determination of traces where solubility errors might otherwise be significant. The precipitate must be free from impurity.

Results are given for the determination of potassium in presence of 13 g. of sodium and lithium nitrates. Single precipitations yielded potassium perchlorate of adequate purity, and results for quantities of 0·1 to 1 g. of potassium normally showed less than 2 per cent. error. Accurate results were not obtained under these conditions for smaller quantities of potassium. H. J. CLULEY

Reviews

COMMERCIAL FRUIT AND VEGETABLE PRODUCTS. By W. V. Cruess. Third Edition. Pp. x + 906. London: McGraw-Hill Publishing Co., Ltd. 1948. Price 51s.

THIS third edition of Professor Cruess's textbook, *Commercial Fruit and Vegetable Products*, brings up to date the industrial and technological information on the subject, and includes a new chapter (XXXV) on Plant Sanitation, which though useful, and valuable in drawing attention to this very important essential in the preparation of any food material, might well have been given more space than the 19 pages allotted to it.

Anyone closely familiar with a particular industry can find some points of criticism in a textbook on that subject, but any such criticism of this work would be more carping than justified. If there is a genuine criticism it is that the book lacks details of adequate testing for, although the author states that the book must be read in conjunction with his *Laboratory Manual of Fruit and Vegetable Products*, that Manual is more for the training of the technologist in understanding the processes of preparing fruit and vegetable products than for providing a scientific study of the processes and scientific testing. Anyone not well acquainted with the industry will find in this new book a surprising wealth of information, useful not only to the food technologist and the factory manager but also to those who have to use and examine the products. Even the experienced technologist or scientist working in these industries will find much valuable information, and moreover will find it in an exceptionally well presented and compact form; every sentence is worth its presence in the book.

The book is divided into 35 chapters, as follows—I. Micro-organisms in Relation to Fruit and Vegetable Products. II. General Principles and Methods. III. Brief History of Canning. IV. Tin and Glass Containers. V. General Considerations in Establishing a Cannery. VI. Washing, Blanching, and Peeling Fruits and Vegetables. VII. Grading Fruits and Vegetables for Canning. VIII. Syrups and Brines Used in Canning. IX. Exhaust and Vacuum. X. Processing of Canned Fruits and Vegetables. XI. Canning of Fruits. XII. Pickling and Canning of Olives. XIII. Canning of Vegetables. XIV. Spoiling of Canned Foods. XV. Unfermented Fruit Beverages. XVI. Fruit and Vegetable Syrups and Concentrates. XVII. Pectin, Jellies, and Marmalades. XVIII. Fruit Jams, Butters, Preserves, and Confections. XIX. Tomato Products. XX. Sun Drying of Fruits. XXI. Dehydration of Fruits. XXII. Dehydration of Vegetables. XXIII. Packing of Dried Fruits and Vegetables. XXIV. Vinegar Manufacture. XXV. Pickles. XXVI. Olive and Coconut Oils. XXVII. Utilisation of Waste Fruits and Vegetables and Disposal of Wastes. XXVIII. Citrus By-products. XXIX. Packing Cases and Other Packages. XXX. Wines. XXXI. Frozen-Pack Fruits and Vegetables. XXXII. Vitamins. XXXIII. Plant Pigments and Related Compounds. XXXIV. Enzymes of Fruits and Vegetables. XXXV. Plant Sanitation.

The chapters that desirably might have been fuller are the last five. Of these, XXXI describes a new industry, rapidly progressing, and of which therefore a description can never be really up to date, and chapters XXXII, XXXIII and XXXIV are, of course, designed for the technologist, not for the specialist in these subjects; chapter XXXV has already been referred to.

The book is well printed and singularly free from printing errors. It is recommended to any chemist or technolgist or factory manager concerned with the food industry. E. B. HUGHES.

PROTEINS AND AMINO-ACIDS IN NUTRITION. Edited by MELVILLE SAHYUN, M.A., Ph.D. Pp. xvi + 566. New York: Reinhold Publishing Corporation. 1948. Price 45s.

This books contains a number of articles on discrete aspects of the proteins and essential amino acids together with an extensive appendix on the composition of American foods.

The individual essays are, on the whole, competent and comprehensive (up to 1946–47). There are four chapters that deal thoroughly with modern views on the use of protein in the treatment of a wide variety of clinical conditions. Of particular interest are the very large amounts of protein that appear to be required in certain circumstances, *e.g.*, after fractures of bones or extensive burns, and the correspondingly enormous amounts of other nutrients that must also be given to ensure the proper utilisation of this protein. The metabolism of protein and the relations between protein metabolism and the vitamins and hormones are well discussed, but a freer use of structural formulae would have clarified much of the interesting paper by Deuel.

There are chapters on the economics of food proteins and nutritive aspects of meat and meat products, but little is said about methods of protein and amino-acid analysis. Nowhere is there any good account of the techniques that may be used for assessing biological value or digestibility, although Mitchell contributes a paper in which current methods are reviewed. Chemical methods of amino acid analysis are not even mentioned.

It is difficult to find any reason why a chapter on filtrable viruses and another on the protein nature of toxins, antitoxins and related substances should be included in a book entitled "*The Proteins and Essential Amino-Acids in Nutrition.*" The space could have been much better used for a good introductory chapter indicating recent trends in the field or to deal more fully with topics such as digestion or analytical methods, which are but briefly mentioned.

This book is one of a type that is becoming increasingly familiar; it is a collection of reviews written by different workers, none of whom attempts to cover more than a fraction of the subject matter. In such publications the editor's responsibility is to ensure that the subject is reasonably well covered and that the articles bear a direct relation to the subject as indicated in the title. This book, like many others of its type, would have been much improved if the separate parts had been more thoroughly integrated.

W. F. J. CUTHBERTSON

PHYSICAL METHODS GROUP

A MEETING of the Group, at which the subject will be "Electrophoretic Analyses," will be held at 3.30 and 5.30 p.m. on Friday, April 1st, in the Chemistry Lecture Theatre of The University, Nottingham. The following papers will be read:

3.35 p.m. "The technique of moving boundary electrophoresis," by R. A. Kekwick, D.Sc.

4.05 p.m. "Electrophoresis in the analysis of serum proteins," by N. H. Martin, M.A., M.B., M.R.C.P., F.R.I.C.

5.30 p.m. "The effect of Antrypol on the electrophoretic pattern of sera," by A. E. Ambler, B.Sc., Ph.D., and J. Madinaveitia, Ph.D.

5.30 p.m. "Ionophoresis of amino acids and peptides," by A. J. P. Martin, M.A., Ph.D.

Members of the Biochemical Society are invited to attend the meeting.

SECOND INTERNATIONAL CONGRESS OF CROP PROTECTION

THE Second International Congress of Crop Protection, under the presidency of the Rt. Hon. The Viscount Bledisloe, P.C., G.C.M.G., K.B.E., F.S.A., will be held in London from the 21st to the 28th of July, 1949.

It will be organised in six Sections as follows—Section 1, Insecticides; Section 2, Fungicides; Section 3, Plant Growth Regulators; Section 4, Toxicology of Crop Protection Substances; Section 5, Methods of Application; Section 6, Analytical Methods and Standardisation.

The charge for membership is £2 0s. 0d. sterling. Forms of application for membership, on which particulars as to Sections in which it is desired to take part and papers it is desired to read are invited, may be obtained from the Honorary Organiser, Lt.-Col. Francis J. Griffin, Second International Congress of Crop Protection, 54, Victoria Street, London, S.W.1.

ANALYST (qualified) required for Analytical Research in connection with pharmaceuticals. The position is particularly suitable for a man aged about 25 years who is looking for an appointment carrying good prospects of personal advancement and scope for independent research, please apply in writing, stating age, qualifications and experience (confidential), and quoting reference No. 2384, to the Personnel Officer, May & Baker Ltd., Dagenham, Essex.

SENIOR ANALYST (male) required, having experience in analysis of pharmaceuticals or fine chemicals. It is intended that the successful candidate shall shortly take charge of one of the Analytical Laboratories, and he must be capable of supervising the work of a considerable group of qualified and unqualified assistants. Applicants should be aged 28–35 years. A progressive salary will be paid and the position will carry sound prospects of advancement. There is a Staff Pension Fund (contributory). Please apply initially in writing, quoting reference No. 2385, to the Personnel Officer, May & Baker Ltd., Dagenham, Essex.

METALLURGICAL CHEMIST wanted for smelting works in Singapore. B.Sc. or equal. Salary according to age and experience. Write Box I.N.W., c/o 95, Bishopsgate, London, E.C.2.

ANALYST required for the Milk and Ice-Cream Division of Food Manufacturers Pilot Plant. London Area. Applicants are invited to apply, giving full details of qualifications, experience, age and salary required, to Box 3704, THE ANALYST, 47, Gresham Street, London, E.C.2.

KENT COUNTY COUNCIL

Applications are invited for an appointment as Assistant on the staff of the County Analyst. Salary A.P.T. Grade VII (£635–25–710). Minimum qualifications, F.R.I.C. (E) or equivalent with experience in foods and drugs, etc. Further particulars obtainable from The County Analyst, County Hall, Maidstone, by whom applications should be received not later than 16th March.

Applications are invited for the post of Analyst, Mineral Nutrition of Plants and Soils, at Long Ashton Research Station.

The appointment will be under a special Scheme of the Agricultural Research Council and will be graded in accordance with the London Scale, less Provincial Differentiation for Assistant Experimental Officers. The salary will be from £220 (at age 18) to £460 p.a. for men, and from £220 (at age 18) to £380 p.a. for women, with superannuation allowance.

Candidates should possess the National Certificate in Chemistry or an equivalent qualification.

The final date for receiving applications, which should be addressed as follows, will be the 19th March, 1949.

The Secretary,
Long Ashton Research Station,
Long Ashton, Bristol.

IMPERIAL CHEMICAL INDUSTRIES LIMITED, Dyestuffs Division, have vacancies in Manchester area for Assistant Analysts for research and routine control of bulk synthetic drugs and processed pharmaceuticals. Minimum qualification Inter. B.Sc. Preferred age limits 18–30. Salary according to qualifications and experience. Applications in writing to Staff Department, Hexagon House, Blackley, Manchester, 9. Ref. AA.

THE DISTILLERS' COMPANY LIMITED RESEARCH ORGANISATION requires a first-class Analyst to organise and run an analytical section. Candidates should possess a B.Sc. Degree, or equivalent, and a number of years' experience of analysis and should apply, in the first instance, to the Controller of Research and Development, The Distillers' Company Limited, 21, St. James's Square, London, S.W.1.

Commencing salary will depend upon age, qualifications and experience. The successful candidate will be required to work at Hull.

ANALYST required by British Oxygen Co., Ltd., to work at their research station in S.W. London. Duties include development of Analytical methods as well as the supervision of general analytical work. Applicants should be between 25 and 30 years of age and possess a first or second class honours degree in Chemistry or A.R.I.C. Apply Senior Research Manager, British Oxygen Co., Ltd., Lombard Road, London, S.W.19.

PHYSICIST required for the Central Research Department of The Morgan Crucible Co. Ltd. Age 21–25, with degree in physics and subsidiary chemistry or mathematics. Applications from men who sit for 1949 finals would be considered. The Company's interests embrace a wide field, including refractories, powder metallurgy and a large variety of carbon and graphite products employed in electrical, mechanical and chemical engineering. Write giving details of age, qualifications, experience, and salary required to the Staff Manager, Battersea Church Road, Lonnon, S.W.11.

JUNIOR RESEARCH ASSISTANT required by The Morgan Crucible Co. Ltd. for a varied range of physical testing in research laboratory, able to work out results accurately. Facilities given for part-time education, a person studying for physics degree preferred, and would be considered for transfer to Senior Staff on graduating and showing sufficient promise. Write giving details of age, experience, qualifications and salary required to the Staff Manager, Battersea Church Road, London, S.W.11.

VACANCIES for Analysts exist in the Pilot Plant Division of the Central Research and Development Department of the Distillers Company, Limited, at Tonbridge, Kent. Applicants should be between 20 and 30 years of age and should possess a B.Sc. in Chemistry or A.R.I.C.

Applications should be submitted to the Controller of Research and Development, The Distillers Company, Limited. 21, St. James's Square, London, S.W.1.

APPLICATIONS are invited for the post of Senior Food Analyst in Research Division. Candidates should have an honours degree in Chemistry and some years' research experience in food chemistry or an allied biochemical field. Initial salary £650. Apply in writing quoting Ref. RS/5 to Staff Division, The Metal Box Company, Ltd., The Langham, Portland Place, W.1.

It is understood that the situation advertised is available only to applicants exempted from the Control of Engagements Order, 1947.

CHIEF CHEMIST required in Public Analyst's laboratory in Central London, Branch E. F.R.I.C. essential, pharmaceutical experience preferable and must be capable analyst and organiser. Commencing salary £1,000, with possibility of salaried partnership later. Write Box No. 3703, THE ANALYST, 47, Gresham Street, London, E.C.2.

A YOUNG RESEARCH CHEMIST is required in the Central Research and Development Department of the Distillers Company, Limited, to work on problems connected with the manufacture of industrial alcohol. Previous research experience is not essential, but applicants must possess a 1st or 2nd class Honours B.Sc. Degree, or equivalent, and preference will be given to those with sound analytical training. Commencing salary will depend upon age, qualifications and experience.

Applications should be submitted to the Controller of Research and Development, The Distillers Company, Limited, Research and Development Department, Great Burgh, Epsom, Surrey.

GLAXO LABORATORIES LTD. have vacancies in their antibiotic factory at Ulverston for chemists and laboratory technicians, qualified and unqualified, for shift and rota day working. Experience in bacteriological assay or analytical work an asset, but not essential. Good salary. Generous shift allowance. Superannuation scheme. Apply, in first instance, to Personnel Manager, Glaxo Laboratories Ltd., Greenford, Middx., stating age, experience and qualifications, if any.

SCIENTIFIC INSTRUMENT CO. is considering the appointment of Travellers able to promote sale of their products to users of laboratory apparatus. Only those with established connections should write with full particulars, in strict confidence, to Box No. 3702, THE ANALYST, 47, Gresham Street, London, E.C.2.

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