THE ANALYST

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THE ANALYST

EDITORIAL

A REFERENCE BOOK FOR ANALYSTS

FOR many years a growing need has been felt by analysts engaged in industry and private practice for a reliable guide to the best standardised methods for the determination of specified constituents in a wide and ever-widening range of materials. To tell the searcher after information of this kind to go to a library and look it up is no solution to the problem; for the search usually creates the further difficulty of choosing from an unwieldy mass of references the method most likely to be best suited to the problem in hand; specialised knowledge combined with critical ability is necessary for a successful solution of the problem.

To meet this need, the Analytical Methods Committee of the Society and a band of enthusiastic and devoted helpers have drawn up lists of tried and tested methods for the analysis and testing of some hundreds of commercial products, ores, metals and miscellaneous materials. These have now been collected and published in the form of a book, under the auspices of the Society, with the title "Bibliography of Standard, Tentative and Recommended or Recognised Methods of Analysis." The book conforms in size and style of cover to the usual bound volumes of *The Analyst*, to the files of which it is expected to prove an indispensable addition. It is sold at a price that is causing considerable anxiety to our Honorary Treasurer—and one that bears no relationship to its real value.

As knowledge accumulates and new and approved methods are devised for the determination of this, that and the other constituent of a sample, further editions of this work will doubtless become necessary and desirable. In the hope that any future edition shall be of even more use to analysts than is the present volume, the compilers welcome suggestions for better methods than those now listed, or additions to the present list; these should be sent to the Honorary Secretary of the Analytical Methods Committee, at the Society's office.

The Analyst accords a hearty welcome to this new companion to its file of volumes in the full assurance that it will soon become an indispensable and a valued helper in furthering the improvement and perfection of analytical chemistry.

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

DEATHS

WE regret to record the deaths of

Kenneth Stuart McManus
Frederick William Richardson
Frank Robert Stephens
William Heaton Thorns.

SCOTTISH SECTION

THE Sixteenth Annual General Meeting of the Section was held in Glasgow on January 24th, 1951, and the following office bearers were elected for the year:—*Chairman*—Mr. H. C. Moir. *Vice-Chairman*—Mr. R. S. Watson. *Hon. Secretary and Treasurer*—Mr. J. A. Eggleston, Boot's Pure Drug Co., Ltd., Motherwell Street, Airdrie, Lanarkshire. *Elected Committee Members*—Messrs. A. R. Campbell, A. Dargie, H. Dryerre, J. M. Leitch, M. Pyke and J. Sword. *Hon. Auditors*—Messrs. J. Andrews and J. Gray.

MICROCHEMISTRY GROUP

THE Seventh Annual General Meeting of the Microchemistry Group was held at the Sir John Cass College, London, E.C.3, on Friday, January 26th, 1951. The following Officers and Committee Members were elected for the ensuing year:—*Chairman*—Dr. Cecil L. Wilson. *Vice-Chairman*—Dr. A. M. Ward. *Hon. Secretary*—Mr. Donald F. Phillips, 10, Richmond Road, Blackpool, N.S., Lancs. *Hon. Treasurer*—Mr. Gerald Ingram. *Elected Committee Members*—Dr. W. T. Chambers, Dr. J. G. A. Griffiths, Dr. G. F. Hodsman, Mr. R. F. Milton, Mr. G. H. Osborn and Dr. J. Sandilands.

After an interval for tea a Symposium on "Radiochemical Techniques in Microchemistry" was presented by members of the staff of the Atomic Energy Research Establishment, Harwell, at which the following papers were read: "The Quartz Ultramicrobalance in Radiochemistry," by Dr. J. K. Dawson; "Micromanipulation of Radio-active Gases," by Dr. W. J. Arrol; "The Determination of Trace Quantities of Elements by Radio-activation," by Mr. A. A. Smales.

An exhibition of microchemical apparatus, arranged by Messrs. R. F. Milton and D. W. Wilson, was held in the Microchemical laboratory; 14 members and firms contributed a very wide range of apparatus, consisting almost entirely of items not previously exhibited.

PHYSICAL METHODS GROUP

THE Sixth Annual General Meeting of the Group was held at 6 p.m. on Tuesday, November 28th, 1950, at the Iron and Steel Institute, 4, Grosvenor Gardens, London, S.W.1. Mr. B. S. Cooper, the Chairman of the Group, presided. The Group Officers and Elected Members of the Committee for the forthcoming year are as follows:—*Chairman*—Mr. B. S. Cooper. *Vice-Chairman*—Dr. W. F. Elvidge. *Hon. Secretary*—Mr. R. A. C. Isbell, Hilger & Watts Ltd., Hilger Division, 98, St. Pancras Way, London, N.W.1. *Members of Committee*—Messrs. C. H. R. Gentry, J. Haslam, G. H. Osborn, J. E. Page, A. A. Smales and F. R. Williams. *Hon. Auditors*—Messrs. C. A. Bassett and D. C. Garratt (re-appointed).

Votes of thanks were accorded to the two retiring members of the Committee, Messrs. L. A. Haddock and J. A. C. McClelland, for their services during the past two years, and to the Hon. Auditors for their work during the past year.

The Annual General Meeting was followed by the Twenty-eighth Ordinary Meeting of the Group, at which Dr. H. Irving delivered a lecture entitled "A Mid-Century Review of Physical Methods of Analysis"; this was followed by a discussion.

BIOLOGICAL METHODS GROUP

THE Sixth Annual General Meeting of the Group was held at 6.15 p.m. on Tuesday, December 19th, 1950, in the rooms of the Chemical Society, Burlington House, Piccadilly, London, W.1. Mr. N. T. Gridgeman was in the Chair.

The following were elected as Officers and Members of the Committee for the forthcoming year:—*Chairman*—Mr. N. T. Gridgeman. *Vice-Chairman*—Dr. H. O. J. Collier. *Hon. Secretary*—Mr. S. A. Price, Walton Oaks Experimental Station, Vitamins Ltd., Dorking Road, Tadworth, Surrey. *Elected Committee Members*—Dr. W. F. J. Cuthbertson, Professor C. H. Gray, Dr. L. J. Harris, Dr. J. Raventos, Dr. G. F. Somers, Mr. G. A. Stewart and *Ex-officio Members*, the President, Honorary Treasurer and Honorary Secretary of the Society and the Editor of *The Analyst*. *Hon. Auditors*—Mr. D. M. Freeland and Dr. J. H. Hamence.

The Annual General Meeting was followed by an Ordinary Meeting at which the following papers were presented and discussed: "Assessment of ACTH Activity," by I. D. K. Halkerston and M. Reiss; "*Euglena gracilis* as an Assay Organism for Vitamin B_{12}," by G. E. Shaw; "The Effect of Interfering Agents on the Vitamin B_{12} Plate Assay (*E. coli* Mutant Method)," by W. F. J. Cuthbertson, Valerie Herbert, H. F. Pegler and C. Quadling.

Analytical Methods Committee

REPORT PREPARED BY THE ANEURINE PANEL OF THE SUB-COMMITTEE ON VITAMIN ESTIMATIONS

The Chemical Assay of Aneurine in Foodstuffs

THE Analytical Methods Committee has received from its Sub-Committee on Vitamin Estimations the following Report based on the work of its Aneurine Panel. The Report has been approved by the Analytical Methods Committee and its publication authorised by the Council.

INTRODUCTION

The following workers assisted in the preparation of this Report: E. R. Dawson (Chairman), F. Wokes (Secretary), D. C. M. Adamson, R. G. Booth, W. F. Elvidge, G. E. Foster, J. Greenbaum, R. A. C. Isbell, J. King, W. Martin, F. W. Norris, T. L. Parkinson, S. A. Price, H. N. Ridyard and S. Y. Thompson. Assistance in some of the investigations was also given by D. J. Finney, J. Houston, B. C. P. Jansen, G. F. Lothian, H. Monk, F. Clermont Scott and Miss S. Weiner.

The Report is concerned with the chemical assay of aneurine in certain specified classes of foodstuffs, namely: cereals and cereal products; malt and malted products; yeast and yeast products; meat and meat extracts.

This Report deals with the determination of aneurine by the thiochrome method. In this method the aneurine is oxidised by alkaline ferricyanide to thiochrome, which fluoresces in ultra-violet light. Under standard conditions the net fluorescence of the thiochrome is directly proportional to its concentration over a given working range. The method originated with Jansen,[1] and has undergone numerous modifications. Many of these are described in "Methods of Vitamin Assay,"[2] which was found by the Panel to be specially helpful in devising the fluorimetric method described below. This method was based on much experimental work too lengthy to describe in detail.

MAIN POINTS STUDIED

Extraction—The Panel experimented with digestion with pepsin, papain, "Taka-diastase" and "Clarase," combined with extraction with diluted hydrochloric acid, sulphuric acid or sodium acetate solution.

Purification of the extract—Washing with *iso*butanol was found to be less effective than adsorption on base-exchange silicate for removal of interfering substances, as shown by the fluorescence of the blanks and the percentage recoveries of added aneurine. The recovery

could vary widely for a given sample according to the level of addition of aneurine.[3] This led the Panel to adopt a cautious attitude to the use of recovery methods to correct for the presence of interfering substances.

Oxidation of aneurine to thiochrome—The use of a premixed alkaline ferricyanide solution gave more uniform results than separate additions of alkali and ferricyanide. Water-saturated *iso*butanol was found more satisfactory than dry *iso*butanol for extracting the thiochrome from the aqueous reaction mixture. A limit has been suggested for the amount of non-specific fluorescence in the *iso*butanol.

Measurement of fluorescence and calculation of results—Calculation of the net fluorescence is preferred to the use of a calibration curve. This calculation facilitated measurement of the fluorescence of the blanks, and was found to be effective with fluorimeters of the null-point type, at present mainly used in this country, as well as with fluorimeters of the direct-reading type.

Results obtained by the recommended method

Table I summarises results obtained by the collaborating laboratories on samples of malt extract, wheat germ, wheat flour, yeast extract and dried yeast. As samples of malted barley and of malt and oil had previously been found to give satisfactory results with the assay methods applied to malt extract, they were not included in the series. All the laboratories agreed in finding no significant amount of aneurine in the meat extract sample, which is therefore not included in Table I. Because of the difficulties that the Panel had encountered when assaying yeast preparations, three samples of dried yeast differing widely in potency were examined. Table II contains results obtained for the samples by biological and microbiological methods.

The results obtained by the proposed method showed a degree of agreement between different laboratories and with biological and microbiological results that seemed to justify the Panel recommending it as a method suitable for use by analysts to determine aneurine in the foodstuffs tested by the Panel. Further trials of the method are needed before it can be finally put forward as an official method of the Society. The Honorary Secretary of the Analytical Methods Committee (Dr. D. C. Garratt) will be pleased to receive comments and criticisms from any analyst using the method.

The results in Tables I and II were obtained in the following laboratories: Boots Pure Drug Co., Nottingham; Cereals Research Station, St. Albans; Distillers Co., Ltd., Research Department, Epsom; the laboratories of D. W. Kent-Jones and A. J. Amos, London, W.5; Glaxo Laboratories, Greenford; Government Laboratory, London, W.C.1; Lyons Laboratories, London; National Institute for Research in Dairying, Shinfield; Nederlandsch Institut voor Volksvoeding, Amsterdam; Ovaltine Research Laboratories, King's Langley; Roche Products, Welwyn Garden City; Vitamins Ltd., Research Laboratories, London; Wellcome Chemical Works, Dartford.

Table I

Collaborative results by the recommended method

	Aneurine found, μg per g						
					Dried yeast		
Laboratory	Malt extract	Wheat germ	Wheat flour (85%)	Yeast extract	Sample 1	Sample 2	Sample 3
A	2·7	22		55	29	17	45
D	4·0	21	3·8	54		20	47
E					24	14	54
F						15	
G	3·9	21		52	27		
I					32		
J	3·7	21	3·8	61	28		
K						16	
L	3·9	20		47	27	19	57
M	3·5	23	3·8	39			
N			3·6		37		47
Means	3·62	21·3	3·75	51·3	29·1	16·8	50

The data of Table I have been examined by Dr. E. C. Wood with a view to assessing the precision of the method. As the amount of aneurine in the various samples differed so

widely, it was first necessary to convert all the individual results into percentages of the mean result for each sample in turn. This has been done in Table IA, omitting references to particular laboratories.

TABLE IA

COLLABORATIVE RESULTS EXPRESSED AS PERCENTAGES OF THE CORRESPONDING MEAN RESULT

Malt extract	Wheat germ	Wheat flour	Yeast extract	Dried yeast		
				Sample 1	Sample 2	Sample 3
74·7	103·1	101·3	107·1	99·5	101·0	93·3
110·6	98·5	101·3	105·2	82·4	118·8	97·4
107·9	98·5	101·3	101·3	92·6	83·2	111·9
102·3	98·5	96·0	118·8	109·8	89·1	97·4
107·9	93·7		91·6	96·1	95·1	
96·8	107·8		76·0	92·6	112·9	
				127·0		

It is apparent that the agreement between laboratories is much better for some materials tested than for others. The coefficient of variation, which is the standard error expressed as a percentage of the mean, is in fact 4·9 per cent. for the two wheat products together, as compared with 13·5 per cent. for the four yeast products together. The appropriate test shows a high probability that this implies a real difference in the precision of the method when applied to these two classes of substance. The mean coefficient of variation for all the samples together is 11·9 per cent., though it is doubtful whether this pooling of all the results is justified. It is safer to say that when a sample of wheat flour or germ is being examined in different laboratories, 19 out of 20 of the results obtained should be found in the long run to lie within ± 10 per cent. of the mean for that particular sample; but if a yeast product is being examined the corresponding range will be ± 27 per cent. Agreement between replicate results in the same laboratory will of course be closer than this; the data available provide no evidence for assessing the "within laboratory" precision.

TABLE II

RESULTS BY BIOLOGICAL AND MICROBIOLOGICAL METHODS

Laboratory	Method used	Aneurine found, μg per g						
		Malt extract	Wheat germ	Wheat flour (85%)	Yeast extract	Dried yeast		
						Sample 1	Sample 2	Sample 3
K	Rat growth					45		
N	Bradycardia		20			27		
P	Rat growth						17	54
F	Yeast fermentation	3·9	21	3·3		38		
J	*Lactobacillus fermentum*	4·1	23		58			
Mean value of the collaborative fluorimetric results (from Table I)		3·62	21·3	3·75	51·3	29·1	16·8	50

RECOMMENDED METHOD

APPARATUS—

Base-exchange tubes—These are to be made of glass, the upper part being not less than 15 cm long and 0·8 to 1·0 cm in internal diameter and the lower part being narrow-bore tubing of suitable length. The lower end may be fitted with a tap or other method of controlling the rate of flow. A reservoir to contain at least 30 ml may be attached to the upper part.

Oxidation vessels—These may be stoppered measuring cylinders, stoppered bottles, separating funnels or large boiling tubes of about 100 ml capacity. Fluorescent grease must not be used to lubricate taps; glycerin or silicone may be used.

Fluorimeter—This may be of the direct reading type, measuring in deflections, or the null-point type, measuring in densities.* The exciting radiation must be within the range 300

*Some null-point type instruments read also in "transmission." Here transmission $= \frac{1}{\text{antilog density}}$.

to 400 mμ; it is most conveniently obtained at suitable intensity by the use of a high-pressure mercury-vapour lamp, type MB, in conjunction with a primary filter. A secondary filter* transmitting mainly light between 400 and 450 mμ is placed between the fluorescent solution and the photo-cell.

REAGENTS—

All chemicals should be of analytical reagent quality or the purest otherwise obtainable.

Ethanol—Redistilled in all-glass apparatus.

Sodium hydroxide, 15 per cent. solution—Dissolve 15 g of sodium hydroxide in water and dilute to 100 ml.

Potassium ferricyanide, 1 per cent. solution—Dissolve 1 g of potassium ferricyanide in water and dilute to 100 ml. This reagent has been found to be stable for at least a week if kept cool and in the dark, preferably in a brown bottle.

Alkaline potassium ferricyanide solution—Dilute 3 ml of 1 per cent. potassium ferricyanide to 100 ml with cool 15 per cent. sodium hydroxide solution. Prepare just before use.

Hydrochloric acid, approximately 0·2 N solution—Dilute 17 ml of concentrated hydrochloric acid to 1 litre with water.

Sodium acetate, 2·5 M solution—Dissolve 205 g of anhydrous sodium acetate (CH_3COONa) or 340 g of $CH_3COONa.3H_2O$ in water and dilute to 1 litre.

iso*Butyl alcohol (water-saturated)*—Steam distil commercial *iso*butyl alcohol in an all-glass apparatus. The fluorescence of the distillate should be not more than that of a 1 in 100 dilution in approximately 0·1 *N* sulphuric acid of the quinine standard (see p. 131), that is, of a diluted solution containing 0·01 μg of quinine sulphate per ml.

Enzyme solution—Prepare a fresh solution daily from a suitable source of phosphatase.† Suspend, with thorough shaking, 6 g of the enzyme preparation in 2·5 *M* sodium acetate solution and dilute to 100 ml with additional sodium acetate solution. Each batch of material used as a source of phosphatase should be tested for aneurine by this fluorimetric method and the necessary correction applied.

Potassium chloride, 25 per cent. solution—Dissolve 250 g of potassium chloride in distilled water and dilute to 1 litre. The reagent is stable indefinitely.

Acid potassium chloride solution—Dilute 8·5 ml of concentrated hydrochloric acid to 1 litre with 25 per cent. potassium chloride solution. The reagent is stable indefinitely.

Base-exchange silicate—This consists of an artificial zeolite in the form of a granular powder of 60 to 90 mesh size, tested for its suitability for adsorbing and eluting aneurine under the given conditions (at least 90 per cent. "recovery" of aneurine should be obtained under the given conditions).‡

Activate the base-exchange silicate as follows: Place a convenient quantity (100 to 500 g) of the base-exchange silicate in a suitable beaker, add sufficient hot 3 per cent. acetic acid solution to cover the material and maintain the temperature at about 100° C for 10 to 15 minutes, stirring frequently. Allow the mixture to settle and decant the supernatant liquid. Repeat the washing three times with hot 25 per cent. potassium chloride solution, and finally wash with boiling water until the last washing gives no reaction for chloride. Dry the material at approximately 100° C and store in a well-closed container.

Stock aneurine solution, 100 μg per ml—Prepare this from the British Standard Preparation, or a sub-standard of equal purity. Dissolve a weighed amount of aneurine hydrochloride equivalent to 50 mg of the International Standard in sufficient 0·2 *N* hydrochloric acid to make 500 ml. This solution is stable for several months if stored in a refrigerator (*i.e.*, below 5° C).

Standard aneurine solution—Dilute 5 ml of stock aneurine solution, warmed to room temperature, to 100 ml with water. Transfer 10 ml of this dilution to a flask containing 200 ml of approximately 0·1 *N* sulphuric acid and 12·5 ml of sodium acetate solution and dilute to 250 ml with distilled water. The final concentration of aneurine is 0·2 μg per ml. This solution is stable for at least a week if stored in the refrigerator.

* Chance OX1, 1·5 to 2·0 mm thick, has been found suitable for the primary filter, and Chance OB2 (blue), 1·5 to 2·0 mm thick, for the secondary filter.

† Taka-diastase (diluted with lactose) (Parke Davis & Co., London) and Clarase (Takamine Laboratories, Clifton, N.J., U.S.A.), have been found suitable.

‡ Decalso F, supplied by the Permutit Co., Ltd., London, has been found suitable.

Stock quinine sulphate solution (100 μg *per ml*)—Dissolve 0·025 g of quinine sulphate, B.P., in sufficient 0·1 *N* sulphuric acid to make 250 ml. This solution is stable for twelve months if stored in a dark brown bottle at a temperature below 5° C.

Quinine standard (1 μg *per ml*)—Dilute 10 ml of stock quinine sulphate solution to 1 litre with 0·1 *N* sulphuric acid. This solution is stable for three months if stored in a brown bottle at a temperature below 5° C. Any solution that has been exposed to ultra-violet light in the fluorimeter should be discarded.

Acetic acid, 3 per cent. solution—Dilute 30 ml of acetic acid to 1 litre with distilled water.

Bromocresol green indicator—Triturate 100 mg of bromocresol green with 7·2 ml of 0·05 *N* sodium hydroxide and dilute with sufficient water, free from carbon dioxide, to make 200 ml.

Nitrogen gas in cylinders—If desired, an air current can be used instead.

Procedure

Extraction—

(*a*) *Sampling*—The material to be assayed, if solid, should pass a No. 30 B.S. sieve or a finer sieve, and should be well mixed just before withdrawal of the sample, to ensure homogeneity. If liquid, the material should be well stirred before sampling.

(*b*) Accurately weigh or pipette into a large boiling tube a sample (not more than 5 g) estimated to contain not more than 50 μg of aneurine. Add 65 ml of approximately 0·1 *N* hydrochloric acid or sulphuric acid. Digest the sample for 30 minutes in a bath of boiling water, with frequent mixing. The liquid must remain at a pH below 4·5 during the digestion. If at the end of the digestion it is not distinctly acid to the bromocresol green indicator, the extract should be discarded and a further quantity of the sample extracted with more concentrated acid.

(*c*) Cool the extract to below 50° C and adjust the pH to between 4 and 4·5 by addition of 2·5 *M* sodium acetate solution, using bromocresol green as external indicator. Add 5 ml of freshly prepared enzyme suspension, mix, and incubate at 45° to 50° C for 3 hours, or at 37° C overnight with addition of a drop of sulphur-free toluene.

(*d*) Cool to room temperature, centrifuge the mixture until the supernatant liquid is clear and quantitatively transfer the supernatant liquid to a 100-ml volumetric flask. Wash the residue by centrifuging successively with 10 ml, 10 ml and 5 ml of 0·1 *N* hydrochloric acid or sulphuric acid. Add the washings to the supernatant liquid and dilute the whole to 100 ml with water. This is the "original extract."

Purification—

(*a*) Plug the bottom of an adsorption column with glass wool, which should be lightly packed, and fill the column with 5 g of activated base-exchange silicate suspended in water. Allow the water to drain almost entirely, but leave enough to cover the base-exchange silicate, and pour in 5 ml of 3 per cent. acetic acid. Allow to drain as before.

(*b*) Transfer 25 ml of the "original extract" to the column by means of a pipette. Discard the filtrate that has percolated through the column. Wash the column with three successive portions, about 10 ml each, of boiling water and discard the washings.

(*c*) After washing the column, pour through 10 ml of almost boiling acid potassium chloride solution from a supply kept hot in boiling water. Collect the eluate in a stoppered 25-ml graduated cylinder. Add a second 10-ml portion when all of the first portion has entered the base-exchange silicate and collect the eluate in the same cylinder. When this second portion has drained through, cool the eluate to room temperature, dilute to 25 ml with acid potassium chloride solution and mix well. This is the "sample eluate."

Oxidation to thiochrome—

In this and all subsequent stages undue exposure of the solutions to direct daylight or other source of ultra-violet light must be avoided.

(*a*) Pipette 5 ml of sample eluate into each of two oxidation vessels.

(*b*) Start a stream of nitrogen or air bubbling through the solution in vessel number 1, add 5 ml of alkaline potassium ferricyanide solution and then add 25 ml* of water-saturated *iso*butyl alcohol, the current of nitrogen or air still being continued. Shake (or continue vigorous bubbling) for 90 seconds.

* See footnote on p. 132.

(*c*) Start a stream of nitrogen or air bubbling through the solution in vessel number 2, then add 5 ml of 15 per cent. sodium hydroxide solution, followed by 25 ml* of water-saturated *iso*butyl alcohol, then continue as in step (*b*). This is the "unknown blank."

(*d*) Repeat steps (*a*), (*b*) and (*c*) with 5 ml of standard aneurine solution in place of the sample eluate. The solution from step (*c*) is the "standard blank."

CAUTION. *To avoid changes in experimental conditions the oxidation of all solutions used in a given assay should be carried out in immediate succession. Similar precautions must be taken in the reading of their fluorescence.*

SEPARATION OF THIOCHROME SOLUTION AND MEASUREMENT OF ITS FLUORESCENCE—

(*a*) After the solutions have stood for a few minutes to allow complete separation, add 1 ml of ethanol to the upper layer in each vessel and stir the upper layer gently until it is clear, taking care to avoid disturbing the aqueous layer.

(*b*) Take off each upper layer into a cuvette and measure its fluorescence against that of the quinine standard, if a null-point fluorimeter is being used, or as direct deflections if a deflection instrument is being used. The blank should exhibit only faint fluorescence.

"RECOVERY" EXPERIMENT—

Repeat the above procedure, including the steps of extraction, purification, conversion to thiochrome, separation of thiochrome solution and measurement of fluorescence, with a "recovery" experiment, made by adding, to another portion of the sample that is the same weight as that previously taken, a volume of the stock aneurine solution containing an amount of aneurine similar to the amount expected in that weight of sample.

CALCULATION—

If the fluorescence has been measured on an instrument provided with a density scale, convert all densities into antilogs and take reciprocals of these.†

Let U = reciprocal for unknown;
U_B = reciprocal for unknown blank;
S = reciprocal for standard;
S_B = reciprocal for standard blank;
V = volume of original solution put through base-exchange silicate.

If the fluorescence intensities have been measured as deflections, these are used instead of the reciprocals of antilogs. Then the aneurine content of the sample in μg per g =

$$\frac{U - U_B}{S - S_B} \times \frac{1}{5} \times \frac{25}{V} \times \frac{100}{\text{g of sample taken}}$$

The factor 1/5 converts the reading to μg per ml instead of μg per 5-ml aliquot. Since the final volume of eluate is 25 ml, the factor 25/V corrects for volume changes during adsorption and elution. If the suggested 25 ml is adsorbed, this factor becomes unity.

Note—This calculation assumes that the fluorescence of the thiochrome solution in the unknown is less than that of the quinine standard. If it is not, the assay should be repeated using a smaller amount of the material.

USE OF RECOVERY EXPERIMENT DATA—

Calculate the percentage recovery of the added aneurine from the following formula—

$$\frac{A_R - A_U}{A_A} \times 100$$

where—

A_U = aneurine content of sample in μg per g calculated as above;
A_R = aneurine content of sample with added aneurine calculated as above;
A_A = μg of aneurine added to each gram of sample.

* If 25 ml of water-saturated *iso*butyl alcohol does not provide sufficient solution for conveniently filling the particular cuvettes in use, this volume can be increased to some precise higher figure, *e.g.*, 30 ml, which must then be maintained throughout all experiments.

† If the instrument reads "transmissions," use those readings instead of reciprocals of antilog density; U, U_B, S and S_B are then the readings themselves.

This percentage recovery provides an indication of the effect of disturbing factors, including the quenching effect of impurities, but should not be relied upon to make a satisfactory correction for interfering factors. In general, if the percentage recovery falls below 80, the result of the assay should be considered unsatisfactory.

REFERENCES

1. Jansen, B. C. P., *Rec. Trav. Chim. Pays-Bas*, 1936, **55**, 1046.
2. "Methods of Vitamin Assay," Association of Vitamin Chemists, Inc., New York, 1947.
3. Ridyard, H. N., *Analyst*, 1950, **75**, 634.

The Assay of Vitamin B_{12}

Part III*

Microbiological Estimation with *Lactobacillus lactis* Dorner by the Plate Method

BY W. F. J. CUTHBERTSON, H. F. PEGLER AND JOAN T. LLOYD

(*Presented at the meeting of the Biological Methods Group on Tuesday, May 23rd, 1950*)

The cup-plate method adapted by Bacharach and Cuthbertson to the rapid microbiological assay of aneurine and riboflavine has been found applicable to the estimation of vitamin B_{12} with a suitable strain of *Lactobacillus lactis* Dorner. The procedure involves the use of a medium similar to those found necessary for other strains of lactobacillus. Ascorbic acid is needed in the assay medium but not in that for preparation of the inoculum.

The technique is similar to that described previously for the method. Factors affecting the "zone" response of this organism to vitamin B_{12} have been investigated; they include the effects of vitamin B_{12c} and the deoxyribosides, times of incubation and standing before incubation, range of concentrations of the vitamin and density of the inoculum. As a result a standardised (2 + 2) assay design has been devised; this permits rapid estimates of potency over a range sufficiently wide and with fiducial limits sufficiently narrow for routine purposes, without the use of an inconveniently large number of assay plates.

A GROWTH factor for *Lactobacillus lactis* Dorner ATCC 8000 has been shown by Shorb[1] to be present in highly refined liver extracts in amounts proportional to the anti-pernicious-anaemia activity. This factor has been isolated by Rickes, Brink, Koniuszy, Wood and Folkers[2] and by E. L. Smith,[3] and shown to be responsible for the clinical activity of liver extracts against pernicious anaemia; it is now known as vitamin B_{12}. We were unable to develop a satisfactory assay procedure based on the data published by Shorb. In our experience the growth responses of this organism were highly irregular and not capable of giving reproducible results in normal microbiological assay procedures. *Lactobacillus fermentum* P 36, which in our hands gave very irregular results in standard techniques, could be used to assay aneurine with fair precision by the cup-plate method of Bacharach and Cuthbertson.[4] We therefore attempted to apply this technique to the estimation of vitamin B_{12} with *L. lactis*.

METHOD

In the cup-plate method a suitable agar medium, deficient in the factor under investigation and inoculated with the test organism, is poured into petri dishes and "cups" are cut out of the agar and filled with appropriate dilutions of standard or test solutions. On

* For particulars of earlier papers in this series (not in *The Analyst*), see reference list, p. 140.

incubation under suitable conditions, growth of the organism may occur in sharply defined circular "zones of exhibition" around the cups. The diameters of these zones have been found to be related to the concentration of the added growth factor and can be measured and used in the determination of the amount of growth factor present in the test solutions. The method developed in the estimation of vitamin B_{12} is very similar to the technique employed in the estimation of aneurine by Bacharach and Cuthbertson,[4] but modifications have been introduced in the light of observations by Cuthbertson and Lloyd[5] on the mode of growth of *L. lactis* and its response to vitamin B_{12}.

MAINTENANCE OF ORGANISM—

The culture of *L. lactis* is maintained by weekly transfer in soya bean medium (10 per cent. suspension of whole soya beans macerated in a Waring Blendor and strained through coarse muslin). The inoculum is prepared from this by subculture in 10 ml of complete basal medium. This medium is the same as that described in Table I except for the omission of agar and ascorbic acid and the addition of 0·005 to 0·02 μg of vitamin B_{12} (as liver extract) per ml. The inoculum is allowed to grow for 15 to 16 hours and is then centrifuged, washed once in saline and diluted with saline to correspond to turbidity 4 on the Burroughs Wellcome turbidity scale. Cultures more than 17 to 18 hours old are frequently unsatisfactory for assay purposes.

PREPARATION OF THE MEDIUM—

The medium is of the composition shown in Table I. The ingredients, except salts, are dissolved in about 800 ml of water; after adjustment of the pH to 6·8, the phosphates

TABLE I

COMPOSITION OF AGAR MEDIA DEFICIENT IN VITAMIN B_{12}

Acid casein hydrolysate	5 g	Nicotinic acid	1 mg
Glucose	10 g	Riboflavine	200 μg
Sodium acetate, A.R.	6 g	Adenine	10 mg
L-Cystine	200 mg	Guanine	10 mg
DL-Tryptophan	100 mg	Uracil	10 mg
Aneurine hydrochloride	200 μg	Tomato juice	50 ml
Biotin	2 μg	Tween 80†	1 g
Folic acid	2 μg	Salt solution C*	5 ml
p-Aminobenzoic acid	100 μg	Ascorbic acid	1 g
Calcium-D-pantothenate	200 μg	K_2HPO_4	0·5 g
Pyridoxamine	400 μg	KH_2PO_4	0·5 g
Water to make	1000 ml		
pH adjusted to	6·8		
Agar (N.Z.)‡	to 15 g		

* Salts C: a solution of 10 g of $MgSO_4.7H_2O$, 0·5 g of sodium chloride, 0·5 g of $FeSO_4.7H_2O$, 2·0 g of $MnSO_4.4H_2O$, and water to 250 ml.

† This amount of Tween 80 may be replaced by 0·39 g of Estax 36 or 29 (Watford Chemical Co.).

‡ From Davis Gelatine Ltd., 29, Mitre Street, E.C.3.

and salt solution are added. The precipitate formed during this procedure is removed by filtration through washed paper pulp on a Buchner funnel. The medium is made up to volume and adjusted to pH 6·8. The agar is added to this solution and dissolved by steaming for 1 hour. The medium is now filtered, while still hot, through washed paper pulp, distributed in 130-ml aliquots and kept in 250-ml flasks. The flasks are plugged and autoclaved at 10 lb steam pressure for 10 minutes. At this stage the medium may be kept for several weeks in a refrigerator.

PREPARATION OF ASSAY PLATES—

The flasks of medium are steamed for 35 minutes to melt the agar and are then cooled to 45° to 50° C in a thermostatically controlled enclosure at 45° C. One millilitre of 0·13 per cent. ascorbic acid solution is now added. Each flask is inoculated with 1 ml of a freshly prepared suspension of *L. lactis* adjusted to opacity 4 on the Burroughs Wellcome scale. The inoculum is well mixed in the medium, which is then distributed in the petri dishes at the rate of 12·5 ml per plate. The plates are cooled in a refrigerator for half an hour to harden the agar; they may be stored in a cold room overnight or even for a week if not needed

at once. The plates are cut with a sharp 8-mm No. 10 cork-borer; the resulting discs of agar are removed with a needle leaving the cups into which standard and sample solutions may be distributed.

TYPE OF PETRI DISHES REQUIRED—

For this technique it is essential to use perfectly flat petri dishes so as to obtain a layer of agar of uniform thickness. The diameters of the zones of exhibition vary widely with differences in thickness of the agar.

TIME OF INCUBATION—

After the plates have been prepared they are incubated at 37° C overnight. Further incubation does not alter the size of the zones of exhibition, but on certain subnormal media further incubation may lead to some improvement in the density of growth within the zones.

CONCENTRATION OF VITAMIN B_{12}—

The diameter of the zones of growth is proportional to the logarithms of the amounts of vitamin B_{12} placed in the cups; this is shown in Fig. 1. This relationship holds true over a wide range of concentrations. The clarity with which the rings are defined decreases

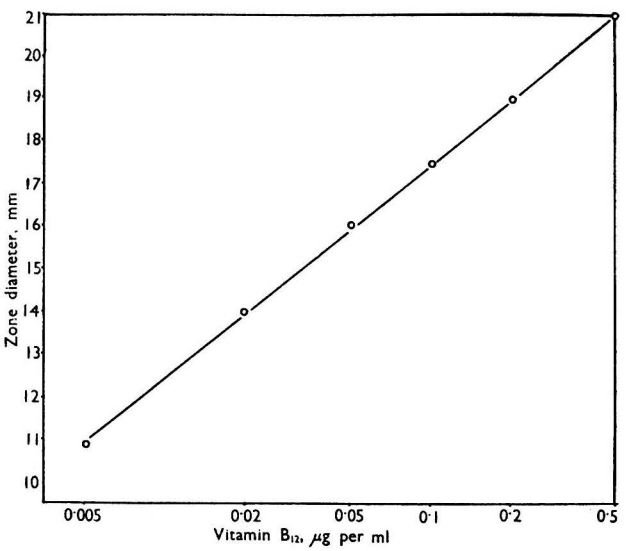

Fig. 1. Response of *L. lactis* Dorner ATCC 8000 to vitamin B_{12} in the cup plate assay. Each point corresponds to the average reading obtained from six cups

slightly as the concentration of vitamin B_{12} decreases; under our conditions well defined rings are obtained with concentrations of vitamin B_{12} in the range 0·01 to 0·5 µg of vitamin B_{12} per ml.

TIME OF STANDING BEFORE INCUBATION—

As in the assays of penicillin and streptomycin, the time the plates are allowed to stand before incubation but after distribution of sample and standard solutions in the cups affects the size of the growth zones obtained. This effect is variable from occasion to occasion, but typical results are shown in Table II.

EFFECT OF INOCULUM DENSITY—

The inoculum used affects both the size and the appearance of the zones of exhibition. In general, the heavier the inoculum the more sharply are the rings defined and the smaller are the zones. The effect of inoculum density is shown in Table III.

TABLE II

RELATIONSHIP BETWEEN ZONE DIAMETER AND TIME INTERVAL BETWEEN FILLING PLATES AND PLACING IN INCUBATOR

Two experiments performed on two different occasions

Concentration of vitamin B_{12} solution			Concentration of vitamin B_{12} solution		
Time interval, minutes	0·05 μg per ml Zone diameter, mm	0·2 μg per ml Zone diameter, mm	Time interval, hours	0·05 μg per ml Zone diameter, mm	0·2 μg per ml Zone diameter, mm
0	17·8	20·2	0	16·12	19·0
15	18·5	20·2	1·5	16·5	19·12
20	19·5	20·4	2·5	17·62	21·0
			3·5	17·75	20·9
			15·0	26·0	30·0

Each diameter is the average from three or four cups.

TABLE III

EFFECT OF INOCULUM DENSITY

Volume of inoculum (turbidity 4) per plate (12·5 ml of agar medium), ml	Diameter of zone of exhibition for vitamin B_{12} concentrations of—	
	0·05 μg per ml, mm	0·2 μg per ml, mm
0·01	21·2	24·4
0·02	20·8	23·6
0·05	19·9	23·5
0·1	19·6	23·6
0·2	17·9	20·4

Each diameter is the average from four cups.

ASSAY DESIGN AND RESULTS

For assay purposes, dilutions of standard vitamin B_{12} and test solutions are chosen to fall within the range 0·02 to 0·2 μg of vitamin B_{12} per ml, over which the relationship between log dose and zone diameter has been found linear (Fig. 1). Experiments have shown that the variance between zone diameters for the same concentration of vitamin B_{12} was much less when the cups used were on the same plate than when cups on different plates were compared; the "within plate" variance was about one-tenth of the "between plates" variance.

Owing to the size of the petri dishes (9 cm diameter) and the size of the zones (13 to 25 mm) normally encountered, not more than 5 or 6 cups may conveniently be used on a plate. Consequently, for routine assays, two cups on each plate are reserved for standard solutions, the concentration of vitamin B_{12} employed being 0·02 and 0·2 μg of vitamin B_{12} per ml. Two cups are normally used for the test solutions, which are diluted to fall within the range of 0·01 to 0·5 μg of vitamin B_{12} per ml. Two different concentrations of test solutions are used in the ratio of 1 to 10. The plates are filled in the following order—

(*i*) Lower test concentration.
(*ii*) 0·02 μg of vitamin B_{12} per ml.
(*iii*) 0·2 μg of vitamin B_{12} per ml.
(*iv*) Higher test concentration.

Three drops, 0·1 to 0·15 ml, of solution, measured with a standard dropping pipette (see Bacharach and Cuthbertson[4]) are placed in each cup; the same dropping pipette is used for all dilutions of test and standard solutions. After the cups have been filled, the plates are left for at least 10 minutes before being put in the incubator. This procedure has been adopted to minimise the effects of standing before incubation and of any differences between dropping pipettes.

After incubation the zone diameters may be measured with a ruler (transparent celluloid rules are suitable) or callipers, or after projection on to a screen.

The normal procedures (Irwin[6]) applicable to the calculation of the results of assays involving a linear relationship between log-dose and response may be applied and fiducial limits may be determined.

TABLE IV

ASSAY OF VITAMIN B_{12} IN LIVER EXTRACT

Variance analysis according to method of Irwin[6]

	Standard vitamin B_{12} (3 drops per cup)		Test preparation (3 drops per cup diluted as shown)	
	0·02 μg	0·2 μg	1/750	1/75
Plate 1	13·0	20·0	14·0	21·0
2	15·0	22·0	15·0	22·0
3	14·0	21·0	15·0	22·0
4	14·0	21·0	15·0	23·0
5	14·0	21·0	15·0	22·0
6	13·0	18·5	14·0	20·0
7	13·0	19·0	14·0	21·0
8	13·5	20·5	15·0	22·0
Average	13·7	20·4	14·6	21·6

ANALYSIS OF VARIANCE—

Source of variation	D.F.	Sum of squares	Mean square	Variance ratio	Significance
Common linear regression	1	374·1	374·7	19·72	significant
Difference due to substances	1	9·6	9·6	50·[illegible]2	significant
Departure from parallel regression	1	0·2	0·2	1·05	not significant
Bias due to plates	7	16·6	2·37	12·47	significant
Error	21	4·0	0·190		
Total	31	405·1			

Activity found: 21·64 μg per ml.

Fiducial range (P = 0·95): 19·49 μg per ml to 24·20 μg per ml; 90 per cent. to 112 per cent.

A typical assay result is shown in Table IV, together with the calculated fiducial limits. On average, fiducial limits of ±15 per cent. (P = 0·05) may be expected in assays of this type employing six plates, although narrower limits can be obtained with a greater number of plates. Assays by the plate method and by a tube technique employing *L. leichmannii* 313 (Lees and Emery[7]) have given comparable results (see Cuthbertson, Lloyd, Emery and Lees[3]).

INTERFERENCE FROM DEOXYRIBOSIDES

Under our conditions *L. lactis* responds to the deoxyribosides as well as to vitamin B_{12} (see Smith and Cuthbertson[9]). The response to thymidine is shown in Table V. Zones

TABLE V

RESPONSE OF *Lactobacillus lactis* TO THYMIDINE (PLATE TEST)

Thymidine concentration, μg per ml	Diameter of zone of exhibition
1	no zone
5	24 mm approx., indistinct zone
10	25·9 mm
20	28·1 mm
50	34 mm

Each diameter is the average from four cups

of exhibition of less than about 25 mm are not formed and only solutions containing 5 μg or more of deoxyriboside per ml give zones of exhibition. Up to 5 μg of deoxyribosides per ml cause only very slight interference in the plate test. Table VI shows the diameters of the zones of exhibition produced by mixtures of thymidine and vitamin B_{12}.

Deoxyriboside zones are readily distinguished from vitamin B_{12} zones, for the growth is much fainter and more diffuse. If vitamin B_{12} is present an inner zone of dense growth may be seen, but when the concentration of thymidine is high this zone is not well defined and cannot be measured accurately.

Samples containing deoxyribosides are readily detected by the appearance of the growth zones and also by the slope of the log-dose - response curve, which is much greater for deoxyribosides than for vitamin B_{12}, as should be clear from Table VI and Fig. 2.

TABLE VI

RESPONSE OF *L. lactis* TO MIXTURES OF THYMIDINE AND VITAMIN B_{12} (PLATE TEST)

Composition of solution		Diameters of zones of exhibition	
Vitamin B_{12}, μg per ml	Thymidine, μg per ml	Inner zone due to vitamin B_{12}, mm	Outer zone due to thymidine, mm
0·02	0	13·1	no zone
0·02	1	13·45	no zone
0·02	5	13·25	24·1
0·02	10	not measurable	26·6
0·02	20	"	28·0
0·02	50	"	30·1
0·2	0	17·05	no zone
0·2	1	17·1	no zone
0·2	5	17·2	23·2
0·2	10	not measurable	25·9
0·2	20	"	28·0
0·2	50	"	29·9

USE OF CHROMATOGRAPHY IN THE PLATE ASSAY

Interference due to the deoxyribosides may be eliminated by a technique previously indicated by Smith and Cuthbertson.[9] Micro-drops of about 1 to 4 μl, containing 0·004 to 0·04 μg of vitamin B_{12}, of test and standard solutions are placed about 1 inch from one edge of a square sheet of Whatman No. 4 filter-paper and about 1 inch apart. Micro-drops of saturated riboflavine solution are placed just beneath but not touching the positions occupied

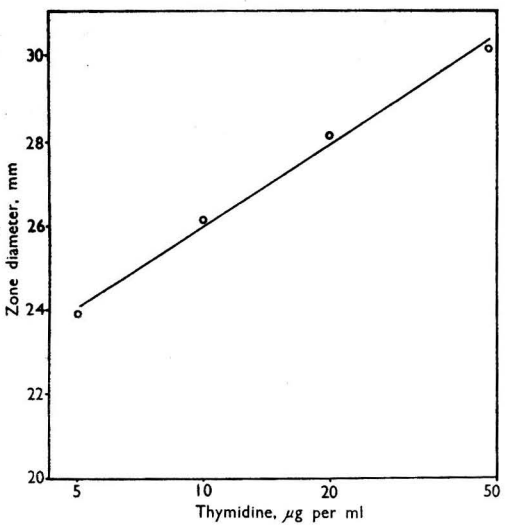

Fig. 2. Response of *L. lactis* Dorner ATCC 8000 to thymidine in the cup plate assay. Each point corresponds to the average reading obtained from four cups

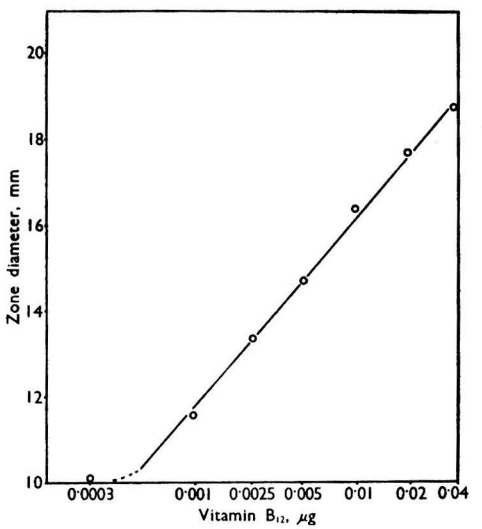

Fig. 3. Response of *L. lactis* Dorner ATCC 8000 to vitamin B_{12} after separation of deoxyribosides by partition chromatography. Each point represents the average zone diameter obtained with four aliquots applied to the filter paper

by the standard and test spots. The paper is dried in the incubator and then rolled into a cylinder and held in this way by metal paper-clips. The filter-paper cylinder is now placed in a beaker containing a layer of water-saturated *n*-butanol about 1 cm deep. Upward development is allowed to take place for about $1\frac{1}{2}$ to 2 hours, *i.e.*, until the riboflavine has passed through and about 0·5 cm beyond the sites at which the vitamin B_{12} and test solutions

had been placed. The paper is then removed and dried in the incubator, and when dried it is cut along the front reached by the riboflavine.

During this brief development the vitamin B_{12} does not move from its site of application, while the deoxyribosides (except cytosine deoxyriboside) travel with and beyond the riboflavine.

When a strip of paper to which the test and vitamin B_{12} solutions have been applied is placed on an inoculated agar test medium and incubated overnight, circular zones of exhibition are produced by the vitamin B_{12}. The type of response obtained is shown in Fig. 3. With only four 2-μl drops of each standard and test solution, fiducial limits ($P = 0{\cdot}05$) of 60 to 150 per cent. were obtained in the assay of samples containing 3 to 5 μg of vitamin B_{12} per ml.

Assay of vitamin B_{12} concentrates from fermentation liquors

In the assay of extracts from streptomyces the results by the plate and tube methods (Lees and Emery[7]) often differed widely, the plate technique almost invariably giving higher results; in some instances they were three times those of the tube assay. It was noticed that the zones of exhibition obtained with these materials were not as sharp as those normally encountered and it was at first suspected that the interference was due to some substance other than vitamin B_{12}; however, it was not possible to separate the vitamin B_{12} activity from the postulated interfering agent.

It has recently been shown that another red cobalt-containing clinically active compound, in addition to the previously described members of the vitamin B_{12} group, is present in streptomyces fermentation products (Smith[10]). This substance, designated B_{12c} in accordance with the suggestion of Buchanan, Johnson, Miles and Todd,[11] itself yields poorly defined zones of exhibition in the plate test under conditions used for vitamin B_{12} assay; weight for weight it appears to be 3 to 4 times as active as vitamin B_{12}, although in tube assays and clinically (Ungley, Mollin and Dacie[12]) its activity is approximately the same as that of vitamin B_{12}. We have not been able to overcome the difficulty that thus appears to be due to vitamin B_{12c}. Until a suitable technique becomes available for doing this, results of plate assays on fermentation liquors may be very unreliable.

Failure to assay solid materials containing adsorbed vitamin B_{12}

Vitamin B_{12} is firmly adsorbed by fuller's earth. The assay of such adsorbates presents some difficulties. It was not found possible to elute completely all the vitamin B_{12} taken up by this material. When fuller's earth adsorbate was placed on plates prepared for vitamin B_{12} assay, growth was noticed around the sites of application. Attempts were therefore made to develop an assay from this observation. Plates were prepared and cut in the normal way and aliquots of aqueous dispersions of the adsorbate were placed in the cups. After incubation the zones of growth around the cups containing the fuller's earth were found to be closely comparable in qualitative appearance to the zones around the cups in which standard vitamin B_{12} had been placed; further, the assay data showed that the nature of the response curves obtained with the fuller's earth adsorbate were not different from those obtained with aqueous solutions of vitamin B_{12} and that assays were statistically valid when tested by the usual mathematical analysis. The results were, however, much lower than those obtained by chemical assay (Fantes and Ireland[13]) or by elution techniques, *e.g.*, one adsorbate gave 25, 48 and 50 μg per g respectively when tested by the plate assay, an elution technique and the chemical method. These observations are reported because with adsorbates the plate method gives results that are very consistent and appear to be valid when checked by methods in frequent use; for instance, another sample of adsorbate was tested on two separate occasions and gave results all within the range of 11 to 13 μg per g, although on other grounds it was believed to contain at least 30 μg of vitamin B_{12} per g.

A fuller account of the assay of adsorbates is to be reported later.

Discussion of the method

The main advantages of the cup-plate technique are its speed and simplicity. Zones of exhibition are formed overnight, but they appear and may be measured after incubation for as little as 6 hours in favourable circumstances, whereas the tube methods require at least overnight incubation for *L. leichmannii*[14] and 4 days for *Euglena gracilis*.[15] With the cup-plate method samples need not be sterilised; in the tube methods strict asepsis is

essential and all samples must be sterilised by filtration through collodion or sintered glass. Autoclaving or seitz filtration may lead to loss of vitamin B_{12}.

The cup-plate method is relatively insensitive. Samples containing less than 50 mμg per ml cannot be assayed satisfactorily, whereas the tube methods employing *L. leichmannii* and *Euglena gracilis* may be used for concentrations as low as 2·0 mμg or 2 μμg of vitamin B_{12} per ml, respectively, in favourable circumstances.

Thymidine and the other deoxyribosides may replace vitamin B_{12} in the nutrition of *L. lactis*[16] or *L. leichmannii*.[17] In the tube techniques growth due to these substances is not readily differentiated from that due to vitamin B_{12}, but in the plate method the appearance of the growth zones due to the different types of substances is at once discernible, because the deoxyribosides give wide diffuse zones of exhibition, while vitamin B_{12} under our conditions gives dense sharply defined zones of growth; hence confusion does not arise and interference is noticed at once. Similarly, though antibiotics and toxic substances in general may interfere in any microbiological test, such interference is readily detected in the plate test by the appearance of zones of partial growth or rings of growth inhibition.

Summary

Lactobacillus lactis Dorner ATCC 8000 requires vitamin B_{12} for its normal nutrition and has been found suitable for assay of the vitamin by the cup-plate method.

Vitamin B_{12c} and the deoxyribosides interfere with the procedure. The effect of the latter, but not of the former, can be eliminated by combining the method with paper chromatography. The presence of deoxyribosides is generally apparent from the type of zone produced.

The combined chromatographic and microbiological procedures make it possible, with a (2 + 2) assay design, dose ratios of 10 to 1 and incubation overnight, to attain a satisfactory degree of precision on a few microlitres of vitamin B_{12} solution.

The method gives results in good accord with those given by the tube method based upon growth of *Lactobacillus leichmannii* 313.

Our thanks are due to Dr. T. G. Brady, Trinity College, Dublin, for a very generous gift of thymidine and to Mr. B. Basil, Glaxo Laboratories Ltd., Greenford, for statistical calculations.

References

1. Shorb, M. S., *J. Biol. Chem.*, 1947, **167**, 455.
2. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.
3. Smith, E. L., *Nature*, 1948, **161**, 638.
4. Bacharach, A. L., and Cuthbertson, W. F. J., *Analyst*, 1948, **73**, 334.
5. Cuthbertson, W. F. J., and Lloyd, J. T., *J. Gen. Microbiol.*, 1951, **5**, 416.
6. Irwin, J. O., *J. Roy. Statist. Soc.*, 1937, Suppl. 4, 1.
7. Lees, K. A., and Emery, W. B., *Biochem. J.*, 1949, **45**, ii.
8. Cuthbertson, W. F. J., Lloyd, J. F., Emery, W. B., and Lees, K. A., *J. Pharm. Pharmacol.*, 1949, **1**, 705.
9. Smith, E. L., and Cuthbertson, W. F. J., *Biochem. J.*, 1949, **45**, xii.
10. Smith, E. L., *Proc. Roy. Soc. Med.*, 1950, **43**, 535.
11. Buchanan, J. G., Johnson, A. W., Miles, J. A., and Todd, A. R., *Chem. and Ind.*, 1950, 426.
12. Ungley, C. C., Mollin, D. L., and Dacie, J. V., *Lancet*, 1950, **1**, 353; *Proc. Roy. Soc. Med.*, in the press.
13. Fantes, K. H., Ireland, D. M., and Green, N., *Biochem. J.*, 1950, **46**, xxxiv.
14. Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, 1948, **176**, 1465.
15. Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L., and Jukes, T. H., *Proc. Soc. Exp. Biol. Med.*, 1949, **70**, 118.
16. Kocher, V., and Schindler, O., *Intern. Rev. Vit. Res.*, 1949, **20**, 441.
17. Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

Note—Reference 5 is to Part I of this series.

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DISCUSSION

MR. K. A. LEES confirmed that when vitamin B_{12c} was assayed by the *L. lactis* plate technique the values obtained were 3 to 4 times as high as those obtained by the tube methods or physical chemical procedures.

In Mr. Lees' laboratories the zones of growth with vitamin B_{12c} in plate tests employing *L. leichmannii* 313 were poorly defined. The type of growth obtained could, however, be readily differentiated from the responses given by thymidine, although the edges of the zones were not sharp, because the growth within the zones was as dense as that observed with vitamin B_{12}. To overcome these disadvantages a plate method employing an *E. coli* mutant[1] has been developed and all pure factors so far tested give clearly defined zones. The *E. coli* technique has the further advantage that a very simple medium is employed.

REFERENCE TO DISCUSSION

1. Bessell, Christine J., Harrison, Eleanor, and Lees, K. A., "Assay of Vitamin B_{12} with a Mutant of *Escherichia Coli*," *Chem. and Ind.*, 1950, 561.

The Assay of Vitamin B_{12}

Part IV*

The Microbiological Estimation with *Lactobacillus leichmannii* 313 by the Turbidimetric Procedure

BY W. B. EMERY, K. A. LEES AND J. P. R. TOOTILL

(*Presented at the meeting of the Biological Methods Group on Tuesday, May 23rd, 1950*)

Details are presented of a microbiological tube assay for vitamin B_{12} with *Lactobacillus leichmannii* 313 as test organism. The procedure employs materials that are readily available in this country.

Statistical analyses of a (3 + 3) assay and a standard response curve are given and show that the method is sufficiently sensitive and accurate for routine use.

Two papers[1,2] from these laboratories describe work on factors affecting the microbiological techniques used for assaying vitamin B_{12}; the cup-plate method is described in detail elsewhere in this issue.[3] In this paper we give the corresponding details for the so-called "tube assay" with a turbidimetric procedure for measuring the growth of the micro-organism. The conditions used are based on the work described in one of the papers cited above.[2]

As was also found by Cuthbertson, Pegler and Lloyd,[3] it has been our experience that *Lactobacillus lactis* Dorner[4,5] gives a very irregular growth response to graded doses of vitamin B_{12}. We therefore carried out further work on the use of *Lactobacillus leichmannii* 313 in the vitamin B_{12} assay, this organism having been recommended by Skeggs, Huff, Wright and Bosshardt[6]; other workers have already reported on its use.[7] As already briefly reported,[8] our assay method depends on the inability of *L. leichmannii* 313 to synthesise vitamin B_{12} under defined conditions, so that there is a direct relationship between the growth of this organism and the concentration of vitamin B_{12} in the test medium over a certain range.

Attempts to devise an assay suitable for routine use, employing the medium of Snell, Kitay and McNutt,[9] were not successful, the chief difficulty being the production of a dense precipitate during the autoclaving of the medium. This medium was modified by the omission of enzymatic casein digest and reduction of the level of salts B. To prevent precipitation in the tube and consequent high blanks, the medium was preheated in bulk and filtered hot.

Hoffman *et al.*[7] reported that thioglycollic acid increased the growth response of *L. leichmannii* to vitamin B_{12}. This was thought to be due to protection of the vitamin

* For particulars of earlier papers in this series (two not in *The Analyst*), see reference list, pp. 145–146.

during the autoclave cycle. Our own work on the effect of reducing agents and oxygenation,[2] as well as the recent work of Koditschek, Hendlin and Woodruff[10] on the nutrition of *L. lactis* Dorner, indicates that vitamin B_{12} functions as an anti-oxidant for the test organism, and consequently there is a direct relationship between the E_H of the medium and the vitamin B_{12} requirement. We have been able to show the mathematical relationship between the response of *L. leichmannii* and the depth of medium in the assay tubes.[2]

Tube assay technique

Preparation of medium—

Solutions A and B are prepared according to the details in Table I and mixed as required, because it has been found that the complete medium does not keep well.

Table I

Assay medium

Solution A (double strength)—	
Sodium acetate, A.R.	20 g
Sodium citrate, A.R.	20 g
Salts A, Salts B } Snell and Wright[11]	12 ml, 120 ml
Acid hydrolysate of casein (based on total solids)	8 g
Tween 80 (purified for T.B. test)	6 g
or	
Crill 10 (Croda Works, Goole)	20 mg
Sodium oleate	—
L-Cystine	400 mg
Glucose	20 g
Glass-distilled water to	1 litre
pH adjusted to	6·8
Solution B (forty times single strength)—	
Adenine, guanine and uracil (of each)	200 mg
DL-Tryptophan	2 g
p-Aminobenzoic acid	2 mg
Asparagine	2 g
Calcium-D-pantothenate	4 mg
Nicotinic acid	20 mg
Riboflavine	8 mg
Aneurine hydrochloride	4 mg
Biotin	40 μg
Folic acid	200 μg
Pyridoxal	4 mg
Pyridoxamine	4 mg
Glass-distilled water to	500 ml

The pH of solution A is adjusted to 6·8. The solution is then heated to boiling and filtered with the aid of kieselguhr and its pH is then re-adjusted to 6·8. It is stored in a refrigerator and may be used over a period of 5 to 6 days.

Solution B is stored in a refrigerator at pH 3·0 to 4·0 and may be used for a period of 10 to 14 days.

Preparation of acid hydrolysate of casein—

Five hundred grams of "vitamin-free" casein (Glaxo Laboratories Ltd.) are heated under reflux with 2·5 litres of 6 *N* hydrochloric acid for 9 hours. The hydrolysate is concentrated to a syrup *in vacuo* and diluted to 4·5 litres with glass-distilled water; after the pH has been adjusted to 3·0 with solid sodium hydroxide, the mixture is filtered and treated twice with 50 g of Sutcliffe Speakman No. 5 charcoal, each absorption involving stirring for 30 minutes. The final solution is diluted to 5 litres. This procedure yields a hydrolysate containing 1·0 to 1·25 per cent. w/v of total nitrogen.

We normally preserve the hydrolysate by freeze-drying to a dry powder; alternatively it may be stored in a refrigerator under toluene. Each batch of acid-hydrolysed casein is tested over a suitable range of concentration.

We have recently found casein hydrolysate supplied by Allen and Hanbury Ltd. to be satisfactory.

CLARIFIED TOMATO JUICE—

Commercial canned tomato juice is filtered, with the aid of kieselguhr, through a Whatman No. 1 filter-paper supported on a No. 54 paper. The filtrate is a clear pale straw colour. The filtered tomato juice is stored in the refrigerator and is used over a period of 3 days only.

Some brands of commercial tomato juice do not contain the factor required by *L. leichmannii*. A large quantity of a tested brand is therefore bought and stored in the cold room: we have found "Crest" brand tomato juice to be satisfactory.

MAINTENANCE OF TEST ORGANISM AND PREPARATION OF INOCULUM—

L. leichmannii is maintained by daily transfer in liquid medium (yeast extract, 1 per cent.; tomato juice, 5 per cent.; separated milk to 100 per cent.). More than three hundred transfers have not resulted in any detectable alteration in the sensitivity of the organism.

TABLE II

ANALYSIS OF DAILY RESPONSE CURVE

Dose, mμg per tube	0·23	0·35	0·53	0·79	1·19	1·78	2·67	4·00	Totals	
Responses ..	0·15	0·28	0·36	0·51	0·68	0·85	1·06	1·21		
Rack I	0·14	0·20	0·36	0·53	0·63	0·80	0·91	1·22		
Sub Totals ..	0·29	0·48	0·72	1·04	1·31	1·65	1·97	2·43	9·89	
Responses ..	0·19	0·23	0·34	0·54	0·64	0·71	0·09	1·29		
Rack II	0·19	0·25	0·37	0·45	0·61	0·85	0·93	1·24		
Sub Totals ..	0·38	0·48	0·71	0·99	1·25	1·56	2·02	2·53	9·92	
Responses ..	0·17	0·23	0·33	0·57	0·65	0·94	1·09	1·18		
Rack III	0·16	0·23	0·38	0·49	0·68	0·83	1·12	1·24		
Sub Totals ..	0·33	0·46	0·71	1·06	1·33	1·77	2·21	2·42	10·29	Regression coefficients
Totals	1·00	1·42	2·14	3·09	3·89	4·98	6·20	7·38	30·10	+0·627,083
Orthogonal Coefficients	−7	−5	−3	−1	+1	+3	+5	+7	+77·88	+0·077,262
	+7	+1	−3	−5	−5	−3	+1	+7	+10·02	+0·009,940
Means	0·167	0·237	0·357	0·515	0·648	0·830	1·033	1·230		
Regression values	0·156	0·251	0·365	0·500	0·655	0·829	1·023	1·237		

ANALYSIS OF VARIANCE FOR HOMOGENEITY—

Source	D.F.	Sum of squares	Mean square	Variance ratio	Significance
Doses	7	6·121,62			
Racks	2	0·006,20	0·003,100	1·251	none
Interaction	14	0·029,87	0·002,134	0·861	none
Error	24	0·059,50	0·002,479		
Total	47	6·217,19			

ANALYSIS OF VARIANCE FOR REGRESSION—

	D.F.	Sum of squares	Mean square	Variance ratio	Significance
Quadratic	1	0·099,60	0·099,60	40·178	very high
Linear	1	6·017,16	6·017,16	2427·656	
Deviation	5	0·004,86	0·000,97	0·391	none
Doses	7	6·121,62			

Standard error = 0·0498

The inoculum is prepared by two daily transfers in basal assay medium enriched with purified liver extract to give a final vitamin B_{12} concentration of 0·015 μg per ml. Volumes of 0·5 ml are subcultured into 10-ml volumes of enriched basal medium in 6 inch × ¾ inch tubes and the cultures are incubated at 37° C for 18 to 20 hours. The cells from the second

transfer in inoculum medium are centrifuged, washed once in sterile physiological saline and re-suspended in sterile saline to Burroughs Wellcome opacity 4. This suspension is diluted 1 to 8 in a mixture of equal volumes of sterile autoclaved physiological saline and clarified tomato juice, the latter having been previously sterilised by filtration through a sintered-glass filter; 0·5 ml of this inoculum is used for each assay tube.

PREPARATION AND DISTRIBUTION OF TUBE MEDIUM—

Solutions A and B are mixed in the following proportions and 8·5 ml are introduced into each of several 6 inch × ¾ inch standard tubes by means of an Ayling filler.

For 500 assay tubes the following are mixed—

Solution A	2500 ml
Solution B	125 ml
Glass-distilled water to	4250 ml

By the additions of 1 ml of suitably diluted test sample and 0·5 ml of inoculum, the volume is increased to 10 ml and the medium reduced to "single strength." The pH is re-adjusted to 6·8 if necessary.

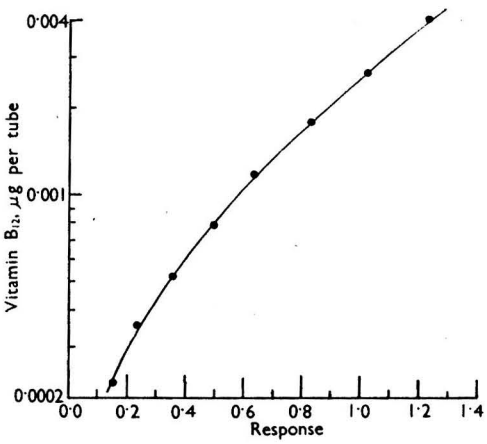

Fig. 1. A typical response curve

The assay tubes are covered by aluminium caps and autoclaved at 15 lb pressure for 10 minutes, the pressure in the autoclave being released quickly and reduced to atmospheric pressure in 2 minutes. We have found that slight differences in the autoclave cycle profoundly influence the slope of the growth response curve. Increasing the autoclave cycle to 30 minutes decreased the accuracy of the test by reducing the range of growth available for the daily response curve. We have not found that increased autoclaving will produce a medium in which *L. leichmannii* no longer requires vitamin B_{12}, as reported by Shaw[12] for *L. lactis* Dorner.

The daily reference curve is obtained by six-tube replicates, each of eight doses of pure crystalline vitamin B_{12}, logarithmically spaced in the range 0·00016 and 0·0027 μg per tube. A few blank tubes are set up as controls each day to indicate what growth, if any, occurs in the absence of vitamin B_{12}. A typical response curve is shown in Fig. 1.

The analysis of a daily response curve is given in Table II; it shows that the standard deviation of a reading is approximately 0·05.

Test samples are diluted according to the estimated potency and sterilised by filtration through sintered-glass filters. One millilitre of each dilution is added to the assay tubes and followed by 0·5 ml of inoculum. The tubes are shaken to ensure thorough mixing of the inoculum and incubated in an incubator room at 37° C for 17 hours. Small laboratory type incubators have not been found suitable. After incubation, the growth is stopped by the addition of 2 drops of 40 per cent. formaldehyde solution to each tube or by steaming; the turbidities are read on a Spekker photo-electric absorptiometer in the normal way.

Dr. W. F. J. Cuthbertson (personal communication) has now found it possible to replace the Tween 80 by Estax 36 (Watford Chemical Company) and the tomato juice filtrate by a

mixture of 250 μg of fumaric acid, 250 μg of sodium ethyloxalacetate* and 100 μg of DL-alanine per millilitre of single strength medium. These constituents may be incorporated in the "forty times strength" vitamin solution. Dr. Cuthbertson has also reported that certain batches of inoculum medium, *i.e.*, basal medium enriched with vitamin B_{12}, that failed to produce satisfactory growth after storage for a few days were greatly improved by the addition

TABLE III

ANALYSIS OF (3 + 3) ASSAY

	Responses of standard (μg per tube)			Responses of unknown			
	0·0008	0·0012	0·0018	4/9U	2/3U	U	
	0·93	1·12	1·38	0·83	1·19	1·33	
	0·83	1·15	1·29	0·87	1·08	1·33	
	0·84	0·98	1·30	0·96	0·95	1·31	
	0·58	0·74	1·28	0·92	1·13	1·34	
	1·06	1·12	1·28	0·80	1·09	1·20	
	0·86	1·05	1·32	1·03	1·05	1·32	
Totals	5·10	6·16	7·85	5·41	6·49	7·83	38·84
Sample difference	−1	−1	−1	+1	+1	+1	+0·62
Linear regression	−1	0	+1	−1	0	+1	+5·17
Departure from parallelism	+1	0	−1	−1	0	+1	−0·33
Combined curvature	+1	−2	+1	+1	−2	+1	+0·89
Opposed curvature	−1	+2	−1	+1	−2	+1	−0·37

ANALYSIS OF VARIANCE—

Source	D.F.	Sum of squares	Mean square	Variance ratio	Significance
Sample difference	1	0·01068	0·01068	0·97	not significant
Linear regression	1	1·11370	1·11370	100·70	$P < 0·001$
Departure from parallelism	1	0·00454	0·00454	0·41	not significant
Combined curvature	1	0·01100	0·01100	0·99	not significant
Opposed curvature	1	0·00190	0·00190	0·17	not significant
Error	30	0·33173	0·01106		
Total	35	1·43755			

Potency ratio: 1·07
Fiducial limits: (P = 0·95) 0·93 to 1·22 (93 to 122 per cent.)

of small amounts of thioglycollic acid or ascorbic acid; this suggested that an accumulation of peroxides had occurred. Excessive amounts of these reducing agents, however, led to the production of unsatisfactory inocula and consequently boiling and cooling of the inoculum tubes immediately before inoculation is recommended.

We wish to acknowledge valuable technical assistance by Miss F. M. Ord and Mr. D. G. Goodinson.

REFERENCES

1. Cuthbertson, W. F. J., and Lloyd, J. T., *J. gen. Microbiol.*, 1951, **5**, 416.
2. Lees, K. A., and Tootill, J. P. R., *in preparation*.
3. Cuthbertson, W. F. J., Pegler, H. F., and Lloyd, J. T., *Analyst*, 1951, **76**, 133.
4. Shorb, M. S., *J. Biol. Chem.*, 1947, **169**, 455.
5. ——, *Science*, 1948, **107**, 396.
6. Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, **176**, 1459.
7. Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, T. H., *Ibid.*, 1949, **181**, 635.

* Commercial brands have not been found suitable and laboratory preparation according to Wislicenus, *Annalen*, 1888, **246**, 315, is recommended.

8. Lees, K. A., and Emery, W. B., *Biochem. J., Proc. Biochem. Soc.*, 1949, **45**, ii.
9. Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.
10. Koditschek, L. K., Hendlin, D., and Woodruff, H. B., *Ibid.*, 1949, **179**, 1093.
11. Snell, E. E., and Wright, L. D., *Ibid.*, 1941, **139**, 675.
12. Shaw, G., *Nature*, 1949, **164**, 186.

NOTES—References 1, 2 and 3 are to Parts I, II and III of this series.

Since this paper was written the following two papers on the microbiological assay of vitamin B_{12} have appeared—

Thompson, H. T., Dietrich, L. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1950, **184**, 175.
Skeggs, H. R., Hepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *Ibid.*, 1950, **184**, 211.

GLAXO LABORATORIES LTD.
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The Chick Assay of Vitamin B_{12} and the Animal Protein Factor

BY M. E. COATES, G. F. HARRISON AND S. K. KON

(*Presented at the meeting of the Biological Methods Group on Tuesday, May 23rd, 1950*)

A method of assay with chicks for vitamin B_{12} and the animal protein factor is described and its accuracy discussed. The method has been applied to several natural materials, and a comparison with microbiological results has been made. Evidence is presented for the existence of other factors besides vitamin B_{12} in the animal protein factor. Difficulty in obtaining satisfactory responses to crystalline vitamin B_{12} is reported.

ALTHOUGH the method now described was originally designed to measure vitamin B_{12}, it has become obvious from our results to date that the test is not specific for vitamin B_{12} alone and that other as yet unidentified vitamins produce a similar growth response in chicks. We prefer therefore to describe the test as an assay of the animal protein factor (APF), although the standard used so far has been a preparation of vitamin B_{12} (Examen, Glaxo) presumed to be free from other factors, and results have been calculated in terms of vitamin B_{12}.

Preliminary accounts of our method of assay have already been presented.[1,2] The present paper describes the application of the method to the assay of a number of natural materials; wherever possible, results have been compared with those obtained microbiologically and it is evident that the two methods are often not in close agreement.

EXPERIMENTAL

PRODUCTION OF THE CHICKS—

As previously reported,[1] normal stock chicks proved unsuitable for this assay, as their reserves of animal protein factor at hatching were high. Chicks were therefore hatched from hens given an all-vegetable diet.[2] It was of major importance in all this work that both hens and chicks should be kept on wire screens to prevent access to their droppings, which have been shown by Rubin, Bird and Rothchild[3] to contain components of the animal protein factor, probably as a result of microbial synthesis after voiding. A deficiency of animal protein factor in hens results in lowered hatchability of their eggs,[2,4] so that to obtain adequate numbers of chicks we found it necessary to include a small but sufficient quantity of vitamin B_{12} in the hen diet to maintain hatchability at a reasonable level without excessive storage in the chicks. About 3 μg of vitamin B_{12} per 100 g of diet proved much too high for this purpose,[2] but 0·3 μg per 100 g was satisfactory. In 9 months, with the addition of this amount of vitamin B_{12}, hatchability of the fertile eggs from 30 hens never fell below 50 per cent., yet the chicks showed marked signs of deficiency of vitamin B_{12} at an early age.

DESIGN OF THE ASSAY—

The assay was designed on similar lines to those already in use here[5] for other members of the vitamin B complex, except that chicks of both sexes were used. The basal diet, with a high content of vegetable protein, had the following percentage constitution: ground maize, 38·6; ground barley, 20; defatted soya grits, 35; dried grass, 3; bone meal, 1·5; limestone flour, 1; sodium chloride, 0·672; $MnSO_4.4H_2O$, 0·028; arachis oil, 0·2. To each 100 g of diet were added 400 i.u. of vitamin A, 50 B.S.I. units of vitamin D_3, 0·15 μg of riboflavine and 1 μg of nicotinic acid; the doses of standard and test preparations were incorporated in the diet.

With chicks produced as described above, a linear relationship was established between their body weights at 4 weeks of age and the logarithm of the dose of vitamin B_{12}. This relationship held over a range of 0·5 to 3·0 μg of vitamin B_{12} per 100 g of diet. As far as possible assays were done with at least three dose levels of both standard and test material, with 5 to 10 chicks on each dose. Calculations were made as recommended in British Standards Specification 911 : 1940,[6] and in most assays the graphs of response to standard and test preparation proved straight and parallel to each other.

ERROR OF THE ASSAY—

The error of estimation unfortunately proved to be high, owing chiefly to the great variation in response among the chicks at each dose level. As the number of chicks available

TABLE I

REPRODUCIBILITY OF RESULTS FOR THE CHICK ASSAY OF THE ANIMAL PROTEIN FACTOR

Sample		Result	T.F.L. at P = 0·95	χ^2	Pooled Result	Pooled Error
		μg of vitamin B_{12} per g			μg. of vitamin B_{12} per g	
Fish solubles..	(*i*)	1·27	0·36 to 2·28 (28 to 180%)	0·36	1·17	0·99 to 1·37 (85 to 118%)
	(*ii*)	1·23	0·68 to 1·90 (55 to 155%)			
	(*iii*)	1·01	0·42 to 1·80 (42 to 176%)			
Fuller's earth adsorbates of *Streptomyces griseus* liquor	(*i*)	134·5	68 to 267 (50 to 198%)	0·23	120·5	102 to 142 (72 to 118%)
	(*ii*)	113·9	70 to 164 (61 to 144%)			

is limited by the special conditions needed to produce them, we find it is impracticable to attempt to reduce the variance either by increasing the size of the test groups or by severely culling the chicks before the beginning of the test. It is possible that the accuracy could be improved if pedigree records of individual birds were kept; the birds could then be distributed over the experimental treatments according to parentage, as is usual with the "litter mate" system adopted in rat assays.

In spite of the error being so high, results were fairly reproducible, as can be seen in Table I. The results for three successive assays of fish solubles and for two of an adsorbate from *Streptomyces griseus* liquor agreed closely among themselves, although the error of each individual test was high. The value of χ^2 for each series of assays was very low indeed, and the pooled results had limits of error well within those usually accepted in a biological assay.

COMPARISON WITH MICROBIOLOGICAL RESULTS

Whenever possible microbiological assays were made of the samples of material tested with chicks. The results are shown in Table II. We are very much indebted to our colleagues F. W. Wilby and J. E. Ford and to Dr. W. F. J. Cuthbertson, of Glaxo Laboratories Ltd., for these microbiological tests. It will be seen from the table that results by the two methods rarely agreed and that the chick assay gave on the whole considerably higher values. These discrepancies could be the effect of (*a*) destruction or incomplete extraction of vitamin B_{12} during preparation of the sample for microbiological assay, (*b*) the existence of vitamin B_{12} in a bound form available to chicks but not to micro-organisms or (*c*) the presence in the

samples of other growth factors essential to chicks but not to *Euglena gracilis* or *Lactobacillus leichmannii*. Two assays of fish solubles and two of whale solubles support the last as the most probable explanation for the following two reasons. (*i*) Samples 1 and 2 of the fish solubles were prepared from the same starting material, but of the two, sample 2 had been

TABLE II

COMPARISON OF THE CHICK ASSAY OF THE ANIMAL PROTEIN FACTOR WITH MICROBIOLOGICAL RESULTS

Sample		Chick assay		Microbiological assay	
		Result	T.F.L. at P = 0·95	Result,	Organism
		μg of vitamin B_{12} per g		μg of vitamin B_{12} per g	
Fish solubles	1	1·17	0·99 to 1·37	0·5	*Euglena gracilis*
	2	0·30	0·16 to 0·46	0·4	„
	3	1·63	1·10 to 2·50	0·7	„
Whale solubles	1 (*a*)	0·14	0·03 to 0·27	0·04	„
	(*b*)	0·71	0·22 to 2·11		
	2	0·43	0·03 to 0·96	0·08	*Lactobacillus leichmannii*
Whalemeat extract ..		0·38	0·10 to 0·61	0·1	*Euglena gracilis*
Fuller's earth adsorbates	1	120·5	102 to 142	about 50	*Lactobacillus leichmannii*
		65	20 to 134	about 37	„

subjected to much more drastic heat treatment. The chick result for it agreed closely with the microbiological figure, whereas for sample 1 the chick value was considerably higher. The presence in fish solubles of a heat-labile growth factor for chicks might therefore be suspected. (*ii*) Two chick results are quoted for one sample of whale solubles. The first,

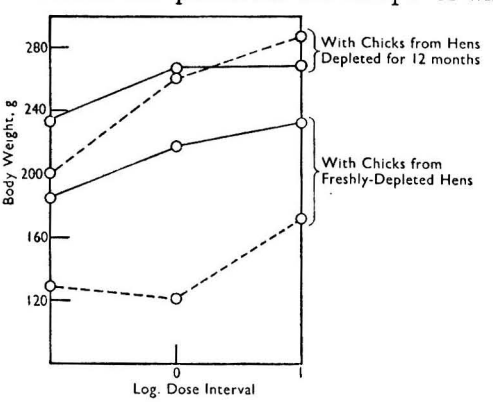

Fig. 1. Assay of whale solubles. Doses of standard: 0·5, 1·0 and 2·0 μg per 100 g of diet.
Response to standard ——
Response to whale solubles – – – –

which agrees fairly well with the microbiological finding, was obtained with chicks from freshly-depleted hens; the second, much higher, result was obtained in a simultaneous assay with chicks from hens that had received the all-vegetable diet for at least 12 months. The dose-response curves for these two assays are shown in Fig. 1. We suggest that the freshly-depleted hens, and consequently their chicks, were deficient only in vitamin B_{12}, but that the hens that had been longer on the diet had become depleted of other components of the animal protein factor as well, and that their chicks responded also to these other growth factors in the fish solubles.

EXPERIENCE WITH CRYSTALLINE VITAMIN B_{12}

Owing to the shortage of crystalline vitamin B_{12} it was impossible to use it regularly as a standard in chick assays and a microbiologically-standardised preparation of Examen

was used instead. When, by courtesy of Glaxo Laboratories Ltd., a small quantity of the crystalline vitamin became available to us, we made several attempts to compare it with the potency of our standard preparation of Examen by chick assay. These attempts invariably failed, as the response of the birds to graded doses of the crystalline material, and occasionally of the Examen, did not pass the test for linearity. The responses were haphazard and showed no particular trend, so it was unlikely that the non-linearity was due to instability of the vitamin in the diets. This lack of linearity has not been encountered with crude materials, although it sometimes happened with Examen. Similar difficulties were experienced with assays of crystalline vitamin B_{12c}, also obtained through the courtesy of Glaxo Laboratories Ltd. It may be that other factors of the animal protein factor complex, present in fish solubles and possibly to a slight extent in Examen, are necessary for the proper utilisation of vitamin B_{12}. It is also possible that vitamin B_{12} alone, without the rest of the animal protein factor complex, upsets the balance of the intestinal micro-organisms and interferes with their contribution to the nutrition of the host.

Discussion of results

This comparison of values found by chick and microbiological assay on crude materials containing vitamin B_{12} shows very clearly that the two techniques are not measuring the same thing. Although it is probable (but by no means certain) that the microbiological methods measure only vitamin B_{12}, it is evident that values found by the chick tests as used by us represent other growth factors as well. This marked difference in results is particularly interesting with assays using *Euglena gracilis*, which is believed to be more specific than *Lactobacillus leichmannii* in its response to vitamin B_{12}. Considerable evidence for the complex nature of the animal protein factor has already been given by Stokstad, Jukes, Pierce, Page and Franklin,[7] by Carlson, Miller, Peeler, Norris and Heuser[8] and by Menge, Combs and Shorb,[9] who demonstrated the necessity for other members of the animal protein factor complex in animal nutrition, but as yet little is known of the importance, if any, of these factors in human pernicious anaemia. In work concerning vitamin B_{12} and its allied vitamins, therefore, it will only be discovered by careful comparison with clinical results whether the microbiological test or the chick test gives the truer assessment of anti-pernicious-anaemia activity, although at present the chick assay is apparently the only means of measuring the whole animal protein factor complex.

We are very much indebted to Glaxo Laboratories Ltd. for gifts of crystalline vitamin B_{12} and standardised Examen, to Dr. J. A. Lovern of the Torry Research Institute, Aberdeen, for the samples of fish and whale products, and to Messrs. D. J. G. Black and J. Getty of the Poultry Department of Reading University for the supply of specially bred chicks for this work.

References

1. Coates, M. E., Harrison, G. F., and Kon, S. K., *Biochem. J.*, 1950, **46**, vii.
2. Black, D. J. G., Getty, J., Coates, M. E., Harrison, G. F., and Kon, S. K., *Ibid.*, 1950, **46**, viii.
3. Rubin, M., Bird, H. R., and Rothchild, I., *Poult. Sci.*, 1946, **25**, 526.
4. Bird, H. R., Rubin, M., Whitson, D., and Haynes, S. K., *Ibid.*, 1946, **25**, 285.
5. Coates, M. E., Kon, S. K., and Shepheard, E. E., *Brit. J. Nutrit.*, 1950, **4**, 203.
6. British Standards Institution, 1940, *Specification No.* 911.
7. Stokstad, E. L. R., Jukes, T., Pierce, J., Page, A. C., and Franklin, A. L., *J. Biol. Chem.*, 1949, **180**, 647.
8. Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C., and Heuser, G. F., *Poult. Sci.*, 1949, **28**, 750.
9. Menge, H., Combs, G. F., and Shorb, M. S., *Ibid.*, 1949, **28**, 775.

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Discussion

Dr. T. Barton-Mann enquired whether Miss Coates had had any trouble, when depleting parent stock, with egg-eating. Did Miss Coates collect eggs for incubation over a period from these depleted parents? His own experience showed that depletion of parents led to egg-eating and that collecting eggs over a period resulted in very great unevenness in growth of chicks.

He also referred to the work of Miller and Groschke,[1] who had abandoned the depletion of parents and fed them a normal ration. After incubation of the eggs the chicks were fed a depleted diet for 14 days and then only those chicks with weights ranging from 75 to 90 g were selected for assay purposes.

Miss Coates replied that she had not herself encountered egg-eating but had occasionally had trouble with cannibalism in some groups of hens. In her laboratory eggs were not individually marked, so that growth of the chicks could not be related to the age of the egg. Parent birds appeared to be thoroughly depleted after 6 to 8 weeks. She agreed that culling the chicks after a preliminary depletion period might be advantageous, but had not had enough birds available to do so.

Reference to discussion

1. Miller, D. C., and Groschke, A. C., *Quart. Bull. Michigan State Coll.*, 1950, **32**, 279.

Some Observations on the Cup-Plate Assay of Vitamin B_{12} Using *Lactobacillus lactis* Dorner 10697

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(*Presented at the meeting of the Biological Methods Group on Tuesday, May 23rd*, 1950)

After many experiments, the routine use of tube assays of vitamin B_{12} with a variety of organisms was abandoned in favour of a cup-plate assay with *Lactobacillus lactis* Dorner 10697. The effects of variation of the pH of the test liquid and of variation in the concentration of cresol present were investigated.

The most reliable results were obtained on solutions containing crystalline vitamin B_{12}, although considerable day-to-day and week-to-week variations were experienced and results between different laboratories showed unexpected variations when assayed against vitamin B_{12} standardised on $E^{1\%}_{1cm}$, 361 mμ measurements; the microbiological assay was found to be particularly useful in determining the stability of samples of vitamin B_{12} of differing purities. With other vitamin B_{12} preparations the microbiological assay was always interpreted in conjunction with organic cobalt determinations; the two assays were complementary because, although the organic cobalt assay gives a theoretically maximum figure, the microbiological assay often exceeds this figure.

Concentrated liver preparations gave, in general, concordant results, although the spread and laboratory deviations were greater than with solutions containing crystalline vitamin B_{12}. When liver concentrates were autoclaved there was often an increase in microbiological activity, sometimes by as much as 70 per cent.

Assays of vitamin B_{12} concentrates obtained from streptomyces fermentation liquors gave results that were difficult to interpret; two anomalous growth zones were obtained, one diffuse, one more dense than with pure vitamin B_{12}, and sometimes the two growth rings were indistinguishable. Results with a single sample could vary from 23 to 56 μg of vitamin B_{12} per ml and it was considered necessary to use the assay in conjunction with a chromatographic procedure, although some difficulty was experienced in getting a quantitative elution of the small amounts present on the chromatogram.

After much trial, tube assays with *Lactobacillus lactis* Dorner ATCC 8000, *Lactobacillus leichmannii* ATCC 4797, *L. leichmannii* 313 ATCC 7830 and *L. leichmannii* NCTC 7854 were considered to be less reliable than a cup-plate assay of the type originally adapted for the determination of vitamin B_1 by Bacharach and Cuthbertson and later for vitamin B_{12} by Cuthbertson; this has now been described in detail.[1] Fewer troubles were experienced in routine work and results were more reproducible, but probably the greatest advantage, as pointed out by the original author, was that the anomalous growth zones produced by growth factors other than vitamin B_{12} made it often possible to recognise erroneous results. In general, the cup-plate procedure was an easier assay, and we did not experience so many

of the seemingly inexplicable results obtainable in many microbiological assays, especially in those for vitamin B_{12}.

A number of cup-plate techniques have been suggested, the most recent by Foster, Lally and Boyd Woodruff[2] with *L. lactis* Dorner 10,697 and by Cohen and Bennett[3] with *L. leichmannii* ATCC 4797. The latter workers used the medium of Skeggs *et al.* with the addition of 0·1 per cent. of ribonucleic acid, 0·1 per cent. of thioglycollic acid and 2 per cent. of potassium sulphate; the resulting medium was slightly alkaline, the pH being 7·4 to 7·9, and the assay could be used over the concentration range 0·05 to 1·6 μg per ml.

Experimental

In practice we have concentrated on the use of the method of Foster, Lally and Boyd Woodruff[2] with a synthetic amino-acid medium for the actual test medium with the addition of agar and 2 per cent. of sodium chloride; the use of this abnormal salt concentration did in our hands eliminate the diffuse growth response of *L. lactis* to growth factors other than vitamin B_{12}. Three strains of *L. leichmannii* were tried and also *L. lactis* Dorner 8000, but our strains would not grow satisfactorily on this synthetic medium. However, as *L. lactis* Dorner 10,697 gave satisfactory results, the work was not extended to include the development in detail of a cup-plate assay with other organisms.

The stock culture medium contained 0·02 per cent. of tomato juice serum, 1 per cent. of yeast extract, 1 per cent. of dextrose and 1·5 per cent. of agar, although in the test medium the tomato juice serum was replaced by fumaric acid and sodium ethyl oxalacetate. Tests showed that the results when these substances were included in the medium were comparable with those obtained when tomato juice serum was included. The culture used, *L. lactis* Dorner ATCC 10,697, was a variant developed by Dr. M. Shorb of the University of Maryland. It is kept as a stab in the stock culture medium and is renewed weekly; if growth is slow, the culture can be renewed by growing it for 24 hours in the liquid medium and again transferring it to the solid medium, although the necessity for such a procedure has been surprisingly rare. Subcultures in the liquid inoculum medium are prepared from the stab cultures and are used after incubation for about 18 hours at 37° C. The liquid culture suspension is washed three times with a weak saline solution (0·25 per cent.) before being used to inoculate the test medium. The final suspension in water or weak saline solution is such that it gives an opacity reading of 0·5 on a Spekker photo-electric absorptiometer, redistilled water set at 1 being used as a control; 2·0 ml of this suspension are added for each 100-ml quantity of liquefied test medium at 45° C. It was found generally that liquid cultures that were more than 24 hours old were unsuitable for assay purposes; storage of a 16-hours old culture at 5° C for 2 days was attempted, but it was unsatisfactory and further attempts to store liquid cultures were not made. Plates of inoculated test medium can, however, be used satisfactorily after refrigeration at 5° C for several days, although in general agar plates were used as quickly as possible.

Assay procedure—

The inoculated test medium is distributed in 15-ml quantities into specially selected flat-bottomed Petri dishes of 90 mm diameter. This quantity produces a thinner agar layer than in the procedure of Foster, Lally and Boyd Woodruff[2] and relatively larger zone diameters were produced. When the agar has hardened, four holes of 8 mm diameter are cut in each plate; two of these holes are used for the standard and the other two for the test solution. The amount of standard or test solution is 2 drops per hole, added from a calibrated pipette.

The dilutions in the range used are usually 0·1, 0·2, 0·4, 0·8 μg of vitamin B_{12} per ml; all solutions, both standard and unknown, are diluted with phosphate buffer at pH 7.

After incubation for 16 hours at 37° C, the diameters of the growth zones are measured in millimetres and calculations are made on sets of four plates with a 1 to 4 dosage ratio, the method of calculation of results being that developed by Knudsen and Randall.[4]

In general, 0·2 μg of vitamin B_{12} per ml gave a growth zone of 19 to 21 mm, and a typical assay gave, for each two-fold increase in vitamin B_{12} concentration, a growth zone diameter increase of 1·7 to 1·8 mm.

The vitamins present in the medium were added in a solution diluted from a concentrated stock solution; the diluted solutions must not be stored, as they are unstable. As an example of this instability, on one occasion the standard vitamin B_{12} solution gave no growth rings,

although a parenteral liver extract on the same plates gave a normal response. It was suspected that a possible deterioration of the vitamin solution had occurred and, by adding fresh solutions of each of the vitamins used in turn to the deficient medium, it was found that it was the biotin which was lacking. This growth factor had apparently been present in sufficient amount in the parenteral liver extract under test to make up for the deficiency in the medium. It may be mentioned that pyridoxine can be used satisfactorily in place of pyridoxamine.

THE EFFECT OF pH—

In examining certain products it was desired that the effect of pH should be known, particularly in the acid range, on the growth rings produced in the assay. Vitamin B_{12} has been found to be stable in aqueous solutions of pH 4 to 7, but unstable in alkaline solutions. Accordingly, two solutions of vitamin B_{12} of the same concentration were prepared, one in a phosphate buffer at pH 7 and the other in an acetate buffer at pH 2. The results are shown in Table I.

TABLE I

COMPARISON OF CUP-PLATE ASSAYS OF VITAMIN B_{12} AT pH 7 AND pH 2

	Microbiological assay results, μg of vitamin B_{12} per ml			
	At pH 7		At pH 2	
Solution	Individual results	Average	Individual results	Average
A	72·0		57·0	
	58·8	64·8	57·6	58·2
	63·6		60·0	
B	60·0		56·4	
	49·2	56·4	62·4	63·6
	60·0		72·0	

The dilutions for testing were made in the usual way with the exception that the solution at pH 2 was diluted in acetate buffer at pH 2 for the test. The growth rings given by the solutions at pH 2 were not uniformly compact but showed a clearer zone spreading outwards from the hole; this zone was not well defined and reached approximately to half-way between the hole and the outer edge of the growth ring. In spite of these anomalies, however, it can be seen that the results obtained at pH 2 are reasonably good.

THE EFFECT OF CRESOL—

For the assay of impure solutions containing vitamin B_{12} and relatively high proportions of cresol, it was necessary to find out whether or not the presence of the cresol was in any way affecting the result. A solution of vitamin B_{12} to which cresol had been added in various amounts was, therefore, assayed; the results are shown in Table II.

With concentrations of cresol of 0·8 and 1·0 per cent. there were slight, unmeasurable, clear zones round the holes. At higher concentrations the clear zones round the holes were well defined and easily measurable. It was, in fact, found possible to determine the concentration of cresol in an unknown solution by this procedure by comparison with cresol solutions of known concentration.

The clear zone due to the presence of cresol appeared after the growth zone due to vitamin B_{12}, and the above results indicate that over this range of cresol concentration the growth response to vitamin B_{12} was not inhibited.

SOME APPLICATIONS OF THE CUP-PLATE ASSAY FOR VITAMIN B_{12}

The results obtained with solutions of pure vitamin B_{12} were, in general, reliable. With impure solutions and concentrates, however, this was not so, and the microbiological assay results were always interpreted in conjunction with chemical determinations of organically combined (in practice, butanol-extractable) cobalt; the result of this determination is referred to below as the "organic cobalt." Although the organic cobalt determination gives a maximum possible figure the microbiological assay often exceeds this figure. The chemical assay process

of Fantes *et al.*[5] is also useful, although it is more satisfactory for streptomyces "concentrates"; we did not find it suitable for preparations containing much organic matter and a low concentration of vitamin B_{12}.

For many impure products, which gave results obviously too high, a chromatographic procedure could be used with advantage. A quantity of vitamin B_{12} dissolved in 5 μl was subjected to paper-strip chromatography with *n*-butanol and water in conjunction with the

TABLE II

THE EFFECT OF CRESOL ON THE CUP-PLATE ASSAY FOR VITAMIN B_{12}

Cresol, per cent. w/v	Microbiological assay results, μg of vitamin B_{12} per ml: Individual results	Average
nil	12·2 13·4	12·8
0·3	12·7 11·8	12·25
0·6	10·7 11·2	10·95
0·8	9·8 12·2	11·0
1·0	12·8 10·5	11·65
1·2	14·0 12·9	13·5
2·4	11·0 12·0	11·5

classical technique of Consden, Gordon and Martin.[6] At the same time a similar chromatogram of riboflavine was run side by side in the same trough. A section of the vitamin B_{12} chromatogram, representing the constituents that had moved more slowly than riboflavine, was cut out and extracted, the extract being assayed microbiologically. Often more satisfactory results were obtained in this way.

CRYSTALLINE VITAMIN B_{12}

In examining samples of crystalline vitamin B_{12}, microbiological assay results obtained on solutions can be checked by spectrophotometric determination of $E^{1\%}_{1cm}$ at 278, 361 and 548 mμ. The results quoted in this work were obtained with a sample of crystalline vitamin B_{12}, standardised on the peak at 361 mμ, using the value for pure vitamin B_{12} of $E^{1\%}_{1cm}$, 361 mμ of 207, as quoted in the 14th edition of the United States Pharmacopoeia. Another criterion for a satisfactory sample is that the ratio of $E^{1\%}_{1cm}$, 278 mμ to $E^{1\%}_{1cm}$, 361 mμ shall fall within certain limits; impure material has a relatively higher light absorption at 278 mμ and this has recently been recognised in the United States Pharmacopoeia.

Results obtained on solutions of pure vitamin B_{12} usually showed a range of ± 15 per cent. from the mean for a single assay (4 plates), variations mainly occurring from one day to another. Hence results ranging from 9·6 to 13·2 μg per ml on the same solution would be regarded as normal on different days, although there was much closer agreement between results obtained on any one day.

VITAMIN B_{12} CONCENTRATES PREPARED FROM LIVER

With impure products containing vitamin B_{12}, diffuse zones were occasionally obtained, although dilution often removed such zones. For vitamin B_{12} concentrates from liver, results were in good agreement with organic cobalt determinations. On occasion, however, treatment of samples of liver concentrates in an autoclave caused an increase of as much as 70 per cent. in microbiological activity, the increase bringing the figure much above the organic cobalt determination. For example, a solution assaying at 33 μg of vitamin B_{12} per ml gave, after autoclaving, a result of 56 μg per ml, although cobalt was present only to an extent sufficient to justify a result of 35 μg per ml.

It was found in general for liver concentrates intended for parenteral injection that the high salt concentration present in the medium did, as claimed by Foster, Lally and Boyd Woodruff,[2] eliminate the growth response to desoxyribonucleic acid, thymidine or reducing substances. Although results could be obtained that varied among themselves to an extent only slightly greater than those for pure vitamin B_{12} solutions, this agreement could not be attained between results by different laboratories. Here variations as large as ± 70 per cent. have been commonly experienced.

Vitamin B_{12} concentrates from streptomyces fermentation liquors

Growth response rings obtained with concentrates from streptomyces fermentation liquors, even of the purer forms, were mainly uniform in density, but the edges were often slightly diffuse and not as sharp as usual. On occasion, especially when the test medium had been freshly prepared, a double zone effect was noted in which the two zones were almost equally dense, the inner one being only slightly denser than the outer one. This effect was markedly different from the diffuse outer growth zones encountered, in particular in the early stages of fermentation processes, which are certainly due to growth factors for *L. lactis* Dorner other than vitamin B_{12}.

As an example, one particular sample of streptomyces concentrate that had an organic cobalt content equivalent to 18 μg of vitamin B_{12} per ml gave widely varying results from day to day, ranging from 23 to 56 μg of vitamin B_{12} per ml; if the outer zone readings were taken, results up to 120 μg per ml were indicated. A particularly disconcertaing effect was that with the same medium and test sample double zone effects were sometimes obtained, sometimes not. It is obvious that chromatographic separation or other differential procedure is necessary to obtain significant results.

The autoclaving of fermentation concentrates yielded, in general, lower results than those for the unheated sample; this effect is thought to be due to the decomposition of the vitamin B_{12} without the appearance of another growth factor, as happens with liver concentrates.

Vitamin B_{12} assays on other materials

A number of other materials, notably products from fish and whale livers, were examined for the presence of vitamin B_{12} by microbiological assay and by organic cobalt determination. The products were moderately highly coloured and growth rings were, in general, dark and coloured; diffuse rings were often present, but these could be eliminated to a great extent by a decrease in concentration in the actual assay.

In a dry fish-liver extract 5·6 μg of vitamin B_{12} per g was found by an organic cobalt determination. In the microbiological assay at an approximate concentration of 0·4 μg of vitamin B_{12} per ml, an inner zone 22·5 mm in diameter was obtained, together with an outer diffuse growth. At one quarter of this concentration, a zone of 19 mm without diffuse growth was obtained, the final average result being 5·9 μg of vitamin B_{12} per g. A whale-liver extract gave 1·2 μg of vitamin B_{12} per g by chemical assay, although microbiologically 2·0 μg per g was obtained. Another powdered fish-liver product gave 6·3 μg of vitamin B_{12} per g chemically and 5·1 μg per g microbiologically.

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References

1. Cuthbertson, W. F. J., Pegler, H. F., and Lloyd, J. T., *Analyst*, 1951, **76**, 133.
2. Foster, J. C., Lally, J. A., and Boyd Woodruff, H., *Science*, 1949, **110**, 507.
3. Cohen, R., and Bennett, R. E., *Communication to the American Chemical Society*, April 11th, 1950.
4. Knudsen, L. F., and Randall, W. A., *J. Bact.*, 1945, **50**, 187.
5. Fantes, K. H., Ireland, D. M., and Green, N., *Biochem. J.*, 1950, **46**, xxxiv.
6. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

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Experiences with the Microbiological Assay of Vitamin B_{12} in an Analytical and Consulting Laboratory

By H. PRITCHARD

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The tube method of microbiological assay of vitamin B_{12} with *Lactobacillus leichmannii* 313, as described by Emery, Lees and Tootill, has been adapted for routine use in a laboratory where demands for the assay are only intermittent.

The vitamin B_{12} potency has been given for a number of materials used in the compounding trade as determined by this test.

Increasing interest in vitamin B_{12} has led the provender milling trade to demand that the potency of concentrates purporting to be rich in this factor should be stated. Analytical and consulting laboratories therefore require a rapid and reliable method of estimation that can be brought into use and laid aside according to need. The microbiological technique will fulfil this purpose if it can be so modified that not too much time and space of a busy and crowded laboratory need be taken up with maintaining culture and medium between tests.

Choice of method

Consideration of the methods of microbiological assay already in use led to the conclusion that a tube method, rather than a plate method, lent itself most readily to work in the routine laboratory. Either *Lactobacillus lactis* Dorner or *L. leichmannii* 313 could be used as the test organism, but discussion with Dr. E. Lester Smith and Dr. W. F. J. Cuthbertson, of Glaxo Laboratories Ltd., indicated that the latter organism was the one of choice.

The method of assay eventually established as routine was that of Emery, Lees and Tootill,[1] with such modifications as were necessary when the method was needed for occasional use only.

Test organism

The first cultures of *L. leichmannii* 313 used in the tests were obtained through Dr. Cuthbertson; they were freeze-dried cultures or organisms grown in the special milk medium described below. As the requirements were intermittent and a period of two or three weeks would sometimes elapse between tests, it was thought that the properties of the organism might be upset by the daily transfer system because of contamination or other accidents. It was desirable, therefore, to have at hand a readily available source of the organism. Enquiries at the Chemical Research Laboratory, D.S.I.R., Teddington, revealed the fact that the Collection of Industrial Bacteria had recently added the organism to its collection there; the number is NCIB 8118. A convenient method of storing the organism was as a stab culture in the following medium—

Casein hydrolysate	0·4 per cent.
Yeast extract	1·0 per cent.
Tomato juice	5·0 per cent. v/v
Dextrose	1·0 per cent.
Agar	2·0 per cent.

Transfer to fresh medium every 14 days.

Once the culture was removed from the refrigerator, the test organism was maintained alive until required by daily transfer into fresh medium consisting of milk with 1 per cent. of yeast extract and 5 per cent. of tomato juice added. This medium was prepared from 10 per cent. of dried skimmed milk powder, 5 per cent. of tomato juice and 1 per cent. of Difco Yeast Extract made up with glass-distilled water and placed in screw-topped vials in 10-ml lots. The medium was autoclaved at 10 lb pressure for 10 minutes and appeared to keep indefinitely after sterilisation. An allocation of milk powder was obtained from the Ministry of Food, the Difco Yeast Extract was obtained from Baird and Tatlock Ltd., and

fresh tomatoes were used. No difficulty has been met in getting supplies of fresh tomatoes during the last twelve months, but several tins of tomato juice were kept as a stand-by. Several brands were found to be suitable. The juice was prepared just before it was needed. The tomatoes were immersed in boiling water for a few minutes and then skinned. The skinned fruits were placed in a piece of cloth and the juice was expressed after mashing them. The juice was then mixed with kieselguhr and filtered with suction through a Whatman No. 1 filter-paper supported on a No. 54.

After incubation at 37° C for 24 hours, the contents of the vial containing the actively growing organism were solid except for about 0·5 ml of clear yellow liquid, which rested on the surface of the clot. To effect the daily transfer, 0·5 ml of the mixed contents of the vial was removed with a sterile pipette and placed in a fresh bottle of medium, which was then incubated in the same way.

BASAL MEDIUM—

Sufficient basal medium for 250 test tubes was made from the constituents shown in Table I.

TABLE I

BASAL MEDIUM

PART 1—	
Sodium acetate (anhydrous)	20 g
Sodium citrate	20 g
Acid casein hydrolysate	8 g
L-Cystine	0·4 g
Crill No. 10	4 g
Sodium oleate	20 mg
Glucose	20 g
Salt solution A*	12 ml
Salt solution B†	120 ml
PART 2—	
Adenine, guanine and uracil	0·02 g each
DL-Tryptophan	0·20 g
p-Amino benzoic acid	0·20 mg
Asparagine	0·20 g
Calcium pantothenate	0·40 mg
Nicotinic acid	2·00 mg
Riboflavine	0·8 mg
Aneurine	0·4 mg
Biotin	4 μg
Folic acid	20 μg
Pyridoxine	4·8 mg

* Salts solution A is 25 g of K_2HPO_4 and 25 g of KH_2PO_4 dissolved in water to 250 ml.

† Salts solution B is 10 g of $MgSO_4.7H_2O$, 2 g of $MnSO_4.4H_2O$, 0·5 g of $FeSO_4.7H_2O$ and 0·5 g of NaCl dissolved in water to 250 ml with 5 drops of hydrochloric acid.

It was impossible to obtain pyridoxal and pyridoxamine, used in the original medium; pyridoxine was therefore used at six times the recommended concentration of the combined weight of these two constituents. Three types of acid-hydrolysed casein were tried from time to time. For a short time the casein was prepared in the manner described by Hawk and Bergheim,[2] but the method was then changed to that described by Barton-Wright.[3] Eventually a good source was found in the dried acid-hydrolysed casein available from Allen and Hanbury Ltd., Ware. Water distilled in glass was used throughout these assays for all purposes. This was made in a special still with an automatic feed, details of which will be published shortly.[4]

Procedure for preparing the basal medium—Dissolve the items in Table I, Part 1, in water and make up to 1000 ml. Adjust the pH to 6·8, and boil the mixture in presence of kieselguhr. Filter by suction through two Whatman No. 1 filter-papers supported on a No. 54 and re-adjust the pH to 6·8. Dissolve the items in Table I, Part 2, in 50 ml of water, with the aid of hydrochloric acid if necessary, and add this solution to the clear filtrate of Part 1 constituents; make up the combined solution to 1625 ml with water.

After the basal medium was prepared, it was found best to keep it in tubes; 6·5 ml were placed in each of a series of 6-inch × $\frac{5}{8}$-inch test tubes with metal caps. The tubes

were placed in wire baskets and sterilised in a low-pressure gas-heated autoclave, which was automatically controlled to maintain the steam pressure at 10 lb per sq. in. The tubes were placed in the autoclave with the water already boiling and the lid was clamped on, steam being allowed to escape through the valve for 5 minutes; the tubes were then heated under 10 lb pressure for 10 minutes and allowed to remain in the autoclave until the pressure had dropped. The tubes were immediately cooled in water and transferred to a refrigerator at −12° C, when the contents froze solid; kept thus, the medium was instantly available for use. Good assays were obtained with medium kept in tubes for over 4 weeks.

Assay procedure

The procedure described below enabled the laboratory to obtain results about 10 days after receipt of routine samples for test. The apparatus was prepared and sterilised by heating at 150° C for 1 to 2 hours or by autoclaving.

Apparatus—

Beakers—50 ml.
Sintered-glass filter—3 × 3.
Buchner flask—500 ml.
Measuring cylinders—100 ml.
Plugged conical flasks—250 ml.
Graduated dropping pipettes—10 ml and 1 ml.
Capped centrifuge tubes—Two 10-ml tubes.

Diluents—

Physiological saline—A sterile 0·9 per cent. w/v solution of sodium chloride in water.
Glass-distilled water—Sterile.
Tomato juice - saline mixtures—A mixture of 4 parts of tomato juice and 3 parts of physiological saline, sterilised by filtration through a Seitz filter.

Preparation of samples for test—

The method of preparing dilutions for the assay tubes depended on the type of material under examination. If it was water-soluble, a portion was accurately weighed and taken up with water in a beaker, by heating if necessary, and then filtered through sintered glass. The dilutions were prepared from the filtered solution and handled aseptically. The method adopted for dealing with material insoluble in water varied; the one found most widely applicable, after attempts with papain digestion, etc., was as follows.

Procedure for water-insoluble samples—Weigh 10 g of the material (for example, liver, fish or meat meal) and place it in a 250-ml flask with 100 ml of water at pH 5. Boil the contents of the flask under reflux for 15 minutes, or heat in an autoclave under free steam for 15 minutes. Cool, add 5 g of kieselguhr and filter. Wash the insoluble matter several times with water, combine the extract and washings and filter them through sintered glass to clarify and sterilise them. Treat the resultant solution in the same way as for water-soluble samples.

Dosing the tubes—

For each sample under test four dose levels were chosen. This number was adopted after experimental procedures involving as many as eight levels. The object was to establish as accurately as possible a few points on the response line, and this was done by using from 7 to 10 tubes at each dose level.

The tubes containing the basal medium were removed from the refrigerator, warmed to room temperature and placed in racks specially designed to fit into the incubator. Each sample had a rack to itself and there was also a rack for the standard and the blanks. The tubes were permanently marked with a diamond. Each of the tubes was dosed aseptically in order with 1 ml of the various test solutions, as were a similar series with the standard solutions. In the tests described here, the standard consisted of a liver extract* that had been standardised by the cup-plate or similar method.

* Kindly provided by Glaxo Laboratories Ltd.

The dose levels of the standard chosen were 0·004, 0·002, 0·001 and 0·0005 μg. In addition, 10 tubes were dosed with 1 ml of water as blanks. After the tubes had been racked, marked and dosed, they were ready for inoculation.

INOCULATION OF THE TUBES—

The organism was prepared two days before being required for the test by taking 0·5 ml of the culture in the milk - tomato juice - yeast medium when 24 hours old and placing it in a tube of basal medium to which has been added about 0·16 μg of vitamin B_{12} and 0·5 ml of tomato juice. The tube was incubated for 24 hours at 37° C, and two lots of 0·5 ml of this culture were then transferred to two tubes containing similar medium. The object of preparing two tubes of inoculum was to guard against accidental breakage in the centrifuge.

After incubation for 24 hours, the contents of the tubes were transferred to the sterile centrifuge tubes and centrifuged for 30 minutes at 2500 r.p.m., with the material at 14·5 cm from the centre of rotation. The clear liquor was decanted and replaced by an equal volume of sterile physiological saline in which the organism was suspended before re-centrifuging; this procedure was repeated two or three times to remove any vitamin B_{12} from the organism. It was found that high blanks were sometimes obtained if the culture was only washed once. After washing the culture in this way, enough saline was added to give the culture suspension an opacity reading of 4 (equivalent to about 1200×10^6 organisms per ml). Then the suspension was made up to eight times its volume with tomato juice - saline mixture (see p. 157) and 0·5 ml was dropped into each tube; the contents were mixed and the racks put to incubate at 37° C overnight.

After 20 hours, or when good gradation was observed, the racks were removed from the incubator, the tubes were free steamed in the autoclave for a few minutes and the degree of growth was measured nephelometrically.

MEASURING THE GROWTH—

Any reliable method can be used for carrying out the readings; we have used the Spekker photo-electric absorptiometer. The contents of each tube in turn were shaken, allowed to stand for 5 or 10 minutes to let the air escape and then transferred for reading to a 1-cm cell. The readings were made against the blanks, which were used in the other cell of the absorptiometer instead of medium only. The absorptions of each tube were read directly on the drum of the instrument, which had been suitably engraved.

GRAVIMETRIC MEASUREMENT OF GROWTH

In order to increase the usefulness and wider application of the test, experiments have been begun on alternative methods of measuring growth. If weighing the amount of bacterial growth would give similar results to those obtained on the absorptiometer, then those laboratories not equipped with an absorptiometer or similar apparatus for nephelometric measurements would, with a little more trouble, be able to carry out the assays. The following experiment gave promising results.

After one of the sets of tubes had completed its period of incubation, the tubes were steamed in the ordinary way and the amount of growth was measured on the absorptiometer. When the readings were completed, the contents of the tubes at each dose level were combined and centrifuged. The clear, supernatant liquor was decanted from each tube and the deposits were washed several times with water. The deposits were then washed into weighed watch-glasses, which were placed in the 37° C incubator for 24 hours to dry out. The weights of the deposits were then determined. The results were as follows—

Tube	Dose, g	Absorptiometer reading	Weight of deposit, g	Ratio of reading to weight
1	0·00424	1·12	0·0640	17·5
2	0·00212	0·81	0·0453	17·9
3	0·00106	0·46	0·0257	17·9
4	0·00053	0·33	0·0180	18·3

This method can be used as a gravimetric method for the rough estimation of the vitamin, or, more usefully perhaps, as a ranging test for the more elaborate assay described above. Three 250-ml conical flasks are each charged with 65 ml of basal medium and sterilised. To flask A, 10 ml of sterile water is added; to flask B, 10 ml of water containing 0·03 μg of

vitamin B_{12} and to flask C, 0·03 g of the sample under test in 10 ml of water. Each of the flasks is then inoculated with the organism in tomato juice saline as for the normal method of test. The flasks are incubated for 20 hours and steamed for 10 minutes, and the contents are transferred to 50-ml centrifuge tubes to collect the organisms. These are washed and weighed as described; alternatively, the growth could be read in graduated conical centrifuge tubes. By comparing the growth of test substance and standard, some idea of the potency can be obtained. For example—

0·03 μg of vitamin B_{12} added to flask 1 $\equiv$ residue weight of 0·043 g.
0·03 g of test substance added to flask 2 $\equiv$ residue weight of 0·053 g.

$$1\text{ g of test sample} \equiv \frac{\text{Antilog}\left(\frac{0{\cdot}053}{0{\cdot}043}\log 0{\cdot}03\right)}{0{\cdot}03} = 1{\cdot}26\ \mu\text{g of vitamin } B_{12}$$

Calculation of results

The results were tabulated; the arithmetic mean of the reading on each set of tubes was calculated and this was plotted against the logarithm of the dose. It was found most convenient to plot the results on two-cycle logarithmic ordinate paper with tenths on the abscissae. In order to illustrate the type of result obtained after the method of assay had become routine, Table II shows results for a sample of fish solubles.

Table II

Example of routine results

Dose, μg	Spekker readings (E)							E, average
Standard—								
0·004	0·69	0·62	0·64	0·70	0·80	0·64	0·73	0·69
0·002	0·46	0·51	0·56	0·46	0·45	0·56	0·50	0·50
0·001	0·31	0·27	0·32	0·37	0·32	0·32	0·32	0·32
0·0005	0·17	0·10	0·12	0·14	0·16	0·10	0·13	0·13
Fish solubles—								
g								
0·0075	0·83	0·84	0·88	0·89	0·91	0·87	0·92	0·88
0·005	0·81	0·81	0·73	0·78	0·76	0·76	0·80	0·78
0·0025	0·59	0·58	0·64	0·61	0·61	0·54	0·56	0·59
0·00125	0·34	0·28	0·44	0·54	0·44	0·41	0·35	0·40

On plotting the results on logarithmic graph paper it was possible to read the potency by comparing the curve of the sample with that of the standard curve. But the following method of calculation, for which I thank Mr. N. T. Gridgeman, enabled the fiducial limits of the result to be found—

An 8-point 56-tube assay

Tube variance	=	1087/48	=	22·65
D	=	861/28	=	30·75
B	=	1487·5/70	=	21·25
I	=	log 2	=	0·3010
log dose-ratio	=	ID/B	=	0·4356
i.e., dose-ratio	=	2·727		

The fish solubles are thus 2·727 times more potent than expected; therefore the assay is: 0·4 × 2·727 μg per g = 1·09 μg per g. The limits of error derive from—

$$\frac{30{\cdot}75 \pm \sqrt{22{\cdot}65/14}}{21{\cdot}25 \pm \sqrt{22{\cdot}65/70}}$$

$$= \frac{30{\cdot}75 \pm 1{\cdot}272}{21{\cdot}25 \pm 0{\cdot}569},\ \textit{i.e.},\ \frac{\pm\ 4{\cdot}137\%}{\pm\ 2{\cdot}678\%},\ \textit{i.e.},\ \pm\ 4{\cdot}928\%$$

Thus—

log dose-ratio	=	0·4356 ± 4·928%
	=	0·4356 ± 9·856% for P = 0·05
	=	0·4356 ± 0·0429 for P = 0·05
Upper fiducial limit	=	1·20 μg per g
Lower fiducial limit	=	0·92 μg per g

Results as good as these are not always obtained, and sometimes the lines are not strictly parallel; a very good idea of the potency can then be obtained by using the slope ratio method.

Lack of parallelism of the curves was sometimes found, particularly when the medium in the tubes was old. Naturally, with liver extracts there is not so much difficulty as with extracts or solubles from other sources, because the standard used was a liver concentrate.

Some results

The main articles of commerce examined in the laboratory for vitamin B_{12} potency by means of this test are connected with animal feeding stuffs and are usually by-products of the fishing industry. The range of potencies in various types of material has been recorded in the following tables.

Table III contains results for fish solubles and fish meal. The former is a by-product of the reduction of herring and other pelagic fish to oil and meal at the fish-meal factories; it is usually in the form of a thick liquid of pale yellow colour. Fish meal is obtained either from white fish by drying the residues from cod-filleting or from the reduction of herring already referred to.

Table III

The vitamin B_{12} potency of samples of fish solubles and fish meal

Fish solubles, vitamin B_{12} potency, $\mu g/g$		Fish meal, vitamin B_{12} potency, $\mu g/g$	
0·66	0·28	0·17	0·11
0·96	0·49	0·20	0·18
1·20	0·33	0·08	0·33
1·07	0·28	0·06	
0·27	0·56	0·17	
0·22	0·30	0·13	

Table IV

The vitamin B_{12} potency of some miscellaneous samples

	Vitamin B_{12} potency, $\mu g/g$
Cattle cubes	0·045
Dried liver meal	0·13
	0·50
	0·43
	0·47
Whale solubles	0·13
	0·11
	0·05
Spray dried fish liver extract	7·1
	8·6
Dried food meal	0·06
Dried pancreas meal	0·09
Raw fish liver	0·06
Fish liver foots	0·09

All the figures emphasise the low level at which this vitamin exists both in natural materials and in those normally used for cattle-feeding and also the differences in potency of various samples of the same types of material. In the fish solubles, for example, the results ranged from 0·22 to 1·20 μg of vitamin B_{12} per g, with an average of 0·55 μg per g.

For fish meals the range was from 0·06 to 0·33 μg per g; the result appeared to depend partly on the type of meal (white fish or herring) and on its previous treatment.

References

1. Emery, W. B., Lees, K. A., and Tootill, J. P. R., *Analyst*, 1951, **76**, 141.
2. Hawk, P., and Bergheim, O., "Practical Physiological Chemistry," Eleventh Edition, J. & A. Churchill Ltd., London, 1938, p. 118.
3. Barton-Wright, E. C., "Practical Methods for the Microbiological Assay of the Vitamin B Complex and Essential Amino Acids," Ashe Laboratories Ltd., London, 1946, p. 56.
4. Wraige, D. R., *Analyst*, 1951, **76**, *to be published shortly*.

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The Determination of Fat in Sweetened Foods

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The method recommended in 1943 for the determination of fat for the Cake and Flour Confectionery Order, 1942, has been found to give troublesome charring with products containing high proportions of sugar. The Soxhlet and Röse - Gottleib methods are inapplicable to a number of sweetened foods, so a modification of the method recommended in 1943 is proposed, namely, that the acid digestion with diluted hydrochloric acid (2 + 1) should be performed at 50° C to avoid charring. The lower temperature also minimises the formation of emulsions in ether extractions. If more than 10 per cent. of sucrose is present, breakdown products interfere.

Examination of seven foods containing large proportions of sugars and fat has shown that fat may be extracted after digestion at 50° C equally as well, and sometimes better, than after digestion at 80° C. The reproducibility of the method was slightly better, drying to constant weight of the fat residue took several hours less by the proposed modification, and the fat obtained appeared to be less adversely affected.

The method for the determination of fat recommended by Nicholls, Colgate, Fraser and Hughes,[1] representing the Society of Public Analysts and Other Analytical Chemists and the Cake and Biscuit Manufacturers War-Time Alliance Ltd., in connection with the Cake and Flour Confectionery Order, 1942, was found to be unsatisfactory with products containing high proportions of sugar, *e.g.*, sweetened powders and fruit cakes. Heavy charring occurred and a non-separable emulsion was formed on shaking the mixture with the mixed ethers.

This difficulty had been encountered previously, and in 1927 the first report of the Milk Products Sub-Committee[2] on the determination of total solids and of fat in sweetened condensed milk recognised that the Werner - Schmid acid digestion method was unsuitable for a product, such as sweetened condensed milk, containing about 40 per cent. of sugar. Wiseman,[3] working in 1930 with chocolate, preferred a modified Röse - Gottleib method. Mitchell,[4] making collaborative studies in 1933 for the Association of Official Agricultural Chemists on the determination of fat by acid hydrolysis of flour, bread and noodles, made an important study of the problem. He used the Hertwig[5] modification of the Werner - Schmid procedure, which became the official A.O.A.C. method for fat in flour. In this method, concentrated hydrochloric acid diluted with water (25 + 11) was used for a 30 to 40-minute digestion at 70° to 80° C, followed by the usual mixed ether extraction. The results were erratic and charring occurred. On trying the acid digestion on ether-washed potato starch at boiling temperature, 71° to 72° C, 57° to 61° C and 51° to 52° C, Mitchell found that carbon-like particles were produced at the boiling temperature and the mixed ether extract was highly coloured. At 71° to 72° C the ethereal layer was amber coloured, at 57° to 61° C it was slightly coloured and at 51° to 52° C it was colourless.

Fat in milk products is usually determined by the Röse - Gottleib procedure when accurate results are required. A modification was adopted by the 1927 Milk Products Sub-Committee.[2] In this method ammonia is used to soften the curd and hot digestion is not employed. Application of this method to an ice-cream powder failed to extract all the fat and gave erratic results (see Table I).

Table I

Determination of fat in ice-cream powder

	S.P.A. condensed milk method, %	Acid digestion at 50° C, %
	16·3	21·95
	16·05	22·05
	14·6	21·95
	17·1	21·90
Average	16·0	21·96

This method is obviously limited to products in which the fat is not too tightly bound in a hard cellular structure. In confectionery products, which have been baked, the fat is tightly bound.

An alkaline digestion was also used by McCance and Shipp[6] and McCance and Widdowson[7] in their researches on the chemical composition of foods. They used a modification of von Liebermann's method, which involved hot digestion with strong sodium hydroxide (saponification) and subsequent acidification and extraction of the fatty acids with ether. For nutritional purposes this is no doubt a useful and sufficiently accurate method, but as a general method for sweetened foods it is open to the objections (*i*) that assumptions as to the constituent fatty acids of the fat are required and (*ii*) that weighing or titrating an aliquot of the ether extract of the fatty acids has not been found to give sufficiently precise results. For an ice-cream powder, a figure of 19·9 per cent. of fat was obtained by weight and 24·9 per cent. by titration.

Direct extraction of the fat, as in the Soxhlet method, has also been known for many years to give inaccurate results with products containing a high proportion of sugar. This was recognised in the Fertilisers and Feeding Stuffs Regulations of 1932[8] and has been mentioned by many workers.[9,10,11,12] McCance and Widdowson[7] called attention to the matter in 1942 and published the table shown here as Table II. Recent work by Hall, Lee, Ormerod and Williams[13] on feeding stuffs gave further confirmation.

TABLE II

Food	Fat, g/100 g: Soxhlet method	Fat, g/100 g: von Lieberman's method	Food	Fat, g/100 g: Soxhlet method	Fat, g/100 g: von Lieberman's method
All-Bran (Kellogg's) ..	1·0	4·5	Grape nuts	0·4	3·0
Biscuits (digestive) ..	13·3	20·5	Malted milk (Horlicks) ..	1·2	8·6
Biscuits (rusks)	5·0	8·4	Ryvita	0·5	2·1
Cornflakes (Kellogg's) ..	0·1	0·8	Shredded wheat	0·9	2·8
Vita Wheat	6·0	10·3	Flour, white	0·5	0·9
Force	0·9	1·9	Flour, wholemeal ..	0·6	2·1

The results shown below were found for two ice-cream powders—

	Powder A	Powder B
Fat by Soxhlet extraction, %	16·35	12·03
Fat by hydrochloric acid digestion at 50° C, % ..	20·8	22·05

An acid digestion method appeared therefore to be preferable, provided that charring could be avoided. In the method recommended by Nicholls *et al.*[1] for Cake and Flour Confectionery, diluted hydrochloric acid was used; the use of this acid was stipulated in the Order. None of the other common acids appears to be suitable; sulphuric acid would probably cause greater charring and would leave a non-volatile residue that would attack the fat if traces were carried over in the ether layer, nitric acid would be too powerful an oxidant, and phosphoric acid would give similar, if less drastic, results to those with sulphuric acid. Hydrochloric acid is non-oxidising, a powerful hydrolyser and volatile at drying-oven temperatures. The method of Nicholls *et al.* uses 10 ml of concentrated hydrochloric acid of sp.gr. 1·18 and 5 ml of water for a 2-g sample.

THE PHENOMENON OF CHARRING—

The charring that occurs with sweetened foods when acid digestion is used requires further definition. Milk solids turn black when boiled with diluted hydrochloric acid (1 + 1), as in the Werner - Schmid method. The breakdown products in this method do not interfere with the fat determination, but remain in colloidal suspension in the acid layer. When much sucrose is present, on the other hand, more troublesome effects occur. These are (*a*) the definite separation of lumps of carbon, (*b*) some thickening of the mixture, (*c*) a yellow colour imparted to the ether layer and (*d*) the production of volatile products that cause fuming when the fat residue is dried and take many hours to remove. Mitchell,[4] as mentioned above, observed similar effects with potato starch. In these experiments flour was found to darken and produce furfural above 55° C, but cornflour, almost pure starch, although darkening at 80° C, did not cause the ether layer to be yellow and gave no fumes in the oven.

For some materials, for example the ice-cream powder and fruit cake initially investigated, the general thickening was so severe that separation of the ether extract layer did not occur even after standing overnight. Other foods containing flour or lactose, or both, without sucrose, have not been found to char sufficiently at 80° C to interfere with the fat determination.

These facts therefore suggest that laevulose is the major cause of the troublesome charring. It is well known that laevulose breaks down at high temperatures with hydrochloric acid. The acid would rapidly invert the sucrose to produce equal amounts of glucose and laevulose; this reaction is the basis of the "rapid" furfural test. Dried fruit in cake would contribute considerable amounts of pre-formed laevulose. Experiment with 2 + 1 hydrochloric acid and digestion for half an hour, as in the method recommended by Nicholls *et al.*, has shown that breakdown begins between 55° and 60° C.

Investigation of variable factors

The three variable factors in the digestion are (*a*) acid strength, (*b*) temperature and (*c*) time. To ensure sufficient structural breakdown of the food to release the fat particles, these three factors should have the maximum possible values. On the other hand, if the acid strength and temperature are too high and the time too long, the fat may be affected. Experiments to find the optimum conditions were therefore made with a mixture of confectionery materials consisting of 40 per cent. of sugar, 20 per cent. of skim milk powder, 20 per cent. of cocoa and 20 per cent. of flour. Two-gram portions in 8-inch × 1-inch boiling tubes were wetted with 2 ml of alcohol and treated with 15 ml of mixtures of concentrated hydrochloric acid and water in the proportions 2 + 1, 1 + 1 and 1 + 2. Digestion was carried out for half an hour at three temperatures, 80°, 65° and 50° C.

Table III

Digestion of experimental mixture with hydrochloric acid

Concentrated hydrochloric acid + water	Temperature, °C	State of mixture	Colour of ether layer	Behaviour of residue in oven
2 + 1	80	Heavily charred; no appreciable flocculent matter	Yellow	Fumed strongly
1 + 1	80	Charred; no appreciable flocculent matter	Yellow	Fumed strongly
1 + 2	80	Dark brown; little appreciable flocculent matter	Yellow	Fumed strongly
2 + 1	65	Charred; little flocculent matter	Paler yellow	Fumed
1 + 1	65	Dark brown; some flocculent matter	Paler yellow	Fumed
1 + 2	65	Light brown; much flocculent matter	Paler yellow	Fumed
2 + 1	50	Dark brown; little flocculent matter	Colourless	No fumes
1 + 1	50	Brown; some flocculent matter	Colourless	No fumes
1 + 2	50	Pink; much flocculent matter unchanged	Colourless	No fumes

The results in Table III show that although charring at 80° C can be prevented by use of a lower strength of acid, volatile extractable products are still produced. Only digestion at 50° C was satisfactory in this respect.

The production of volatile products was then investigated between the temperatures of 50° and 65° C, the acid strength being kept at 2 + 1 and time at half an hour. At 55° C furfural could just be detected by smell, but the ether layer was not yellow and there were no fumes from the fat residue in the oven. At 60° and 65° C the ether layer was again coloured and the residue fumed in the oven.

It is apparent that temperature is a more important factor in the production of volatile products than acid strength, and 55° C appears to be near the limit under the condition

of the tests. To allow a margin of safety, 50° C was adopted as the temperature that should be used in a method involving acid digestion of sugars.

Table III suggests that no advantage is gained from the use of a weaker acid at this low temperature. The problem now was whether the 2 + 1 acid strength, as recommended by Nicholls *et al.*, was sufficient, at the lower temperature, to release the fat particles in the wide variety of natural and processed foods that may be analysed.

The third variable, time, was therefore studied, bearing in mind that increase in time was to be avoided if possible, and results are shown in Table IV. A mixture of ground-nut oil, sugar and dextrin was analysed.

TABLE IV

EFFECT OF TIME ON PRODUCTION OF FURFURAL OR CHARRING

Temperature, 50° C; other details as for the method of Nicholls *et al.*[1]

Time of digestion, minutes	Weight of oil taken, g	Weight of oil recovered, g	Recovery, %	Colour of ether layer	Behaviour of residue in oven	Aniline acetate test for furfural in aqueous layer
30	0·4900	0·5005	102·1	Colourless	No fumes	Negative
45	0·4450	0·4595	103·2	Pale yellow	Fumes	Positive
60	0·4850	0·4995	103·0	Pale yellow	Fumes	Positive
75	0·4280	0·4395	102·7	Yellow	Fumes	Positive

The results for this mixture show that no advantage was gained by prolonging the time and that digestion for more than 30 minutes produced sufficient furfural to cause some interference; moreover, darkening of the solution progressively increased with time. Charring is avoided, therefore, if the time of digestion is not increased beyond the original half hour.

THE MINIMUM SUGAR CONTENT TO CAUSE CHARRING

The lowest percentage of sucrose that would cause troublesome charring was ascertained. One gram of skim milk powder was made up to about 2 g with varying proportions of sugar and fat, and the fat determined by the method of Nicholls *et al.* at 80° C. The results are shown in Table V.

TABLE V

EFFECT OF VARIOUS PROPORTIONS OF SUGAR

Sucrose, %	Fat added, %	Fat recovered, %	Recovery, %	Colour of ether layer	Behaviour of residue in oven
2·5	50·4	49·6	98·4	Colourless	No fumes
5·0	46·8	46·5	99·4	Colourless	No fumes
10·0	43·8	44·0	100·4	Pale yellow	Fumes
20·0	52·5	50·9	—	Yellow	Strong fumes

It appears that the breakdown products become excessive when the percentage of sugar is between 5 and 10. At higher percentages of sugar the charring gives rise to products that necessitate much longer oven-drying of the fat residue—up to 13 hours on occasion—and may lead to abandonment of the determination owing to the formation of unbreakable emulsions at the ether extraction stage. With simple fat, sugar and milk powder mixtures 3 to 5 hours' drying is sufficient and no unbreakable emulsions were formed (see Table VI).

TABLE VI

EFFECT OF HIGHER PROPORTIONS OF SUGAR

Sucrose, %	Fat added, %	Fat recovered, %	Recovery, %	Time to obtain constant weight of fat, hours
15	33·3	33·5	100·5	3
25	30·9	30·7	99·3	3
30	31·6	31·7	100·5	3
30	32·6	33·1	101·5	3
40	35·5	35·9	101·0	3
50	36·5	37·2	101·8	5
60	34·2	34·4	100·6	5

Fairly consistent results are obtained, although the recovery is somewhat high, possibly owing to the formation of non-volatile breakdown products.

USE OF LOWER TEMPERATURE DIGESTION—

Previous work having shown that undesirable charring did not occur at a temperature of 50° C (Table III), a comparison was made of results on various products by the method

TABLE VII

COMPARISON OF METHODS AT 50° AND 80° C

Product	Fat found at 50° C, %	Fat found at 80° C, %	Drying time at 50° C, hours	Drying time at 80° C, hours
Ice-cream powder	21·95	A	3	—
	22·05	A	2	—
	21·95	A	2	—
	21·90	A	2	—
Average	21·96			
Standard deviation	0·09			
Rock cakes	22·35	21·47	1	2
	21·28	—	1	3
	21·88	22·06	1	3
	21·92	21·41	1	3
Average	21·86	21·65		
Standard deviation	0·64	0·60		
Bourn-Vita	7·54	6·85	1	2
	7·16	6·94	1	3
	6·89	7·18	1	3
	6·89	7·26	1	3
Average	7·12	7·09		
Standard deviation	0·53	0·36		
Fruit cake	20·70	A	2	—
	20·82	A	2	—
	18·48	A	2	—
	21·67	A	2	—
Average	20·42	Sample not homogenous		
Standard deviation	0·57			
Confectionery products—				
Chocolate couverture	37·31	37·87	1	13
	37·17	37·33	1	13
	37·21	37·19	2	13
Average	37·23	37·45		
Standard deviation	0·11	0·57		
Shortbread biscuits	26·70	27·20	1	4
	26·44	27·60	1	4
	26·93	27·36	1	4
	26·32	26·85	1	4
Average	26·60	27·25		
Standard deviation	0·50	0·53		
Marzipan (ground almonds and sugar)	30·01	29·02	2	9
	30·40	29·37	2	10
	29·79	29·07	1	10
	30·03	29·25	2	9
Average	30·06	29·18		
Standard deviation	0·39	0·31		

of Nicholls *et al.* at temperatures of 50° C and 80° C. The work was carried out without a centrifuge, 8-inch × 1-inch boiling tubes and bark corks being used. As the capacity of these tubes was slightly less than the cylinder recommended, four extractions were made using 40, 30, 30 and 30 ml of the mixed ethers and 10 ml of alcohol instead of 50, 40 and 40 ml of mixed ethers and alcohol as recommended. If after standing overnight the ether emulsion had not separated, the determination was abandoned, as were those marked A in Table VII.

Conclusions

A number of conclusions can be drawn from these results—

(*a*) In the seven products studied—the fat contents of which would all be difficult or impossible to determine by the Soxhlet or Röse - Gottleib method—the fat was equally, and sometimes better, extracted at 50° C than at 80° C.

(*b*) There was little to choose between the reproducibility of the methods. The mean of the standard deviations is 0·40 for the acid digestion at 50° C and 0·47 for that at 80° C. For the chocolate couverture, the reproducibility at 50° C is distinctly better than at 80° C.

(*c*) The time required to bring the fat residue to constant weight was invariably less, sometimes considerably less, with the 50° C digestion than with the 80° C digestion. Often 1 hour sufficed for the former, while the latter required 10 and 13 hours in two instances.

(*d*) The appearance of the fat was cleaner and paler from the 50° C digestion than from the 80° C. There is every likelihood that the constants would be less affected at the lower temperature.

Mention should also be made of studies on the acid digestion method by Schall and Thornton[14] and Randle[15,16] in the United States, who worked on animal feeds containing cereals. Schall and Thornton found somewhat high results by an acid digestion method, but, in spite of determinations of unsaponifiable matter, nitrogen and furfural on the extracts, could obtain no clue to the cause.

Randle,[15] working for the A.O.A.C., confirmed Schall and Thornton's results and made a further study of the effect of different concentrations of acid. Using 2 + 1 acid (strictly 25 + 11) and 1 + 2 (11 + 25) an average of 19 results on cereal-containing animal feeds showed that the weaker acid gave a result 0·14 per cent. higher. He said this weaker acid seemed to facilitate the fat determinations since emulsions were less likely to form, and the extraneous materials settled below the side-arm of the Röhrig tube better than with the strong acid.

Randle[16] continued his study in 1945 and found that the presence of the alcohol appreciably affected the amount of fat extracted. From six cooked animal feeds he obtained an average content that was 1·76 per cent. lower without alcohol than with it. Compared with the Bailey - Walker direct extraction method, the results with alcohol were 1·84 per cent. higher, although the results without alcohol were only 0·08 per cent. higher.

Van Hoorst[17] appears to be the only worker who has published a paper on this subject since 1945; in this paper he discarded Weibull's method in favour of a coagulation-extraction method. Hence it appears that there is need for thorough official study of the determination of fats in sweetened foods.

References

1. Nicholls, J. R., Colgate, R. T., Fraser, L. S., and Hughes, E. B., "Methods of Analysis for the Purposes of the Cake and Confectionery (Control and Maximum Prices) Order, 1942," *Analyst*, 1943, **68**, 48.
2. Analytical Methods Committee, "Report of the Milk Products Sub-Committee," *Ibid.*, 1927, **52**, 402.
3. Wiseman, C. E., *Analyst*, 1930, **55**, 684.
4. Mitchell, Lloyd C., *J. Ass. Off. Agric. Chem.*, 1933, **16**, 514–18.
5. Hertwig, C., *Ibid.*, 1923, **6**, 508.
6. McCance, R. A., and Shipp, H. L., "The Chemistry of Flesh Foods and their Losses on Cooking," Medical Research Council, Special Report Series, No. 187, H.M. Stationery Office, London, 1933.
7. McCance, R. A., and Widdowson, E. M., "The Chemical Composition of Foods," Medical Research Council, Special Report Series, No. 235, H.M. Stationery Office, London, 1942.
8. S.R. & O., 1932, No. 658.
9. Button, Forrest C., *J. Milk Technol.*, 1937, **1**, 30.
10. Lockwood, H. C., *Analyst*, 1938, **63**, 705.
11. Stoldt, W., *Z. Unters. Lebensm.*, 1938, **76**, 228.
12. Voris, Stephen S., *J. Ass. Off. Agric. Chem.*, 1941, **24**, 624.
13. Hall, E. M., Lee, W. V., Ormerod, O., and Williams, E. T., *Analyst*, 1949, **74**, 438.
14. Schall, E. D., and Thornton, M. H., *J. Ass. Off. Agric. Chem.*, 1943, **26**, 404; abstracted in *Analyst*, 1943, **68**, 376.
15. Randle, Stacy B., *J. Ass. Off. Agric. Chem.*, 1943, **26**, 340.
16. ——, *Ibid.*, 1945, **28**, 768.
17. van Hoorst, F. T., *Chem. Weekblad.*, 1949, **45**, 295; abstracted in *Brit. Abs. C.*, 1949, 352.

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The Detection of Palladium, Platinum and Rhodium with *p*-Nitrosodiphenylamine

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p-Nitrosodiphenylamine can be used to detect palladium, platinum and rhodium in the presence of one another and common accompanying elements. Interference from strongly coloured ions in the detection of palladium is prevented by extracting the palladium complex with an organic solvent; preliminary investigation suggests that use might be made of this procedure to separate palladium quantitatively from other platinum metals. The interference of palladium in the test for platinum or rhodium is prevented by adding dimethylglyoxime. Interference by rhodium in the test for platinum is obviated by extracting the platinum complex with an organic solvent. Platinum, rhodium and iridium do not interfere with the detection of palladium. The effect of salts and mineral acids on the sensitivity and the reactions of many other ions with *p*-nitrosodiphenylamine have been studied.

ALTHOUGH various methods have been reported for detecting each of the platinum metals, there is a need for tests that will not be made useless by the presence of large amounts of the other platinum metals or of metals that frequently accompany them. Compounds containing the *p*-nitrosophenylamine group, $p\text{-}NOC_6H_4N<$, have been used for detecting[1,2] and determining[2,3] palladium and are highly selective. The present investigation has shown that solutions of platinum and rhodium, when *heated* with *p*-nitrosodiphenylamine, react to give colours that can be used for their detection. This paper presents a method for detecting palladium, platinum and rhodium in the presence of large amounts of one another and of other metals; it is based on the large difference in the rate of reaction of palladium as compared with that of platinum or rhodium.

REAGENTS—

p-Nitrosodiphenylamine—This compound was obtained from the Eastman Kodak Company (No. 1127) and was recrystallised from 50 per cent. ethyl alcohol. A solution of the reagent was prepared by dissolving 50 mg of the recrystallised material in 500 ml of 95 per cent. ethyl alcohol and diluting to 1 litre with water.

Platinum metal chloride solutions—These solutions were prepared by dissolving the pure metal or suitable pure salt in aqua regia, hydrochloric acid or water; nitric acid, if present, was removed by evaporating to dryness in the presence of hydrochloric acid and dissolving the residue in a solution containing 1 ml of concentrated hydrochloric acid per litre.

p-NITROSODIPHENYLAMINE AND PALLADIUM

REACTION ON PAPER—

It has been reported that 0·01 μg of palladium can be detected with *p*-nitrosodiphenylamine by the drop-reaction technique on paper.[2] This limit has now been confirmed and the use of this reagent for the detection of palladium in the presence of other platinum metals has been investigated.

Procedure—Moisten a piece of Whatman No. 120 filter-paper with the reagent solution and allow to dry. From a glass hair capillary, run 1 drop of a feebly acid solution containing palladium on to the prepared paper. A purple or reddish-purple spot, due to the formation of the complex $Pd(NOC_6H_4NHC_6H_5)_2Cl_2$, indicates palladium. If the procedure is carried out *at room temperature* the reagent is very selective, the only interference noted being that due to gold.

No noticeable reaction was obtained under these conditions from solutions containing 50 μg per ml of the following ions: $Fe^{\cdots}$, $Fe^{\cdot\cdot}$, $Co^{\cdot\cdot}$, $Ni^{\cdot\cdot}$, $Al^{\cdots}$, $Sb^{\cdots}$, $Ba^{\cdot\cdot}$, $Be^{\cdot\cdot}$, $Bi^{\cdots}$, $Cd^{\cdot\cdot}$, $Li^{\cdot}$, $Ag^{\cdot}$, $Ca^{\cdot\cdot}$, $Cu^{\cdot\cdot}$, $Ce^{\cdot\cdot\cdot\cdot}$, $Mn^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, $Pb^{\cdot\cdot}$, $Ti^{\cdot\cdot\cdot\cdot}$, $Hg^{\cdot\cdot}$, $Zr^{\cdot\cdot\cdot\cdot}$, $UO_2^{\cdot\cdot}$, AsO_4''', SeO_3'', CrO_4'', MoO_4'', TeO_4'', and WO_4''.

Gold ($AuCl_4'$) gives a purple spot similar to that given by palladium, although the reaction is not as sensitive; 1·5 μg of gold per ml are required to give a noticeable reaction.

Although no reaction is obtained with the other platinum metals even after 6 hours, both platinum and rhodium react to give a purplish red spot after 24 hours. It is thus possible to detect palladium in the presence of large quantities of platinum, rhodium and iridium, alone or present together. Solutions containing 0·1 μg of palladium per 0·05 ml give a positive test in the presence of as much as 500 parts of platinum, iridium and rhodium.

Salts and mineral acids have a marked effect on the sensitivity of the test. With a palladium concentration of 0·1 μg per 0·05 ml the colour intensity decreases markedly in solutions having a salt concentration of 0·025 *M*; solutions 0·5 *M* in sodium chloride, ammonium chloride, sodium nitrate, sodium sulphate or potassium chloride still give a faint reaction. Similar results are obtained with mineral acids, solutions 0·05 *M* in hydrochloric acid showing a definite decrease in colour intensity; with a palladium concentration of 0·1 μg per 0·05 ml, however, a faint reaction is still obtained from solutions 0·5 *M* in hydrochloric acid.

REACTION IN TEST TUBE—

The detection of palladium with *p*-nitrosodiphenylamine can be carried out easily with solutions in an ordinary test tube; this procedure offers some advantages over the drop-reaction method. The *p*-nitrosodiphenylamine complex can be readily extracted from

TABLE I

DETECTION OF PALLADIUM WITH *p*-NITROSODIPHENYLAMINE

Pd present, μg per ml	Other metal present, μg per ml	Remarks
0·3	1300 Pt	Extraction not necessary to detect palladium
0·3	3200 Pt	Difficult to detect palladium without extraction with ethyl acetate; extraction gave a strongly pink acetate layer; a solution of platinum only similarly treated gave a yellow acetate layer (reagent)
0·3	32000 Pt	Excellent positive test after ethyl acetate extraction
0·3	800 Rh	Difficult to detect palladium without extraction; excellent positive test after extraction
0·3	8000 Rh	Pink acetate layer after extraction showed the presence of palladium
0·3	300 Ir	Difficult to detect palladium without extraction; excellent positive test after extraction
0·3	3000 Ir	Pink acetate layer after extraction showed the presence of palladium

aqueous solution with ether, ethyl acetate, or chloroform. Although an extraction procedure is not necessary, it does possess the important advantages of eliminating the usual masking interferences of cobalt, iridium and rhodium and of providing a method of concentrating the palladium with a resultant gain in sensitivity. Furthermore, the application of this procedure for separating trace amounts of palladium from concentrated platinum metal solutions shows definite possibilities.

Extraction of palladium complex—Solutions containing 3 μg of palladium per ml were treated with hydrochloric acid and 1 ml of reagent solution, and after 5 minutes, extracted with ethyl acetate. A deep red colour developed in the acetate layer derived from solutions containing hydrochloric acid up to approximately 0·1 *M*; the aqueous layer was completely colourless after extracting with 10 ml of ethyl acetate, and no palladium could be found in it by spot tests after evaporation. Similar results were obtained with ether or chloroform as extractant.

Effect of salts—The intensity of the colour produced by 0·3 μg of palladium per ml was lower in 0·02 *M* sodium chloride solution and decreased further with increasing salt concentration until in 0·6 *M* solution no colour was obtained. Potassium and ammonium salts had a similar effect.

Procedure—To 5 ml of a feebly acid solution add 4 or 5 drops of the *p*-nitrosodiphenylamine reagent. Development of a red-to-purple colour indicates the presence of palladium. If the colour is masked by that of the solution itself, extract with 5 ml of ethyl acetate or chloroform. Table I shows some of the results obtained by this procedure. These results were confirmed by at least two other members of this laboratory.

The test for palladium was successful in solutions containing 1 per cent. of vanadate, chromate, or cobalt. Rhodium and iridium, quoted as "masking interferences" by West and Amis,[1] can also be eliminated by the extraction procedure.

Preliminary experiments indicate that the extraction of the *p*-nitrosodiphenylamino complex of palladium may be useful in the separation of trace amounts of palladium from other platinum metals. In a few experiments the palladium complex was extracted with ethyl acetate or chloroform, the extract was evaporated to dryness and the residue treated with sulphuric and nitric acids to destroy organic matter. The palladium was determined colorimetrically with 2-mercapto-4:5-dimethylthiazole.[4] Results so far have been satisfactory, but further experiments are necessary to complete this investigation.

p-NITROSODIPHENYLAMINE AND PLATINUM

p-Nitrosodiphenylamine reacts with $PtCl_6''$ solutions to give a slight red coloration *only after several hours at ordinary temperatures*. Increased temperature has such an effect on the reaction rate, however, that an intense red colour is obtained by boiling a feebly acid solution for 10 minutes. This reaction permits the detection of platinum in the presence of large amounts of palladium, iridium, rhodium and other metals.

Procedure—Dilute a few drops of the feebly acid test solution in an ordinary test tube to approximately 5 ml with distilled water, add 5 drops of the reagent solution, and place the test tube in a beaker of boiling water for 15 minutes. A pink-to-red coloured solution shows the presence of platinum. The limit of platinum detectable is 1 μg per ml.

Reactions of various ions—No reactions with *p*-nitrosodiphenylamine were noted when this procedure was applied to solutions containing 100 μg per ml of the following ions: $Pb^{\cdot\cdot}$, $Ag^{\cdot}$, $Cu^{\cdot\cdot}$, $Co^{\cdot\cdot}$, $Ni^{\cdot\cdot}$, $Sb^{\cdot\cdot\cdot}$, $Bi^{\cdot\cdot\cdot}$, $Hg^{\cdot\cdot}$, $Al^{\cdot\cdot\cdot}$, $Ba^{\cdot\cdot}$, $Be^{\cdot\cdot}$, $Cd^{\cdot\cdot}$, $Ca^{\cdot\cdot}$, $Ce^{\cdot\cdot\cdot\cdot}$, $Li^{\cdot}$, $Mg^{\cdot\cdot}$, $Mn^{\cdot\cdot}$, $Sr^{\cdot\cdot}$, $Tl^{\cdot}$, $Th^{\cdot\cdot\cdot\cdot}$, $Sn^{\cdot\cdot}$, $Zn^{\cdot\cdot}$, $Zr^{\cdot\cdot\cdot\cdot}$, $UO_2^{\cdot\cdot}$, AsO_4''', CrO_4'', MoO_4'', PO_4''', SeO_3'', TeO_3'', WO_4'' and VO_3''.

Ferric iron gave a turbid orange coloured solution, rhodium an orange-pink colour, and iridium a light yellow-brown. Palladium and gold reacted as previously described.

Effect of salts and mineral acids—Salts have a definite effect on the sensitivity of the test for platinum. With a platinum concentration of 2·5 μg per ml, little difference in the colour could be detected in solutions 0·02 *M* in sodium chloride, but with increasing salt concentration the intensity decreased until at 0·07 *M* a colourless solution was obtained.

Mineral acids also have a marked effect on the colour intensity. With a platinum concentration of 6 μg per ml, maximum intensity was obtained in solutions 0·005 to 0·01 *M* in hydrochloric acid; a faint pink colour was detectable, however, in solutions 0·1 *M* in hydrochloric acid. Colourless solutions were obtained at higher acidities.

DETECTION OF PLATINUM IN THE PRESENCE OF OTHER PLATINUM METALS—

Under the conditions of the reaction with platinum, palladium and rhodium must both be considered as positive interferences. These interferences, however, are readily eliminated and the procedure outlined below permits the detection of platinum in the presence of palladium and rhodium in addition to iridium and common accompanying elements.

Palladium interference is obviated by one of two possible procedures. (i) Dimethylglyoxime prevents the formation of the palladium complex with *p*-nitrosodiphenylamine but does not interfere with the platinum reaction; this was shown in numerous experiments carried out on platinum, palladium and platinum - palladium mixtures. The procedure followed was to evaporate the solutions (which varied greatly in hydrogen ion concentration) to dryness on a steam-bath, dissolve the residue in distilled water, add 5 drops of 0·1 *M* hydrochloric acid, and dilute to approximately 5 ml. The amount of dimethylglyoxime added depended, naturally, on the amount of palladium present; a large excess was avoided because of the sensitivity of the test to salt concentration. (ii) Palladium can be allowed to react with *p*-nitrosodiphenylamine *at room temperature* and the complex extracted with ethyl acetate before testing for platinum. Since the platinum complex is not formed during this time, little, if any, platinum is extracted in the procedure, but the usefulness of the method

is limited by the number of extractions necessary to complete the removal of large concentrations of palladium. One extraction with 5 ml of ethyl acetate completely removes palladium from a solution containing 3 μg per ml, so this method is, therefore, recommended only for solutions containing palladium at such concentrations.

Under the conditions for detection of platinum, rhodium in excess of 10 μg per ml gives a colour similar to that given by platinum. Interference from rhodium above this concentration, however, can be readily eliminated by extraction of the platinum complex with ethyl acetate or chloroform, which do not extract the rhodium complex even from solutions containing 150 μg of rhodium per ml. Complete extraction of the platinum complex was obtained from acid solutions containing 150 μg of platinum per ml by shaking with 10 ml of ethyl acetate. This procedure also avoids interference of iridium and other metals that ordinarily would cause a masking effect because of their colour.

TABLE II

DETECTION OF PLATINUM WITH *p*-NITROSODIPHENYLAMINE

Pt present, μg per ml	Other metal present, μg per ml	Remarks
2·5	12 Pd	Excellent test for platinum; palladium reaction prevented by dimethylglyoxime
2·5	700 Pd	Pink solution showed the presence of platinum; palladium reaction prevented as above. Palladium solution treated similarly with dimethylglyoxime gave no reaction on adding *p*-nitrosodiphenylamine
2·5	3 Pd	Palladium removed before testing for platinum by adding reagent and extracting. Good positive test for aqueous extract from solutions containing platinum; negative test from solutions containing only palladium
nil	5 Rh	Pale yellow solution
2·5	nil	Pink solution
nil	15 Rh	Pink solution obtained shows that rhodium will interfere at this concentration unless extraction procedure is used
nil	130 Rh	Acetate layer after extraction of the pink solution was colourless; acetate layer after extraction of solution containing 2·5 μg of platinum was pink
2·5	1300 Rh	Pink acetate layer on extraction showed the presence of platinum; extraction of solution containing 1300 μg of rhodium similarly treated gave a colourless acetate layer
2·5	500 Ir	Platinum difficult to detect without extraction
2·5	1000 Ir	Pink acetate layer after extraction; extraction of solution containing 1000 μg of iridium gave a colourless acetate layer
2·5	200 Pd 200 Rh 200 Ir	Palladium reaction prevented with dimethylglyoxime; extraction of solution after reaction gave a pink acetate layer, a good positive test

A series of unknown solutions, prepared by two members of this laboratory, was analysed and the platinum was detected in all. The compositions of these unknown solutions varied, but a typical one contained 5 μg of platinum in the presence of 100 μg each of rhodium, arsenic, lead and lithium. Other solutions contained antimony, zinc, vanadium, manganese, tin, nickel, mercury or silver. Detection of platinum in the minerals braggite and sperrylite was also easily possible.

The possibility of using *p*-nitrosodiphenylamine for the colorimetric determination of platinum was investigated. The intensity of the colour produced, however, was so dependent on the boiling period used to develop the colour in the solution that the work was abandoned. Results obtained by use of a Spekker absorptiometer with a 4-cm cell and Ilford No. 604 filter for 8 μg of platinum per ml after boiling for various times are shown below—

Boiling time, minutes	10	15	30	60
Extinction	0·568	0·504	0·384	0·243

p-NITROSODIPHENYLAMINE AND RHODIUM

Neutral rhodium solutions react with *p*-nitrosodiphenylamine *when heated* to give an orange to orange-red colour. The sensitivity of this reaction is dependent on the acidity of the solution. With a rhodium concentration of 2·5 μg per ml, solutions containing 1 drop of 0·1 *M* hydrochloric acid in a volume of 5 ml gave only a faint reaction; solutions containing even 1 drop of 0·1 *M* sodium hydroxide gave no reaction.

Procedure—To 5 ml of a neutral solution in an ordinary test tube add 5 drops of the reagent and place the test tube in a beaker of boiling water for 5 minutes. The appearance of an orange to orange-red colour indicates the presence of rhodium. The limit of rhodium detectable is 0·5 μg per ml.

Effect of salts—The salt concentration is not so important in the test for rhodium. With 2·5 μg of rhodium per ml, a strongly positive test was obtained from solutions 1·0 *M* in sodium chloride, and a reasonable positive test in 2 *M* sodium chloride.

Interferences—Palladium can be eliminated as described above for platinum. Platinum causes no interference below 5 μg per ml under the conditions described, but higher concentrations of platinum give a colour similar to that given by rhodium. Attempts to detect rhodium in the presence of platinum by extraction procedures were unsuccessful because the platinum complex was only partially extracted from neutral solution. Iridium does not interfere except at such concentrations that its colour masks the reaction.

TABLE III

DETECTION OF RHODIUM WITH *p*-NITROSODIPHENYLAMINE

Rh present, μg per ml	Other metal present, μg per ml	Remarks
3	6 Pd	Palladium reaction prevented with dimethylglyoxime; excellent positive test for rhodium
3	170 Pd	Excellent test for rhodium; similar test on solution containing only palladium, in the presence of dimethylglyoxime, was negative
3	700 Pd	Good positive test for rhodium; palladium reaction prevented with dimethylglyoxime
3	14 Ir	Good positive test for rhodium; solution of iridium treated similarly was light brown in colour
3	140 Ir	No difficulty in detecting rhodium at this iridium concentration
3	280 Ir	Rhodium detected only with difficulty because of the strongly coloured iridium solution

The author desires to record his sincere appreciation to Mr. L. S. Theobald for his encouragement and help in this work. Appreciation and thanks are expressed to Lord Beaverbrook for providing the scholarship, through the Beaverbrook Overseas Scholarship Fund, that enabled this work to be completed.

REFERENCES

1. West, P. W., and Amis, E. S., *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 400.
2. Yoe, J. H., and Overholser, L. G., *J. Amer. Chem. Soc.*, 1941, **63**, 3224.
3. Overholser, L. G., and Yoe, J. H., *Ibid.*, 1939, **61**, 2058.
4. Ryan, D. E., to be published shortly.

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LONDON, S.W.7

July, 1950

A Photo-electric Micro Nephelometer

By A. C. MASON

A photo-electric nephelometer is described which is suitable for small volumes, 0·1 to 0·5 ml. The apparatus may be used over a much wider range of concentrations than the usual design of nephelometer.

Nephelometric and turbidimetric methods are not widely applied in chemical analysis chiefly because the precision attained with them is not of a high order; on the other hand they are sometimes useful for rapid routine determinations, or when there is no suitable alternative. Nephelometric methods are particularly useful in biological work, where the sampling errors may be much greater than the analytical errors. It is then advantageous to use a rapid but less accurate method and to analyse a correspondingly larger number of samples.

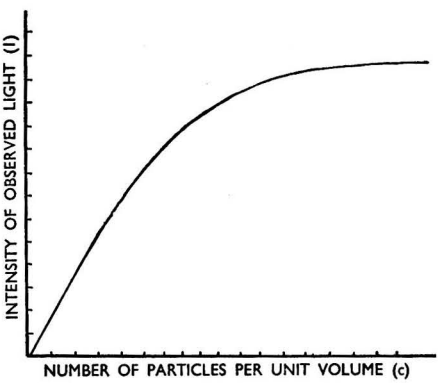

Fig. 1. Diagram showing that the intensity of observed light, I, is not in linear proportion to the number of particles in the suspension when viewed in the usual type of nephelometer

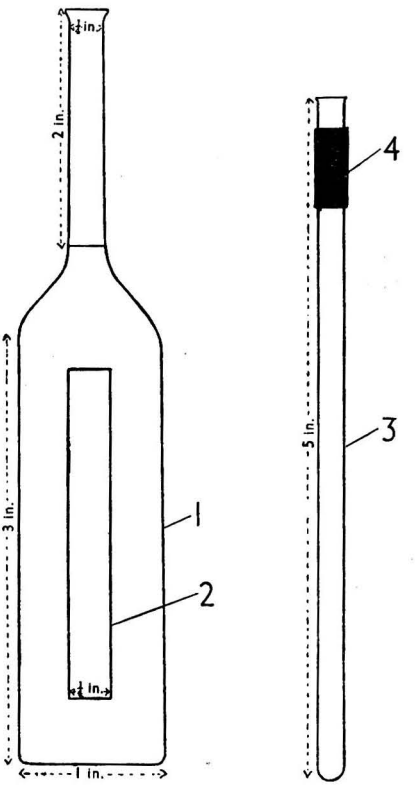

Fig. 2. Glass components of the nephelometer cell
(1) Outer tube, (2) window, (3) inner tube to fit into neck of tube (1), and (4) roll of thin paper wound around (3) and shellacked

Although the nephelometric technique is potentially applicable to any reaction in which a finely divided precipitate is produced quantitatively, the method depends critically on the physical condition of the precipitate (*e.g.*, the size and number of the primary particles and the degree of aggregation). It is often possible, however, by laying down strictly defined conditions for precipitation, raising the viscosity of the medium and using protective colloids, to obtain precipitates that are suitable for nephelometric work.

The apparatus described in this paper was designed for use with small volumes of solution, 0·1 to 0·5 ml.

Basic principles

In the usual type of nephelometer the turbid suspension is placed in a vessel illuminated from the side and the amount of light scattered is measured in a direction at right angles, usually vertically. The relation between the intensity, I, of scattered light and the concentration, C (amount per unit volume), of the insoluble substance for such an apparatus is shown in Fig. 1. As C increases, I increases disproportionately, and a stage is reached beyond which further increase in C produces no appreciable increase in I. This is because light scattered from the upper layers of the suspension is prevented from reaching the observer by the optical density of the lower layers of suspension. The same difficulty arises with turbidimeters in which transmitted light is measured (see Fig. 5). The design of the apparatus described below minimises this defect and thus greatly extends the concentration range over which measurements may be made. At the same time, owing to the small volume that can be employed, the sensitivity of the method is greatly increased.

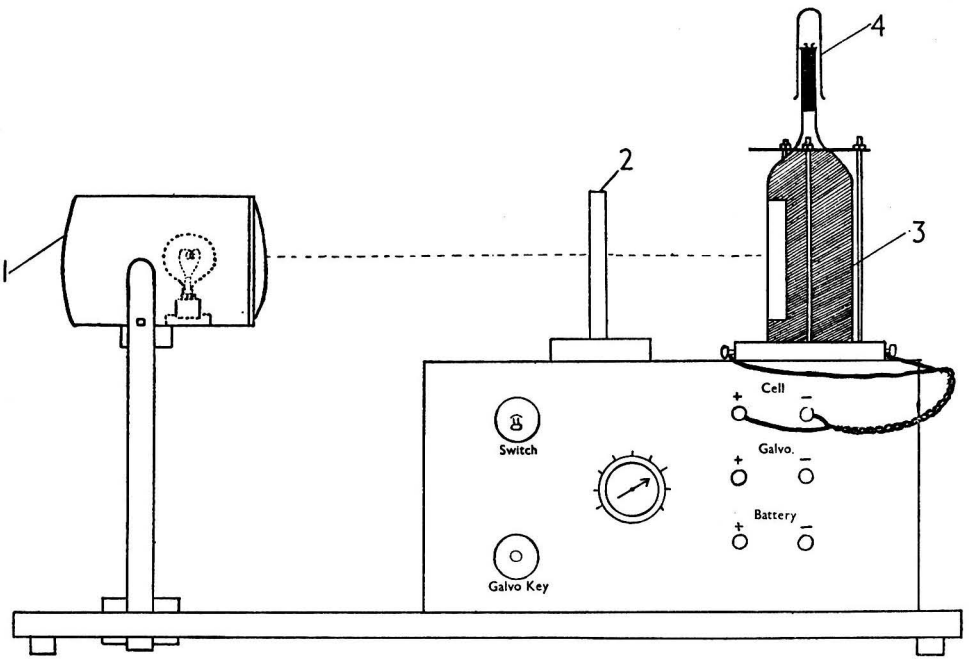

Fig. 3. Nephelometer stand and instrument panel
(1) Microscope lamp fitted with 12-volt bulb, (2) holder for filters, (3) nephelometer cell, and (4) cover consisting of a small black-enamelled test tube

The apparatus consists essentially of a narrow tube mounted concentrically inside a 1-inch diameter tube, the outer surface of which is silvered except for a narrow window at the side to admit the incident light and another window at the bottom to allow the scattered light to fall on a photo-electric cell mounted below. The current produced by the light falling on the photo-electric cell may be measured directly by means of a micro-ammeter or indirectly by developing a potential difference across a fixed resistance and balancing it against a potentiometer.

The apparatus

Construction of the nephelometer cell—

An ordinary 1-inch diameter boiling tube is shaped to the dimensions shown in Fig. 2. After thorough cleaning inside and out, the tube is allowed to stand in a bath of silvering solution.[1] The tube is removed, washed with distilled water, allowed to dry and painted with shellac varnish (5 per cent. solution of shellac in industrial spirit), leaving the area for the window and the bottom unvarnished. When the varnish is dry, the silver is removed from the unvarnished areas by rubbing with a cloth. A final coat of black paint over the varnish helps to exclude extraneous light. A narrow tube (3), of suitable dimensions, is

prepared with a rounded bottom. Thin typing paper (4) is wrapped round the upper part of this tube until a tight push fit into the neck of the silvered tube is obtained. The paper is then coated with shellac varnish to hold it in position. Sufficient water is placed in the silvered tube to bring the level almost to the neck when the centre tube is inserted. The neck is then gently heated and the shellacked centre tube and paper wrapping pushed home. More shellac applied to the neck ensures a water-tight joint. (If at some subsequent date it is necessary to withdraw the tube, this can be accomplished quite readily after heating the neck of the silvered tube in a flame to soften the shellac.) The reason for filling the silvered tube with water is to prevent any difficulty that might arise through condensation of moisture on the inner surface of the silvered tube.

The three fixing screws of a 1-inch diameter mounted selenium photo-electric cell (as supplied by Messrs. Megatron, London) are discarded and replaced by three rods tapped at both ends. The nephelometer and photo-cell are then assembled. The assembly fits on a stand, as shown in Fig. 3, by means of locating pins, so that it may be easily removed for filling and cleaning. The stand carries a microscope lamp, which is focussed on the nephelometer. Provision is made for the interposition of filters if required.

THE PHOTO-ELECTRIC CELL CIRCUIT—

Direct measurement of current output from the photo-electric cell by means of a micro-ammeter was considered unsatisfactory because a considerable amount of the incident light is reflected into the photo-electric cell even with water in the centre tube. The difficulty of this high zero reading is overcome by making use of the circuit described by Campbell and Freeth[2,3] that is shown in Fig. 4.

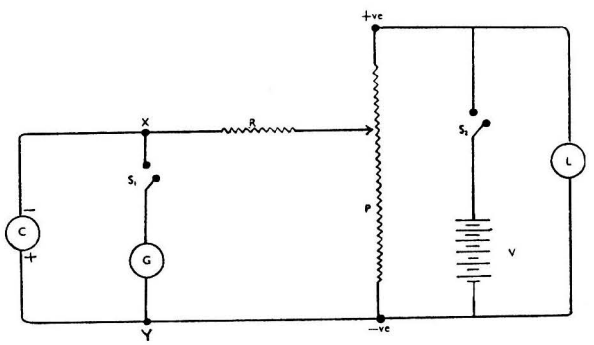

Fig. 4. Circuit diagram

(C) Photo-electric cell, (G) low resistance galvanometer or micro-ammeter, (L) lamp, source of illumination, (P) wire-wound radio-type 10-watt potentiometer, resistance small compared with (R), (R) fixed resistance, radio-type, about 100,000 ohms (see text), (S_1) galvanometer key or switch, (S_2) on-off switch, and (V) 12-volt car battery

The potentiometer is adjusted to give no deflection with water in the centre tube. Thereafter the potentiometer is left set while the turbid solutions are introduced into the centre tube and the corresponding deflections read on the galvanometer or micro-ammeter.

It will be seen from the circuit diagram that the potentiometer is connected, not in the usual manner, but positive to negative, and that the galvanometer is not in series with the cell but in parallel. Such an arrangement is suitable for the measurement of current output because at balance the potential difference between X and Y is zero, and the cell is operating under ideal short circuit conditions.

At balance no current flows through G and the fall of potential across R due to the current output, i, of the photo-electric cell is iR. This, together with the applied negative potential, −P, sum to zero, *i.e.*—

$$i\mathrm{R} - \mathrm{P} = 0, \text{ or } i = \mathrm{P}/\mathrm{R}$$

This equation enables R to be chosen to suit the maximum values of i and P.

The apparatus was designed with due regard to the factors, discussed by Preston,[3] that influence the performance of selenium type photo-electric cells.

SOURCE OF ILLUMINATION—

The intensity of illumination need not be high but it must remain constant during a series of determinations; for this reason mains operated lamps are not satisfactory. It is found that a microscope lamp fitted with a 36-watt motor car bulb is quite suitable. A 12-volt

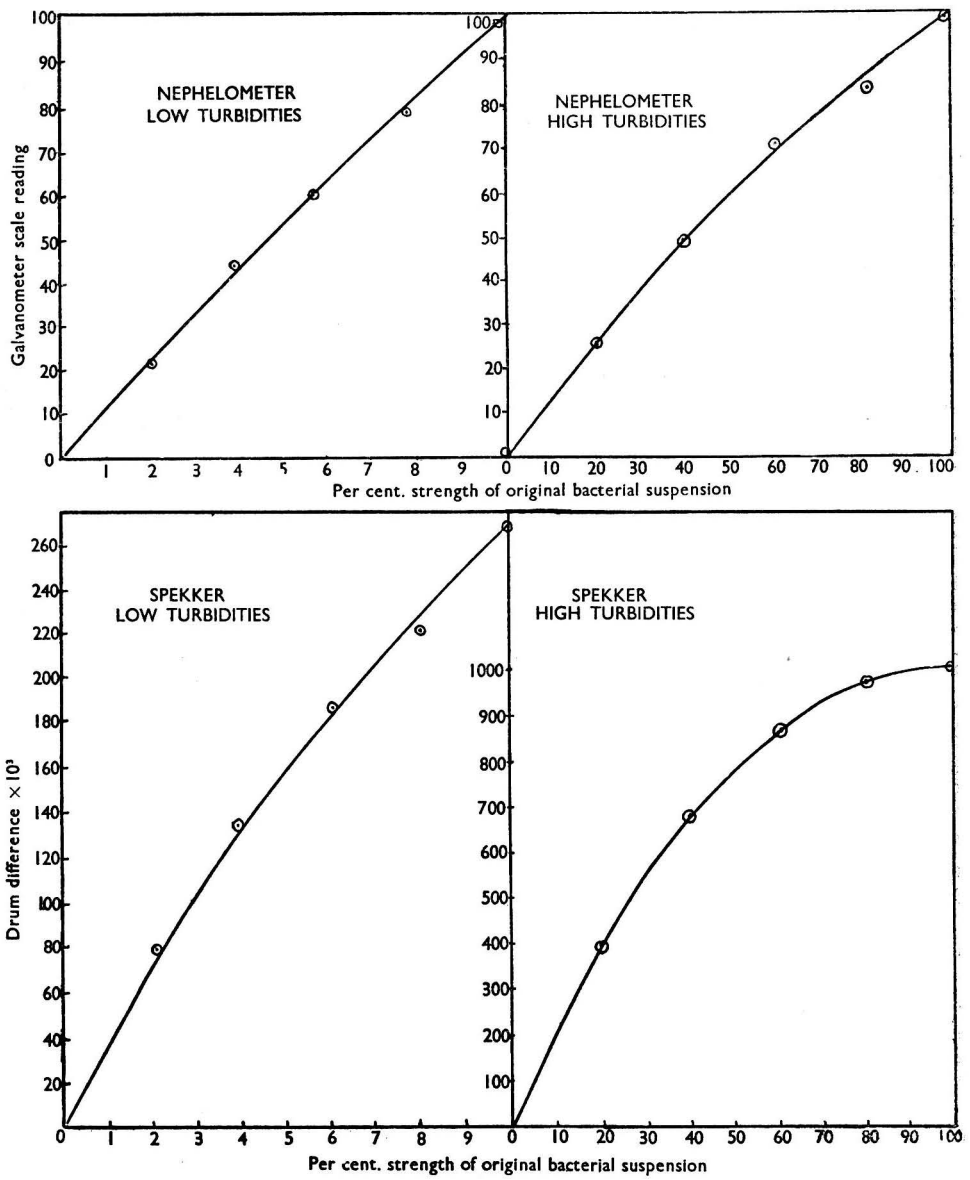

Fig. 5. Comparison of micro-nephelometer and the Spekker absorptiometer. The same series of bacterial suspensions was used, 0·25 ml with the nephelometer and 0·5 ml with the Spekker

car battery is used to operate both the lamp and the potentiometer. Before work is begun, the battery should be fully charged and the voltage allowed to reach a steady value by leaving the lamp switched on for 15 to 30 minutes.

The light intensity may be varied by adjusting the iris diaphragm of the microscope lamp or by any convenient alternative such as varying the resistance in the lamp circuit.

Operation of the nephelometer

The centre tube of the nephelometer is cleaned thoroughly by rinsing with water. This is conveniently done by means of a teat pipette drawn out to a long capillary tube, which can be introduced into the centre tube. The centre tube may also be cleaned by means of a piece of stiff wire around which cotton is wrapped to form a kind of fluffless pipe cleaner.

Water is placed in the centre tube and with various settings for light intensity the values of P that give zero deflection of the galvanometer are found. Then the most turbid suspension is placed in the nephelometer and the light intensity adjusted so that the full scale of the galvanometer is used. The potentiometer, P, must, of course, be adjusted at the same time so that the galvanometer reading will be zero for water in the centre tube. The apparatus is then ready for a series of readings, which are made by merely placing the turbid suspension in the centre tube and reading the galvanometer. The apparatus is calibrated by means of a series of standards.

Performance of the instrument—

In order to test the apparatus and to avoid the difficulties that arise with chemical precipitates, a suspension of killed bacteria was used and a series of dilutions prepared. The concentration of the turbidities was then expressed in arbitrary units that were in strict proportion covering the range 0 to 100. The results, Fig. 5, show that departure from linearity is not great. For comparison, the light transmittance for the same series of suspensions was determined with the Spekker absorptiometer and the results plotted in Fig. 5. With the Spekker, 1-cm micro cells were used with neutral heat absorbing filters.

The author's thanks are due to Miss B. M. Kidwell and Mr. R. Neve for assistance in constructing and testing the apparatus and to Miss M. E. Bunyard for making the drawings.

References

1. Hodgman, Charles D., "Handbook of Chemistry and Physics," 27th Edition, Chemical Rubber Publishing Co., Cleveland, Ohio, 1943, p. 2433.
2. Campbell, N. R., and Freeth, M. K., *J. Sci. Inst.*, 1934, **11**, 125.
3. Preston, J. S., *Trans. Illuming. Eng. Soc.*, 1943, **8**, 121.

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Notes

AN IMPROVED TECHNIQUE FOR PRECIPITATION OF POTASSIUM WITH SODIUM COBALTINITRITE

Sodium cobaltinitrite continues to be the commonest reagent for the precipitation of potassium, both in qualitative and quantitative analysis, in spite of the well known instability of the reagent in solution. Generally, great care must be exercised in preparing the reagent and it must be kept cold and in the dark. Kramer and Tisdall[1] kept the reagent in a refrigerator and filtered it before use. Tischer[2] prepared a fresh solution from the solid immediately before use. But invariably the reagent deteriorates rapidly when the aqueous solution is used in the laboratory under ordinary conditions. The reagent is, however, quite stable in the solid form. This fact is utilised in the technique to be described.

AnalaR sodium cobaltinitrite, 2·5 g, is dissolved in 5 ml of water in a 100-ml conical flask and then precipitated in a finely divided form by adding 10 ml of 99 per cent. alcohol slowly and with shaking. After centrifugation the supernatant liquid is decanted away and the precipitate is suspended in 50 ml of 99 per cent. alcohol. This should give a finely divided even suspension that can be used in the same way as a solution of the reagent, provided it is shaken before use.

If the suspension is run slowly into water, while stirring, the suspension passes instantly into solution, and if it is run into a solution of a potassium salt, the potassium complex is precipitated. So long as the ratio of volumes of reagent to aqueous solution does not exceed 1 to 2 there will be no excess of undissolved sodium cobaltinitrite.

The suspension may be stored for months without any special precautions with regard to temperature or light exclusion. It is important that 99 per cent. alcohol be used in preparing the suspension because the presence of moisture leads to decomposition of the reagent and loss of sensitivity.

The reagent has been used in this laboratory for the estimation of potassium both by a nephelometric method and by a modification of the method described by Ismail and Harwood[3] in which the silver potassium cobaltinitrite complex is precipitated by adding silver nitrate to a buffered solution of the potassium salt, followed by addition, while stirring, of sodium cobaltinitrite suspension. The precipitate is centrifuged, washed, dissolved in acid and the cobalt determined colorimetrically by means of potassium thiocyanate.[4,5]

An estimate of the precision of this method can be made from the following figures which relate to eight independent estimations of potassium in a sample of plant leaves—

Number of estimations	Mean	Standard error	Standard error as percentage of mean
8	106 μg	2·5 μg	2·4

REFERENCES

1. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, **1921, 46, 339.**
2. Tischer, J., *Biochem. Z.*, **1931, 238**, 148.
3. Ismail, A. M., and Harwood, H. F., *Analyst*, 1937, **62**, 443.
4. Mason, A. C., "Report of East Malling Research Station for 1949," 1950, pp. 111–115.
5. Sandell, E. B., "Colorimetric Determination of Traces of Metals," Interscience Publishers Inc., New York, 1944, p. 202.

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July, 1950

MANGANESE SULPHATE AS A CATALYST IN CERIC SULPHATE TITRATIONS

THE use of ceric sulphate as an oxidising agent in acid solutions is becoming increasingly popular and the two most convenient ways of standardising the ceric sulphate are against the primary standards sodium oxalate and arsenious oxide. In the first of these, and also with the second if in acid solution, iodine chloride is used as a catalyst. As iodine chloride is itself an oxidising agent, the amount added must be small and the quantity of reducing agent comparatively large so as to reduce the error to a minimum.

In the standardisation of potassium permanganate against sodium oxalate the reaction proceeds slowly until manganese sulphate has been formed; this then catalyses the reaction. Hence the use of manganese sulphate as a catalyst in the above reactions is suggested. As its part in the reactions is purely that of a catalyst, no error is introduced.

It is still necessary to maintain the temperature of the solution being titrated between 45° and 50° C.

The following figures were obtained when 1 drop of *o*-phenanthroline ferrous complex, 0·025 *M* solution, was used as indicator. The use of crystal violet[1] as the indicator is not recommended.

Solutions—

Ceric sulphate, 0·05 *N*, standardised against electrolytic iron.
Iodine chloride, 0·005 *M*.
Manganese sulphate, 0·01 *M*.

Weight of sodium oxalate taken, g	Weight found by titration with catalysts— 1 ml of $MnSO_4$, g	2 ml of ICl, g
0·02040(8)	0·02041	0·01992
0·06122(4)	0·06118	0·06081
0·1052(6)	0·1052	0·1050

Each result is an average of four readings.

REFERENCE

1. Murthy, T. K. S., and Raghava Rao, S. V., *J. Indian Chem. Soc.*, 1950, **27**, 37.

GUILD OF UNDERGRADUATES
UNIVERSITY OF WESTERN AUSTRALIA
CRAWLEY, WESTERN AUSTRALIA

J. P. WATSON

July, 1950

Apparatus

AUTOMATIC DETERMINATION OF RATE OF MERCURY FLOW FROM DROPPING ELECTRODE

For use in polarographic analysis, Lingane[1] has described the construction of a dropping mercury electrode that permits the automatic timing of the interval during which a definite mass of mercury flows from the capillary.

Three tungsten contacts are sealed one above the other through the wall of a vertical Pyrex tube, which communicates with the capillary at its lower end and with a reservoir for mercury by way of a side arm and flexible tube. The reservoir is raised until the mercury reaches the uppermost contact, after which any further flow from the reservoir is prevented by closing a clip on the flexible tube. Whilst mercury flows from the capillary and its meniscus passes down the tube, an electric clock automatically records the interval between the circuit breaks at the upper and middle contacts. If the mass of mercury flowing during this interval is once determined, the rate of flow of mercury can be measured subsequently, with the tip of the capillary in any desired supporting electrolyte, merely by observing the interval registered automatically by the clock.

An apparatus based on Lingane's description has been in use in the writer's laboratory for three years, and some details that have contributed to its successful operation are noted below and illustrated by reference to Fig. 1.

Design and position of contacts—

The contacts, P, Q, R, of 26 S.W.G. tungsten wire, are sealed directly through the wall of a Pyrex tube of 3·5 mm internal diameter, but the upper and middle contacts both project for a distance of 7 mm down the axis of the tube. Since the tungsten-to-glass seals are several millimetres distant, the walls of the tube surrounding the tips of contacts P and Q are perfectly cylindrical and the mercury meniscus is therefore disposed symmetrically around these contacts at the instant of breaking circuit.

Cleaning and sharpening the contacts—

As Lingane points out, the contacts must be clean and they should terminate in fine points to minimise the tendency of mercury to adhere to them. It will be found that the contacts, after sealing through the Pyrex tube, will be coated with a blue-grey film. This film is conveniently removed by electrolysis, and by the same process the tips of the contacts can be sharpened to needle points. For this purpose the upper end of the tube is temporarily closed, the tube is inverted and sufficient 10 per cent. sodium hydroxide solution introduced to cover all the contacts, of which P and Q must then point vertically upwards. Contacts P and R are connected across a 4-volt accumulator but their polarity is reversed at 1-minute intervals. After several reversals the blue-grey film either peels from both contacts, or dissolves in the electrolyte. Having reached this stage, P is connected to the positive pole for 5 minutes, during which time the tip of the contact dissolves rapidly leaving it sharply pointed. Lastly, the current is reversed for 1 minute to remove from contact P by cathodic treatment any trace of an orange coloured film which the 5-minute anodic treatment often produces. Contact Q is similarly cleaned and polished to a point. The bore of the tube is washed with a stream of cold water, then with acetone, and finally a stream of clean, dry air is drawn through it.

Protection of contacts from moisture—

The method of assembly of the dropping mercury electrode has been described elsewhere,[2] although for the most part Fig. 1 is self-explanatory. When in use the assembly is situated above a large water-bath kept by a thermostat at 25·0° C, and it has been found advisable to protect the tungsten contacts (as well as the mercury in the reservoir) from the surrounding moist atmosphere by means of tubes J and K, containing silica gel.

Notes on operation—

When the mercury meniscus stands at the level S, mid-way between the points of contacts P and Q, the rate of flow from the capillary used by the writer approximates to 1·90 milligrams per second, and the interval to be timed automatically is about 21 minutes. The self-starting "Industrial" Synclock Time Interval Meter manufactured by Messrs. Everett Edgcumbe and Co.,

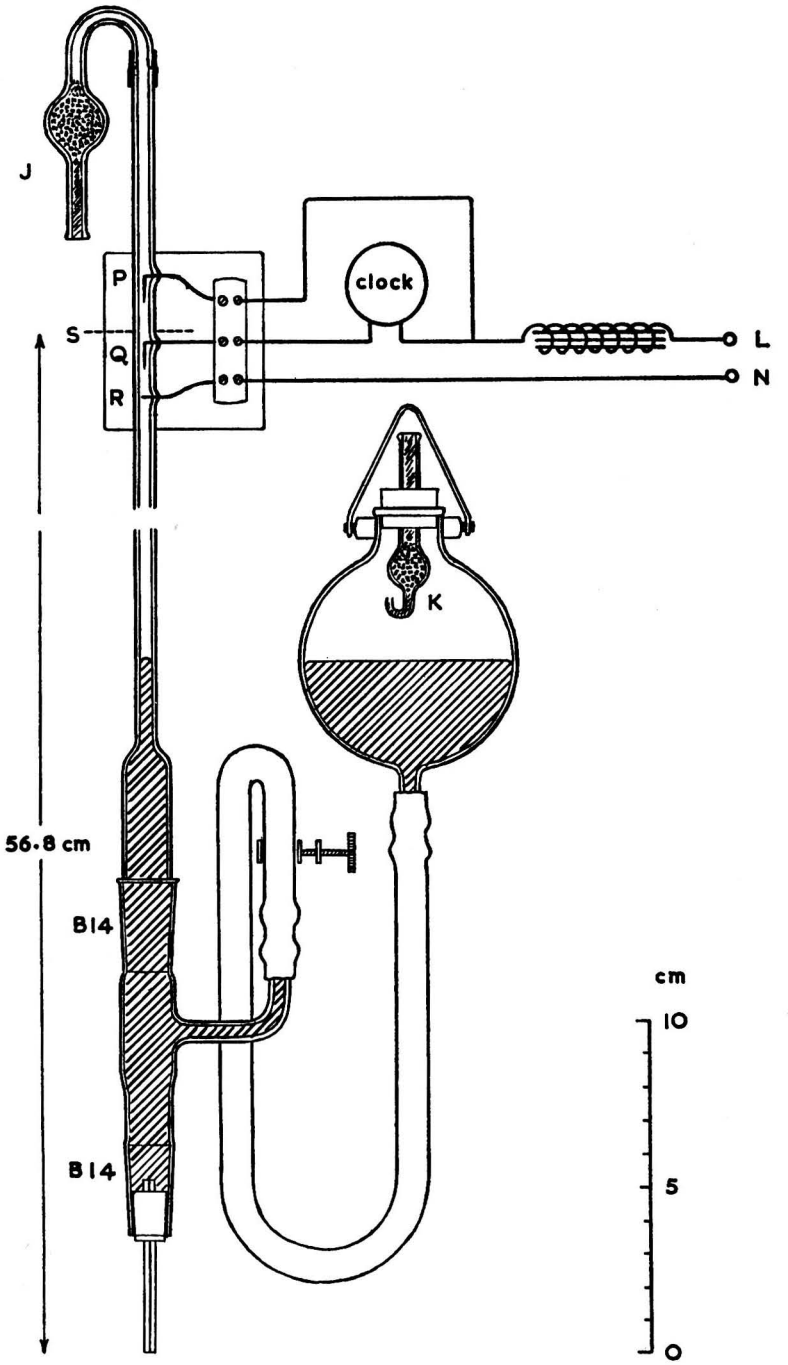

Fig. 1. Dropping mercury electrode assembly

Ltd., is used. This clock, which *must* be mounted on a vertical panel, is operated from the A.C. mains through a choke provided by the makers. The choke should be connected directly to the live side of the mains, two leads being taken from it, one of which passes directly to the upper contact whilst the other is connected through the clock to the middle contact. The neutral side of the mains is connected directly to the lower contact.

With attention to these details the interval between the circuit breaks at the upper and middle contacts is reproducible to 2 or 3 seconds, and the accuracy of the automatic timing is dependent upon the maintenance of the nominal mains frequency.

Tungsten metal can be rendered passive by contact with the common mineral acids, and especially with chromic and nitric acids.[3] In the care and cleaning of apparatus having tungsten-to-glass seals these reagents should be avoided if possible, otherwise, through passivity, very high contact resistances might be introduced. Passive films on tungsten contacts can sometimes be removed by mechanical polishing, but in less accessible positions they can often be removed by alternate anodic and cathodic treatment in 10 per cent. sodium hydroxide solution followed by washing and drying as described above. It is a wise precaution always to test the resistance at tungsten contacts before assembling such apparatus.

This note is based upon a contribution to a meeting of the Polarographic Discussion Panel of the Physical Methods Group on July 19th, 1950, under the chairmanship of Mr. J. Haslam.

REFERENCES

1. Lingane, J. J., *Ind. Eng. Chem., Anal. Ed.*, 1944, **16**, 329.
2. Furness, W., *J. Soc. Dyers and Colourists*, 1950, **66**, 270.
3. Mellor, J. W., "A Comprehensive Treatise on Inorganic and Theoretical Chemistry," Vol. XI, Longmans, Green and Co., London, 1943, pp. 719 and 720.

CENTRAL RESEARCH DEPARTMENT
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W. FURNESS
July, 1950

Official Appointments

PUBLIC ANALYST APPOINTMENTS

NOTIFICATION of the following appointments has been received from the Ministry of Food since the last record in *The Analyst* (1951, **76**, 118).

Public Analyst	*Appointments*
CAHILL, Terence John (Deputy)	County Borough of Carlisle.
CHALMERS, Frederick Grant Duncan ..	County Borough of Walsall.
JONES, William Elwyn (Deputy)	City and County Borough of Worcester.
JONES, William Elwyn (Deputy)	Borough of Oldbury.
RYMER, Thomas Edward (Deputy)	Borough of Barnes.
RYMER, Thomas Edward (Deputy)	Borough of Mitcham.
RYMER, Thomas Edward (Deputy)	Borough of Surbiton.
RYMER, Thomas Edward (Deputy)	Urban District of Coulsdon and Purley.
RYMER, Thomas Edward (Deputy)	Borough of Wimbledon.
RYMER, Thomas Edward (Deputy)	Borough of Sutton and Cheam.
RYMER, Thomas Edward (Deputy)	Urban District of Esher.

OFFICIAL AGRICULTURAL ANALYST APPOINTMENTS

NOTIFICATION of the following appointments has been received from the Ministry of Agriculture and Fisheries since the last record in *The Analyst* (1951, **76**, 118).

Agricultural Analyst	*Appointments*
CHALMERS, Frederick Grant Duncan ..	County Borough of Walsall.
JAMES, George Vaughton (Deputy)	Administrative County of Wiltshire.
JONES, William Elwyn (Deputy)	County Borough of Worcester.

British Standards Institution

DRAFT SPECIFICATIONS

A FEW copies of the following draft specification, issued for comment only, are available to interested members of the Society, and may be obtained on application to the Secretary, Miss D. V. Wilson, 7–8, Idol Lane, London, E.C.3.

Draft Specification prepared by Technical Committee PVC/1—Pigments, Sub-Committee PVC/1/11—Extenders.

CM(PVC)7945—Draft B.S. for Extenders for Paints.

Book Reviews

PHYSICAL CHEMISTRY. By (the late) E. D. EASTMAN, Ph.D., and G. K. ROLLEFSON, Ph.D. Pp. viii + 504. New York and London: McGraw-Hill Book Company, Inc. 1947. Price 42s. 6d.; $5.00.

It has been an agreeable task discovering what the authors imply when they say that "The material in this book is designed to meet the requirements of the year course in physical chemistry usually presented to third- or fourth-year college students (in an American university) and to serve as a reference book in later work."

The book is, in fact, a commendable attempt to present the basic concepts of physical chemistry, together with discussion and illustrative examples, in a severely limited space. The material included is up to date and is likely to remain so. Established principles do not change; new ones are discovered from time to time, but nothing less than a cataclysm in physical chemistry would be needed to justify a substantial revision of even one of the twenty-six chapters, which, incidentally, cover the following fields of physical chemistry: the states of matter, phase equilibria, surface phenomena, kinetic theory, the laws of thermodynamics, thermochemistry, atomic and molecular structure, radio-activity and nuclear reactions, chemical reaction kinetics, interactions of light with matter, electrochemical phenomena.

However, this book is not without some avoidable faults and failings. The reader occasionally finds sentences in which each word needs to be pondered, and also rather more than an elementary knowledge of physical chemistry has to be invoked before the authors' statement is recognised as a too concise presentation of the matter under discussion. Rare lapses in phraseology (p. 476, lines 17, 18; p. 485, line 27) imply the application of a line of reasoning, not acceptable in science, that because certain theoretical concepts are true, therefore the phenomena specified are observed. The authors' aim of making the book serve for reference in later work would have been brought nearer fulfilment were there adequate references to textbooks on specialised fields that the authors survey; further, the indexing and cross-referencing could be a little more generous. For example, "colloids" (p. 13) and the "Gibbs - Helmholtz equation" (pp. 260, 459) receive no mention in the index, and the statement on p. 261 that "The relationship between fugacity and pressure is discussed more fully in a later chapter" should have included a precise location.

Some scientific books are out of date before they are published, others have years of useful currency before them. The book under review takes a high place in the latter class and is commended as a good investment (although rather highly priced since devaluation of sterling) to any analytical chemist, or for that matter any chemist, who is seeking a compact and not too mathematical presentation of physical chemistry.

J. G. A. GRIFFITHS

BIOLOGICAL STANDARDISATION. By J. H. BURN, D. J. FINNEY and L. G. GOODWIN. Second Edition. Pp. vii + 440. London: Oxford University Press. 1950. Price 35s.

In 1928, Professor Burn wrote a little book called "Methods of Biological Assay." In 1937 he replaced this by his well-known "Biological Standardisation"—so much more complete and detailed as hardly to be called a revision. This book was reviewed in *The Analyst* (1938, **63**, 462). The senior author, whose name still stands alone on the covers, has now been joined by Mr. D. J. Finney, whose contribution is the new Chapter III entitled "Statistical Analysis," and Mr. L. G. Goodwin, who is responsible for five new chapters at the end. The theory and practice of biological assays have been developed greatly in the last 13 years, and the present revision of the 1937 book is certainly welcome.

It is an eloquent tribute to the extent to which the statistician is now regarded as an essential collaborator with the bio-assayist that, whereas in 1937 Burn's own Chapter III, "Mathematical Treatment of Results," occupied 29 pages out of 288, Finney's now takes up 151 out of 440. This chapter is an admirable attempt to cover the whole ground, starting from scratch, of statistics as applied to bio-assays. Whether one can, or should try to, teach statistics *en passant* is a moot point; but Finney's very readable and lucid style should at least inculcate in his readers the fundamental principles involved, and some may even be led to make a deeper study of the subject, in which event this chapter will have been well worth while.

The entirely new chapters, both those by Burn (XIV to XX) and those by Goodwin (XXI to XXV), provide very practical accounts of some interesting modern techniques—involving the breeding and maintenance of all sorts of "small deer," from snails to mites—for evaluating the merits of chemotherapeutic agents. Although many of the tests described hardly come under the heading of biological standardisation, they could readily be adapted to the form of comparative assays.

Chapters IV to XIII of the 1937 edition have been reprinted with little change except for the addition of new paragraphs on methods published since 1937. In consequence, assay designs and methods of computation are still given in the words of 1937, and this has led to many inconsistencies with Finney's Chapter III.

The chapters on vitamins in the 1937 edition have been omitted, which is a pity; bio-assays for vitamins A, B_1 and B_2 (to name no others) are still the final court of appeal where other methods of estimation have given discordant or improbable results. Exceptionally, the chapter on vitamin D has been retained quite unchanged, and this is an even greater pity, for it required modernising more, perhaps, than any other.

The book is not comprehensive; microbiological assays, the standardisation of antibiotics and serological techniques are not mentioned. But with all its limitations, it still fills a real need. It is much to be hoped that when a third edition is called for—as doubtless it soon will be—Professor Burn will take the opportunity of raising the early chapters to the high level of the rest of the book by completely revising them, and of supplying, either personally or through a co-author, new chapters dealing with the missing topics, particularly the bio-assay of the vitamins. The book would then be as pre-eminent in its field as was—in its time—that excellent book, the edition of 1937.

ERIC C. WOOD

ADVANCED CHEMICAL CALCULATIONS. By SYLVANUS J. SMITH, M.A. Pp. viii + 454. London: Macmillan & Co., Ltd. 1950. Price 17s.

Chemical calculations form a smaller part of the college syllabus than formerly. The pressure of increased subject-matter makes this necessary, but most would agree that it is regrettable. Consequently, it is left to the student—and how strong-willed he must be—to make up the gap. This book meets the student half way by being eminently digestible, which is an achievement indeed. This is partly realised by the inclusion of the relevant subject-matter so that the problems do not involve mere substitution in a set of equations.

Criticisms of the book fall on the reading matter, rather than the problems themselves. It is to be regretted that the author did not comply with the recommendations of the Joint Committee on Symbols, for he might have avoided the use of F for the Gibbs free energy, which apparently necessitated the mysterious ϕ for the Faraday; or the use of T in Chapter 19 (to avoid confusion with n, used for valency instead of z) and n in Chapter 9 for the transport number. Again, it is often necessary to simplify physical chemistry in order to produce a problem, but in certain sections, particularly that on overpotential, the simplifications are not pointed out, and a very erroneous impression may be given to the student. Of the rest, the author favours the use of cycles to obtain thermodynamic equations rather than the direct approach, so that some of the treatments, though clear, are rather cumbersome.

The author has included a considerable proportion of intermediate work in order to preserve continuity with his "Introductory Chemical Calculations." Even so, it is somewhat surprising to find volumetric calculations appearing as late as p. 205, although experience shows that many degree students would benefit from the use of this chapter. The scope and standard of the remainder of the book, however, is entirely adequate, and the problems are sufficiently varied for the needs of an Honours degree student. The author has provided a very useful book, in which Messrs. Macmillan & Co., Ltd. maintain their high standard of production.

J. F. HERRINGSHAW

COLORIMETRIC METHODS OF ANALYSIS. Third Edition. By FOSTER D. SNELL, Ph.D., and CORNELIA T. SNELL, Ph.D. Volume II, Inorganic. Pp. x + 950. New York: D. Van Nostrand Company, Inc. London: Macmillan & Co., Ltd. 1949. Price 90s.; $12.00.

In the preface to this massive volume it is pointed out that during the year 1946 no less than 23 per cent. of all analytical papers published were concerned with colorimetric methods, and it is therefore no exaggeration to say that the appearance of a new edition of this work is an event of considerable significance to all analysts. The colorimetric determination of 45 metals is discussed in as many chapters, the remaining third of the text being devoted to non-metals and acid radicles, while the indexes alone occupy 62 pages. In general, after a brief introduction to each chapter, the methods of preparation of samples are given in the following sequence: metals, minerals, liquid samples, solid organic samples; then follow the preparation of standards and finally the descriptions of available colorimetric procedures in approximate order of their importance.

The authors state that they have chosen a middle course in the matter of critical selection: procedures considered to be of minor importance are briefly described and fewer than half of the available methods are given in detail.

However one studies this book, whether by rapidly turning over the pages or by carefully reading a chapter, the same impression of the painstaking thoroughness exercised by the authors is given. It is indeed difficult to find any just ground for criticism. One might remark that occasionally useful procedures have been overlooked: thus, the excellent modification of the phenoldisulphonic acid method for nitrates proposed by R. C. Frederick (*Analyst*, 1919, **44**, 281), rendering it applicable in the presence of an appreciable concentration of chloride, is not mentioned; again, the careful work of M. B. Richards (*Analyst*, 1930, **55**, 554) on the determination of manganese in plant and animal tissues, although alluded to, is not described. It is perhaps natural that the authors should favour methods appearing in American literature, but it is a pity that many valuable papers published in British journals have not, perhaps, received as much attention as they merit. The arrangement, already alluded to, of describing the preliminary treatment of samples under a series of headings in the first portion of each chapter and the colorimetric procedures proper in the latter part, although effecting an economy of space, is sometimes a little disconcerting. Such an extensive survey would hardly have been possible on any other plan, but in certain instances where the character of the samples varies so widely as, for example, tungsten steel and butter, a less rigid adherence to the scheme, even at the expense of some repetition, might have been an improvement.

The above criticisms are offered with some trepidation, particularly as they are not of a constructive character, and it is therefore only fair to add that, considering the work as a whole the reviewer, at any rate, is impelled to admiration. Although the cost of this handsomely bound book is considerable, it is good value at the price and every analyst would be well advised to ensure that he has a copy available.

N. L. ALLPORT

REPORTS ON BIOLOGICAL STANDARDS: VI, THE DESIGN OF TOXICITY TESTS. By W. L. M. PERRY. M.R.C. Special Report Series No. 270. Pp. vi + 51. London: H.M. Stationery Office. 1950. Price 1s. 6d.

The best method of estimating toxicity is based on the concept of the mean lethal dose (M.L.D.), the amount of a substance necessary to kill exactly half the animals of a particular species (preferably a particular strain of that species). Not only may it well prove impossible to submit the whole existing population of those animals to test, but it would certainly also be most extravagant to do so even were it feasible. A sample is therefore chosen so as to be as representative as may be; from the variations in response of the individuals in the sample, statistical theory enables us to extrapolate the experimental results to give an estimate of the M.L.D. for the whole population as well as a measure of the limits within which this M.L.D. is likely to lie for any preferred level of probability.

Hitherto it has been customary to collect the necessary data by submitting groups of animals to graded doses of the substance under test and to plot the percentage mortality in each group against the dose of substance. There then appears the S-shaped curve so familiar to physiologist and pharmacologist; the more animals that are used, the smoother the curve. For easy comparison of one M.L.D. with another, and for estimation of errors, a simple conversion of the dose to its logarithm and expression of the percentage mortality as its normal equivalent deviation (or probit) leads to linearity of relationship and much facilitates calculation of the required values.

Dr. Perry has studied the method and the accepted mathematical treatment of its results. He has shown excellent reason for accepting the view that more precision with a given number of animals—or the same precision with a smaller number—is obtainable by abandoning the use of "percentage mortalities" in favour of a figure expressing the mean responses of individual animals. This is to be expected, for it is recognised that "quantal" (all-or-nothing) responses are inherently less capable of providing from a given number of observations as much information as an equal number of quantitative (continuous variate) responses.

How, then, can we get a series of quantitative results from groups of lethally and sub-lethally dosed mice or rats? Dr. Perry has answered this question clearly and convincingly, while at no time attempting to burke the issue that there are certain practical difficulties attached to the recording of survival times, which are what in fact he advocates as suitable "metameters" for inversely measuring the toxic effects of a drug.

Experience alone can show whether or not this recommendation has even more to be said for it than Dr. Perry modestly claims and whether or not the disadvantages of the necessarily more frequent observations are as serious as he frankly suggests they may be. At least he is to be thanked and congratulated for having studied and expounded what may well prove to be one of the most important advances in biological assay methods since the classical work of Gaddum and his colleagues—also, by the way, published as "green reports" by the Medical Research Council.

Meanwhile, it is probable that all concerned will accept Dr. Perry's suggestion to use the term "(2 + 2)" instead of "four-point" in describing assays with two doses of standard and two of test substance and the corollary procedure of replacing "six-point" by (3 + 3), (2 + 4) or (1 + 5), as the case may be. Some will also welcome Dr. Perry's proposals because, besides the advantages he mentions, they will make it possible to investigate whether the increased precision usually conferred on continuous variate assay methods by the use of "pure line" animals is also legitimately expected in toxicity tests.

A. L. BACHARACH

INTRODUCTION TO SEMI-MICRO QUALITATIVE CHEMICAL ANALYSIS. By LOUIS J. CURTMAN. Second Edition. Pp. xvi + 391. New York and London: Macmillan and Co., Ltd. 1950. Price 28s. 6d.

The first edition of this book was published in 1942; it was reprinted several times, and has now been revised. The lengthy theoretical section, really a treatise on elementary physical chemistry, which occupies practically one-third of the book, has been left untouched in any radical way. The amount of elementary detail included here, seemingly inseparable from American textbooks of qualitative analysis, presumably arises from the American system of teaching by which it is possible to study analytical chemistry, so to speak, *in vacuo*, without any collateral training in the other branches of chemistry. In consequence, much of this matter would be redundant in a textbook of the same level in this country.

The descriptions of the reactions of the common ions, dealt with in the following 80 pages, are also largely unaltered, as are the 20 pages dealing with calculations.

In the laboratory sections modifications have been made in the schemes for both cations and anions where more satisfactory procedures have been developed since the appearance of the first edition. The description of the semi-micro techniques appears to be sound, clear and orthodox for this range. This part provides an excellent introduction to small-scale procedures for anyone not already familiar with them. It tends, perhaps, to include too much that should surely be left to the individual instructor—or to the intelligence of the student. Thus, in the general directions we find (4) *Do not converse with your neighbour while working.* (11) *Consult your instructor when a result is obtained which you cannot explain even with the aid of all the information in your textbook.* It is curious, also, to find that the ferric chloride method for interfering phosphate has now been included as an alternative to the zirconium separation, "for those who may prefer the classic method," although it was omitted in the first edition. This, particularly in semi-micro work, seems to be a retrograde step.

The book is probably too expensive to commend itself generally as a working textbook in qualitative analysis for elementary classes. It would not be suitable, however, for more advanced courses, since its scope is distinctly limited. It could serve as a useful supplement to the normal text for those students who can be encouraged to practise the laudable habit of consulting more than one source of information.

CECIL L. WILSON

PROGRESS IN CHROMATOGRAPHY, 1938–1947. By L. ZECHMEISTER. Pp. xviii + 396. London: Chapman & Hall Ltd. 1950. Price 45s.

The publication by Zechmeister and Cholnoky in 1937 of the first textbook of chromatography was indicative of the growing importance of a technique that until then had been used by relatively few chemists. During the next ten years chromatography came into much more general use, owing in part no doubt to the stimulus of Zechmeister and Cholnoky's book, of which an English translation appeared in 1940. The "classical" application of chromatography to the separation of animal and plant pigments, vitamins, sterols and hormones was followed by its successful use in the separation of a much greater variety of organic and even inorganic substances; and, still more important perhaps, the chromatographic principle was extended far beyond its original concept, so that partition chromatography and ion exchange chromatography are now available to supplement the older method of adsorption chromatography with its quite definite limitations, whilst frontal analysis and displacement development have still further extended the scope of this now indispensable weapon. There must be few research laboratories in which one form of chromatography or another is not in regular use.

All these important developments inevitably meant that sooner or later the original textbook would have to be revised, and for various reasons Professor Zechmeister has chosen to write a supplementary volume, the general arrangement of which follows that of "The Principles and Practice of Chromatography." The two volumes together give a complete account of all the important work that has been done in this field up to the end of 1947. It is most unfortunate that delays in publication made it impossible to bring the subject-matter more nearly up to date, since several important advances have been made in the intervening three years.

Many readers will turn first to the section on "Principles" in order to ascertain how far the theoretical treatment of the subject has advanced since 1937. They will doubtless be very disappointed that so little is yet known about the mechanism of the chromatographic separation process, although considerable light on the subject—as well as some heat—resulted from a recent discussion arranged by the Faraday Society which unfortunately took place after the book under review had gone to press.

A study of the rest of the book clearly indicates how far theory has lagged behind practice, and how diverse are the variations in procedure that may be used in any particular instance. In a field in which so few general principles can apparently be enunciated, it is imperative to have available skilled guidance in the choice of the methods to be used. Here in this book is the requisite information. Here the author has described, with adequate experimental details, the separation of typical representatives of various classes of organic compounds, and has supplemented these descriptions with brief summaries, frequently in the form of tables, of the conditions required for the separation of other compounds belonging to the same groups, together with references to the original publications where more detailed information can be found. Obviously, within the limited space available, it would have been impossible to provide detailed descriptions of every important application of chromatography, and the book must therefore be used as a guide to the original literature rather than as a substitute for it. With its aid, research workers will save themselves a considerable amount of time and labour in making literature surveys, and will find an abundance of ideas to help them in solving their own problems. To the analyst the book offers a challenge as well as guidance for, in spite of its phenomenal success in qualitative analysis, chromatography has given less satisfactory results in quantitative analysis, although there are signs that interest in this aspect of chromatography is increasing. Perhaps the next decade will witness important developments in this direction. At all events this book is to be commended to all analysts as a useful account of what has been achieved and as a reminder of what still remains to be done.

The book is well printed and bound and is provided with very full author and subject indexes. Special reference must be made to the frontispiece, which is a hitherto unpublished photograph of Tswett, the inventor of adsorption chromatography. F. A. ROBINSON

Publications Received

ORGANIC SYNTHESES. Volume 30. Edited by A. C. COPE. Pp. vi + 115. New York: John Wiley & Sons, Inc. London: Messrs. Chapman & Hall. 1950. Price 20s.

THE BIOCHEMISTRY OF B VITAMINS. By ROGER J. WILLIAMS, ROBERT E. EAKIN, ERNEST BEERSTECHER, JUN., and WILLIAM SHIVE. Pp. x + 741. New York: Reinhold Publishing Corporation. London: Messrs. Chapman & Hall. 1950. Price 80s.; $10.00.

COLLOID CHEMISTRY, THEORETICAL AND APPLIED. Collected and edited by JEROME ALEXANDER. Volume VII. THEORY AND METHODS. BIOLOGY AND MEDICINE, TECHNOLOGICAL APPLICATIONS. Pp. xiv + 736. New York: Reinhold Publishing Corporation. London: Messrs. Chapman & Hall. 1950. Price 120s.; $15.00.

REPORT UPON THE WORK OF THE DEPARTMENT OF THE GOVERNMENT CHEMIST FOR THE YEAR ENDING 31ST MARCH, 1950. Pp. 36. London: H. M. Stationery Office. 1950. Price 1s.

SOYBEANS AND SOYBEAN PRODUCTS, Volume 1. Edited by KLARE S. MARKLEY. Pp. xx + 540. London and New York: Interscience Publishers Inc. 1950. Price 88s.; $11.00.

METHODS IN FOOD ANALYSIS APPLIED TO PLANT PRODUCTS. By MAYNARD A. JOSLYN. Pp. x + 525. New York: Academic Press Inc. 1950. Price $8.50.

BRITISH CHEMICALS AND THEIR MANUFACTURERS. Pp. 169. London: Association of British Chemical Manufacturers. 1951. *Gratis.*

SAFETY IN THE CHEMICAL LABORATORY. By H. A. J. PIETERS and J. W. CREYGHTON. Pp. xii + 258. London: Butterworth's Scientific Publications Ltd. 1951. Price 15s.

Biological Methods Group

IT is proposed to hold a Summer Meeting at a country venue on Friday, June 1st, 1951, at which short, original communications will be presented and laboratory demonstrations will be given. The success of this meeting—the first of its kind to be held by the Biological Methods Group—will depend very largely on the support forthcoming from members. Those who will be able to contribute papers or give demonstrations are invited to communicate with the Honorary Secretary of the Group, S. A. Price, Esq., Walton Oaks Experimental Station, Vitamins Ltd., Dorking Road, Tadworth, Surrey, as soon as possible, so that arrangements can be made. Suitable contributions from non-members of the Society will also be welcome.

Decennial Index to THE ANALYST

THE Decennial Index to *The Analyst* for Volumes LXI–LXX, covering the years 1936 to 1945, is now ready. Members of the Society who have not yet ordered a copy should do so as soon as possible. Members should order from The Editor; non-members should order from Messrs. W. Heffer and Sons Ltd., Petty Cury, Cambridge, or through their usual bookseller.

A few copies of the Decennial Indexes for 1926–35, 1916–25, 1906–15 and 1896–1905 are still available from the publishers, Messrs. W. Heffer and Sons Ltd.

THE RESEARCH ORGANISATION of The Distillers Company Limited requires a CHEMIST, aged up to 26 years, for research and development work on the recovery of antibiotics and other fermentation products of pharmaceutical interest from fermented media. Applicants should possess a 1st or 2nd Class Honours B.Sc. degree, A.R.I.C. or equivalent, and preferably previous appropriate research experience. The appointment, which offers excellent opportunities with good prospects of advancement for a young chemist wishing to enter the antibiotics industry, will be tenable at Bromborough, Cheshire. Commencing salary will depend upon experience and qualifications. Non-contributory pension scheme. Apply to The Controller of Research and Development, Room 176, Distillers House, 21, St. James's Square, London, S.W.1.

THE RESEARCH and Development Department of the Distillers Company Limited, Great Burgh, Epsom, Surrey, requires a CHEMIST for training in antibiotics research work. The initial duties will be biochemical analyses. Applicants must possess a University degree or equivalent in chemistry but experience whilst desirable is not necessary. Age up to 26 years. This vacancy offers excellent opportunities for a young graduate wishing to enter the antibiotics research field, and good prospects of advancement. The commencing salary will depend upon experience and qualifications. Non-contributory pension scheme. Apply to The Controller of Research and Development.

THE QUEEN ELIZABETH HOSPITAL FOR CHILDREN, HACKNEY ROAD, E.2
APPOINTMENT OF BIOCHEMIST

APPLICATIONS are invited from Analytical Chemists and Biochemists for the post of Biochemist to work under the direction of the Pathologist. Applicants should have an honours degree in Chemistry (or equivalent) and have had a wide experience of Analytical Chemistry or in Hospital Bio-Chemistry. The post affords an opportunity for research. Salary scale £650 × £25—£800 per annum. National Health Service conditions applicable. Applications, with copies of available testimonials and/or names of referees, should be addressed to the Secretary to arrive not later than 26th March, 1951.

ROCHE PRODUCTS LIMITED have an opening for a man of B.Sc. standard with experience in analytical work. Letter stating qualifications, experience and salary required to the Secretary, Roche Products Ltd., Welwyn Garden City, Herts.