# The ANALYST

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# THE ANALYST

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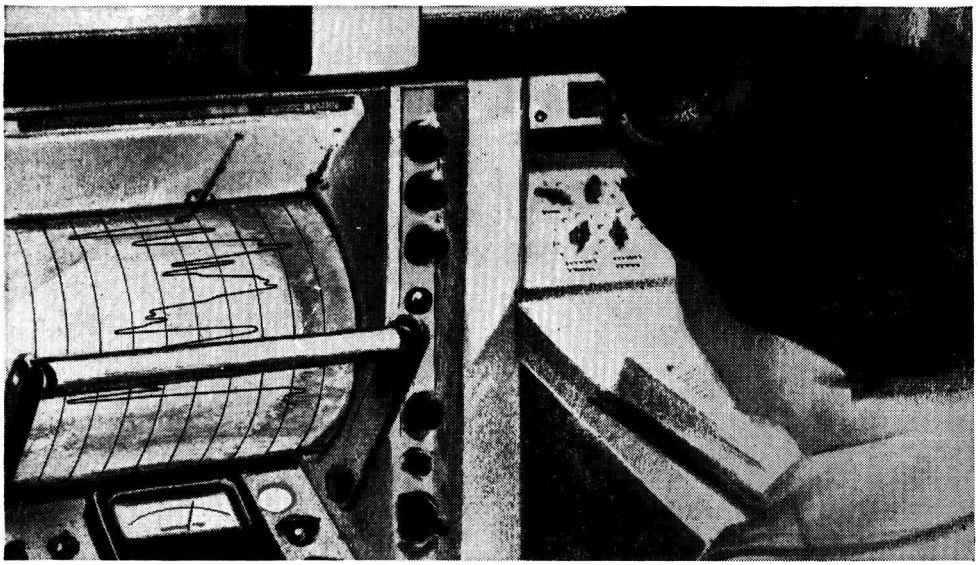

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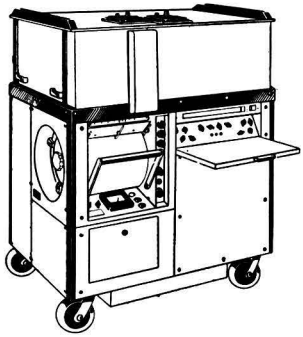

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# THE ANALYST

## PROCEEDINGS OF THE SOCIETY FOR ANALYTICAL CHEMISTRY

### JOINT MEETING

A JOINT Meeting of the Society with the Iron and Steel Institute and the Institute of Metals was held on Tuesday and Wednesday, May 3rd and 4th, 1960, at Denison House, 296 Vauxhall Bridge Road, London, S.W.1. The subject of the meeting was "The Determination of Gases in Metals." The Chair was taken by Mr. H. A. Sloman, M.A., F.I.M., F.R.I.C., of the National Physical Laboratory.

The following papers were presented and discussed in four sessions: "Sampling of Liquid Metals," by Professor T. B. King (read by Mr. R. D. King); "The Determination of Oxygen in Metals: A Review," by W. T. Elwell, F.R.I.C.; "The Determination of Gases in Metals by Vacuum Fusion," by J. E. Still, B.Sc., F.R.I.C.; "The Determination of Gases in Metals by the Semi-micro Vacuum-fusion Technique," by A. Parker, B.Sc., A.R.I.C.; "An Assessment of Carrier-gas Methods for the Determination of Gases in Metals with Particular Reference to Steels," by C. E. A. Shanahan, B.Sc., F.R.I.C., F.I.M.; "The Determination of Oxygen in Beryllium by Activation Analysis," by R. F. Coleman, B.Sc., A.C.T., A.R.I.C.; "Emission Spectrometric Determination of Oxygen, Hydrogen and Nitrogen in Metals," by Professor V. A. Fassel (read by Mr. J. F. Duke); "The Determination of Oxygen and Nitrogen in Iron and Steel by an Isotope-dilution Method," by M. L. Pearce and C. R. Masson; "A Gas Measurement System for Vacuum-fusion Equipment Yielding Both High Precision and Wide Range," by A. Bacon, A.Met.; "The Determination of Nitrogen in Metals: A Review," by J. D. Hobson, Ph.D., F.R.I.C., F.I.M.; "The Determination of Nitrides in Metals," by H. F. Beeghly, B.S., Chem.E.; "The Determination of Hydrogen in Metals: A Review," by R. Eborall, M.A. (read by Dr. C. E. Ransley); "The Determination of Hydrogen in Cast Iron," by J. V. Dawson and L. W. L. Smith, B.Sc.; "An Improved Carrier-gas Technique for the Determination of Hydrogen in Steel," by F. R. Coe, B.Sc., and N. Jenkins, A.Met.; "X-ray Emission Analysis and the Determination of Gases in Metals," by T. Mulvey; "Application of Internal Friction Measurements to the Study of Gases in Metals," by G. M. Leak, B.Sc., Ph.D., F.Inst.P., F.I.M.

There was an attendance of over 330 registered delegates, including most of the recognised experts in the field from many parts of the world. The full proceedings, including all papers and the discussions on them, will be published by the Iron and Steel Institute as a Special Report before the end of the year.

### NORTH OF ENGLAND SECTION

A DEMONSTRATION and Ordinary Meeting of the Section was held on Friday and Saturday, April 8th and 9th, 1960, at the Nuclear Chemical Laboratories, Liverpool College of Technology, 2 Blackburne Place, Liverpool. The Chair was taken at both meetings by the Chairman of the Section, Dr. J. R. Edisbury.

At 7 p.m. on April 8th, an introductory lecture to the exhibition and demonstration on radiochemical methods was given by J. W. Lucas, B.Sc., F.R.I.C. The Ordinary Meeting was held at 2.15 p.m. on April 9th, when the following paper was presented and discussed: "The Application of Isotopes to Analysis," by D. Gibbons, B.Sc., Ph.D., A.R.I.C.

### SCOTTISH SECTION

AN Ordinary Meeting of the Section was held at 7.15 p.m. on Friday, April 29th, 1960, at the Central Hotel, Glasgow, C.1. The Chair was taken by the Vice-Chairman of the Section, Mr. A. F. Williams, B.Sc., F.R.I.C.

The following paper was presented and discussed: "Chemical Services on British Railways," by G. H. Wyatt, B.Sc., Ph.D., F.R.I.C.

## MIDLANDS SECTION

AN Ordinary Meeting of the Section was held at 6.30 p.m. on Tuesday, April 26th, 1960, in the Mason Theatre, The University, Edmund Street, Birmingham, 3. The Chair was taken by the Honorary Assistant Secretary of the Section, Mr. R. Adkins, B.Sc.

The following paper was presented and discussed: "The Analytical Chemistry of Titanium and Zirconium," by W. T. Elwell, F.R.I.C., and D. F. Wood, B.Sc., A.R.I.C.

## PHYSICAL METHODS GROUP

THE seventieth Ordinary Meeting of the Group was held at 6 p.m. on Tuesday, April 5th, 1960, at the Northern Polytechnic, Holloway Road, London, N.7. The Chair was taken by the Chairman of the Group, Dr. G. W. C. Milner, F.R.I.C., A.Inst.P.

The following automatic analytical instruments were described and demonstrated: "Auto Analyzer," by A. L. J. Buckle, M.Sc., Ph.D., A.R.I.C.; "E.I.L. Model 24 Automatic Titrimeter," by G. Mattock, B.Sc., Ph.D., A.R.I.C.; "D.C.L. Null-balance Magnetic Oxygen Analyser," by C. W. Munday, B.Sc., A.R.I.C.; "Mervyn - CERL Automatic Sulphur Dioxide Recorder," by Mrs. M. W. Redfearn (see brief descriptions below).

### AUTO ANALYZER

Described by Dr. A. L. J. Buckle (*Techicon Instruments Co. Ltd.*)

THE Auto Analyzer is a completely automatic system that records the level of concentration of a given component in the test solution against a known concentration of that component in a standard control solution. No gravimetric or volumetric measurement is involved, but there is a continuous plotting of ratios (the concentration of the sought material in the unknown against its known concentration in the standard control). For this reason it is never necessary to bring a reaction to completion. The complete system may employ colorimetry, conductance, pH, flame spectrometry or the use of temperature-sensitive elements as criteria of concentration. The Auto Analyzer system consists of a number of units or modules, each of which performs one of the various basic functions into which any chemical analytical procedure can be broken down. There are modules for sampling, metering, heating and cooling, digestion, separation, mixing, solvent extraction, end-point measurement and recording and so on. The Auto Analyzer can be used sequentially to perform analyses on large numbers of samples—it can handle analyses at up to sixty samples per hour—or it can be used continuously to monitor process streams and even to control them and the processes they serve.

### E.I.L. MODEL 24 AUTOMATIC TITRIMETER

Described by Dr. G. Mattock (*Electronic Instruments Ltd.*)

THIS titrimeter consists of a control unit and a head unit, the former controlling the addition of titrant by the latter. The control unit consists essentially of a pH-meter amplifier (it may in fact be used as a pH meter) plus a relay system governing the opening and closing of circuits in the volumetric head unit; the necessary signals are derived from a pair of potentiometric electrodes (*e.g.*, glass or platinum electrode + reference electrode), the e.m.f. from which is fed to the amplifier for eventual operation of the relays.

The head unit incorporates the burette and solenoid-operated all-glass tap, together with a stirrer motor and the electrodes. In the rest position, the tap is closed by a glass-enclosed soft-iron slug. When the titration begins a field from one coil of the solenoid lifts the slug clear, at the same time lifting an upper capillary so as to permit free flow of titrant into the sample. Completion of the titration is effected at a pre-set pH or millivolts signal from the electrodes corresponding to the titration end-point. At a pre-set number of millivolts before this end-point, the first solenoid coil is de-energised, and a second energised. This causes the slug to drop slightly—not to close the tap but to permit the flow to be controlled by the capillary only. The object of this procedure is to reduce the rate of titrant addition just before the end-point to prevent overshoot. At the end-point e.m.f. this second coil is de-energised, and the slug drops to its rest position, thereby stopping titrant flow. The burette reading is then the titre value.

The provision of two head units allows an operator to set up one titration while another is being performed automatically, so permitting continuous application. Any type of potentiometric titration can be performed, including acid - base, redox, precipitation and complex-forming.

The demonstration illustrated the potentiometric titration of metal ions by EDTA—particularly the determination of water hardness.

## D.C.L. Null-balance Magnetic Oxygen Analyser

Described by Mr. C. W. Munday (*The Distillers Co. Ltd.*)

The physical property of oxygen that distinguishes it from other common gases is its paramagnetism. This was discovered by Michael Faraday, who in 1851 demonstrated that a hollow glass sphere at the end of a horizontal rod supported by silk fibres was attracted to a magnet when filled with oxygen.

In practical oxygen analysers based on this experiment, the convenience and sensitivity of Faraday's arrangement are increased by having a sphere at both ends of a bar, forming a "dumb-bell," which is sealed, the gas under test surrounding it. The dumb-bell is suspended at its centre and biased so that the diamagnetic spheres are repelled to an equilibrium position on opposite sides of a permanent-magnetic field most intense at its centre. When the oxygen content of the surrounding gas changes, the equilibrium position of the dumb-bell changes also, and this movement is detected by a light beam deflected across a double photocell by a mirror on the dumb-bell.

The D.C.L. Null Balance Magnetic Oxygen Analyser has been evolved as a result of research to attain the greatest refinement of this type of instrument. The analysis cell has been designed to give high sensitivity to oxygen changes, combined with extreme robustness, rapid response and minimal flow and level-sensitivity. A novel feedback circuit ensures strictly linear indication on each range and minimum electronic drift effects.

The application of the instrument in the chemical industry was also briefly described.

## Mervyn-CERL Automatic Sulphur Dioxide Recorder

Described by Mrs. M. W. Redfearn (*Central Electricity Research Laboratories*)

The Mervyn-CERL Automatic Sulphur Dioxide Recorder was developed initially at the Central Electricity Research Laboratories for monitoring the concentration of sulphur dioxide in the atmosphere. The instrument is intended for long periods of continuous operation at a fixed site and is sensitive to 1 part of sulphur dioxide in 100 million parts of air over the range 0 to 50 parts per hundred million. The range can be extended by simple adjustment.

The sulphur dioxide in the air is absorbed by a hydrogen peroxide reagent in a continuous counter-current absorption column. This results in the formation of sulphuric acid in the reagent solution, and the increase in conductivity of this solution is measured and recorded on the chart. The recorded sulphur dioxide concentration depends on the ratio of flow of air to flow of reagent in the absorption column and the temperature at which the conductivity measurements are made. To check that the instrument works properly when unattended, these three variables are monitored continuously, but to avoid the expense of a multi-pen chart recorder, switching arrangements have been designed so that characteristic marking lines appear on the chart of the one-pen recorder.

The Mervyn-CERL instrument is now being used for atmospheric pollution work in many parts of the world.

# BIOLOGICAL METHODS GROUP

An Ordinary Meeting of the Group was held at 7 p.m. on Wednesday, April 13th, 1960, in the Meeting Room of the Chemical Society, Burlington House, London, W.1. The Chair was taken by the Chairman of the Group, Dr. J. I. M. Jones, F.R.I.C.

The following papers were presented and discussed: "The Assay of Gonadotrophins in Body Fluids," by J. A. Loraine, M.D., Ch.B., M.R.C.P.; "Insulin Assay in Plasma by the Rat Diaphragm Method," by P. H. Wright, M.Sc., M.B., Ch.B.

# Obituary

## FRANCIS WILLIAM FREDERICK ARNAUD

FRANCIS WILLIAM FREDERICK ARNAUD died on March 15th, 1960. He was 81. He was born in Chester, his father, Samuel, having come from Paris and adopted British nationality. Francis was sent to school in Paris and then to the Sorbonne, but he came back to London and continued his studies at King's College. His professional life began with W. F. Lowe, public analyst and assayer at Chester, and continued with C. H. Cribb, with whom he was joint author of several papers.

In 1906 he was appointed Borough Analyst for Portsmouth, where he stayed until 1913, when he came to Maidstone as the first full-time County Analyst for Kent, which post he occupied until his retirement in 1946.

His published work, alone or as co-author, dealt with the action of alkaline waters on iron, the determination of butter-fat and coconut oil in margarine, the determination of boric acid, losses in meat on cooking and oiled apple wraps.

At the time of his death he was one of the oldest members of our Society, to which he was elected in 1903. Thus his connection with the Society of Public Analysts was long and also distinguished: he was Vice-President in 1922–23, Honorary Secretary, 1926–31, and President in 1932–33, and in addition to these periods of office he served on the Council for a total of 21 years. With such a background it is not perhaps surprising that he was among those who were opposed to the transmutation into the Society for Analytical Chemistry. Arnaud also served for two periods on the Council of the Institute of Chemistry, as it then was.

Apart from his profession he had many interests, of which perhaps Freemasonry and agricultural matters were the chief. In Freemasonry he became outstanding, being at one time Deputy Provincial Grandmaster of Kent: in gardening he was talented and enthusiastic and was prominent in many sides of the agricultural activities of Kent, the "garden of England." So far as can be discovered he originated, and he certainly fostered, the sale of shoddy and other rough nitrogenous manures at unit value, a method which, although not officially recognised by the Fertilisers and Feeding Stuffs Act or Regulations, is the only logical way of buying and selling these varied and variable products. After his retirement he acted for some time as adviser to a commercial group of farms in Kent.

He was an all-round athlete and Victor Ludorum in 1900 and 1901 of King's College and Hospital. In those days he excelled most at running, but he remained very fit and could still play tennis at the age of seventy. During the first World War he served as a lieutenant in the Volunteer Battalion, Royal West Kent Regiment. In 1950 a stroke left him with badly affected speech and some paralysis, in spite of which he remained cheerful and active until the very last day of his life, when he died quite suddenly sitting in his chair watching television. He had married Hilda Betts in 1906: she died in 1957, and they are survived by their only child, Commander Dudley Arnaud, D.S.C., V.R.D., R.N.V.R.

Arnaud served on many Committees, such as those of the local Conservative Party, museum and cricket clubs. He was at one time President of the Mid Kent Natural History and Philosophical Society and Chairman of the Loose Parish Council. He will thus be long remembered by his host of friends—for his absolute integrity, good company, simplicity, devotion to small children and great charm to all.

H. E. MONK

# The Determination of Vitamin $B_{12}$

## A Critical Review*

BY W. H. C. SHAW AND CHRISTINE J. BESSELL

*(Glaxo Laboratories Ltd., Greenford, Middlesex, and Sefton Park, Stoke Poges, Bucks.)*

SUMMARY OF CONTENTS



IN the 12 years since the independent, but almost simultaneous, isolation of crystalline pure vitamin $B_{12}$, by Rickes, Brink, Koniuszy, Wood and Folkers[1] in America and by Lester Smith and Parker[2] in England, research on $B_{12}$† and the many related compounds now known to exist has been intense.

Various aspects of the field have been covered in nine symposia held from 1948 to date, and the proceedings of the later ones in London (1955),[3] Hamburg (1956),[4] Rome and New York (1958), in so far as reports have yet been published, together with recent papers, reviews and monographs,[5,6,7,8,9,10,11] contain much valuable and detailed information. In particular, these publications cover aspects not directly related to the subject of this review, and these will not be considered further here.

The earliest observations on the curative effect of raw liver in pernicious anaemia were made some 20 years before isolation of the anti-pernicious anaemia factor, $B_{12}$, in 1948. Why, it is pertinent to ask, should the task of isolation have taken so long, extending over a period in which several other vitamins were recognised, isolated, characterised and finally synthesised? The answer lies partly in the extremely small amounts occurring in liver (about 1 p.p.m.), to some extent in the absence, particularly at the early stages, of suitable extraction techniques and, by no means least, in the lack of any analytical methods (including animal tests) by which the various stages of purification could be controlled.

At the time, the only known method for checking the partly purified fractions was clinical evaluation, with all its attendant inaccuracies, on cases of pernicious anaemia in relapse. The fact that pure $B_{12}$ was obtained with only this test as a guide is a great tribute to the care with which the clinical work was carried out. A step forward was made with the recognition by Shorb[12,13] in America that *Lactobacillus lactis* Dorner would not grow satisfactorily in a medium free from $B_{12}$. All current microbiological methods derive in principle from this observation. Although it is now known that *L. lactis* responds to many

* Reprints of this paper will be available shortly. For details, please see p. 456.

† For brevity, the term "$B_{12}$," unqualified, will be used here for vitamin $B_{12}$ (cyanocobalamin).

substances besides $B_{12}$, Shorb's method of assay greatly facilitated the later stages of the American work.

A search for more economic sources of $B_{12}$ than liver led to the discovery of its presence in the liquors derived from the commercial production of streptomycin by the then established deep-fermentation methods for its large-scale production. It is now generally preferred, however, to carry out separate fermentations for $B_{12}$ with *Streptomyces* or some other organism specially selected for its ability to produce $B_{12}$ free from analogues (see below). This is now the major commercial source of $B_{12}$.

To the analyst, $B_{12}$ poses problems ranging from an evaluation of the compound itself to the determination of the almost infinitesimal amounts in sea water. For a proper understanding of these problems, some preliminary consideration of the chemistry of $B_{12}$ is essential.

In the later stages of isolating $B_{12}$ from liver and fermentation liquors, it became clear that a number of compounds closely similar in properties to $B_{12}$, but distinguishable from it by partition chromatography, were present in the original materials or were formed during the extraction processes then in use. These compounds, designated $B_{12a}$, $B_{12b}$ and $B_{12c}$, were later shown to be readily convertible to $B_{12}$, when it was realised that cyanide, in addition to co-ordinated tervalent cobalt, formed an essential part of the $B_{12}$ molecule. The cyanide can be displaced from $B_{12}$ by photolysis or by certain reagents. Replacement with hydroxyl gives $B_{12a}$ and $B_{12b}$, subsequently shown to be identical. Replacement with nitrite gives $B_{12c}$. These substances, originally isolated from natural sources, may be converted to $B_{12}$ by treatment with cyanide. The term "cobalamin" was coined to refer to the intact $B_{12}$ structure without the cyanide group. Hence $B_{12}$ itself became cyanocobalamin, $B_{12a}$ and $B_{12b}$ hydroxocobalamin and so on. Other cobalamins may be formed by treatment with different reagents.[14] Subsequently it was recognised that another series of compounds, differing more fundamentally from $B_{12}$, existed in such materials as fermentation liquors, bovine rumen contents, calf faeces and sewage sludge.[15,16,17,18,19,20] These compounds differ from $B_{12}$ in having in the nucleotide a base different from 5:6-dimethylbenziminazole. Much confusion arose as to the identity of the various factors (known collectively as "$B_{12}$ analogues") described by different workers; this cleared as their structures, summarised in Table I (see p. 398), became established. These analogues are without the haematopoietic activity of $B_{12}$, except, notably, factor I ($B_{12}$ factor III), which was obtained from sewage sludge[21] and shown to have 5-hydroxybenziminazole as the nucleotide base.[22,23] Apart from these natural analogues, others may be prepared by adding suitable precursors to the fermentation medium (biosynthesis). Of the limited number evaluated clinically, some, particularly those with benziminazole derivatives as the nucleotide base, have the full clinical activity of $B_{12}$.

The nomenclature of the $B_{12}$ group of substances was considered by a commission set up at the European Symposium on Vitamin $B_{12}$ and Intrinsic Factor, Hamburg, 1956.[24] Under the "Tentative Rules" for nomenclature subsequently recommended[25] and, apart from the numbering of the nucleus, since adopted, $B_{12}$ itself, for example, becomes $\beta$-(5:6-dimethylbenziminazolyl) cobamide cyanide.

### Crystalline vitamin $B_{12}$—

Vitamin $B_{12}$ (see Fig. 1),[26,27,28] official as cyanocobalamin in the British, United States and other Pharmacopoeias, occurs as a tasteless, odourless red crystalline powder or as needle-like crystals, which after drying possess refractive indices $\alpha$ 1·616, $\beta$ 1·62 and $\gamma$ 1·664. It is hygroscopic and can take up appreciably more than the permitted 12 per cent. of moisture; this may be removed quantitatively by drying under reduced pressure at 105° C to give the anhydrous substance, which is extremely hygroscopic. When heated, the crystals darken, but do not melt below 300° C. Vitamin $B_{12}$ is soluble in water to the extent of 1·25 per cent. at 25° C; it is also soluble in lower alcohols and phenols, but insoluble in most other organic solvents. The optical rotation in aqueous solution has been recorded as $[\alpha]_{6563}^{23} = -59° \pm 9°$,[29] and $[\alpha]_{6438}^{20} = -110° \pm 10°$,[30] but the intense colour makes observation difficult.

Complete identification of a substance as complex as $B_{12}$ presents some special problems. Cobalt and phosphorus may be detected and determined by conventional chemical methods after ashing or fusion with potassium hydrogen sulphate, and various degradation products may be identified after more or less vigorous treatment with acids, alkalis or oxidising agents. Physical methods are more promising. Infra-red absorption[30,31] serves to differentiate $B_{12}$ from other cobalamins,[32] but would only detect relatively large amounts of them in admixture

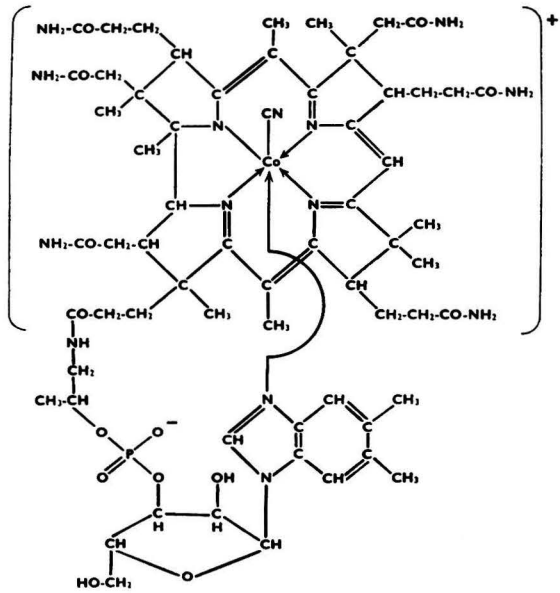

Fig. 1. Vitamin $B_{12}$ ($C_{63}H_{88}O_{14}N_{14}CoP$); molecular weight 1355·42

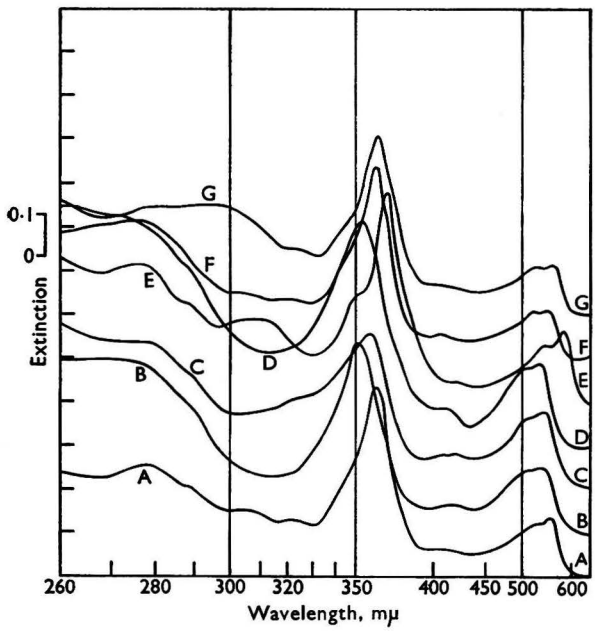


Fig. 2. Absorption spectra: curve A, 0·002 per cent. of vitamin $B_{12}$ in water; curve B, 0·003 per cent. of hydroxocobalamin in phosphate buffer (pH 3·0); curve C, 0·003 per cent. of hydroxocobalamin in phosphate buffer (pH 10); curve D, 0·003 per cent. of nitritocobalamin in water; curve E, 0·002 per cent. of vitamin-$B_{12}$ dicyanide complex; curve F, 0·002 per cent. of *pseudo* vitamin $B_{12}$ in water; curve G, 0·002 per cent. of vitamin $B_{12}$ III (factor I) in water.

For clarity, each curve has been displaced by 0·1 extinction unit above the one below

with $B_{12}$. The ultra-violet and visible absorption curve in water shows maxima at 278, 361 and 550 m$\mu$ (see Fig. 2), and this is probably the best single identification test, particularly when strengthened by the imposition of limits for the ratios of extinction values at these three wavelengths. However, the existence of $B_{12}$ analogues with similar absorption spectra must not be overlooked, and for further differentiation reliance must be placed on other methods; these are considered in more detail below. Clearly, existing official monographs are by no means exclusive for $B_{12}$, and they could well be amplified by the inclusion of at least one additional test. Biological assay with *Ochromonas malhamensis* (see p. 404), which most clearly approaches higher animals in the specificity of its requirement for $B_{12}$, will differentiate $B_{12}$ from the clinically inactive factors, but not from factor I ($B_{12}$ III). By electrophoresis in dilute acetic acid on paper, the presence of any basic or acidic substances can be detected, but electrophoretically neutral factors, in common with $B_{12}$ itself, are not moved from the point of application. Since the main object of any additional test is the detection of $B_{12}$ analogues, paper chromatography would appear to be the technique most deserving of official recognition. Although certain $B_{12}$ analogues cannot be separated from each other by this means, all the known natural ones can be separated as a group from $B_{12}$ and detected in trace amounts by application of the bio-autograph technique (see p. 401) on a medium seeded with an organism, such as *Escherichia coli* mutant 113–3, that responds to analogues as well as to $B_{12}$. By careful selection of the strain of organism for use in the commercial fermentative production of $B_{12}$, the formation of analogues can be minimised, and crystalline $B_{12}$ substantially free from them can be obtained. However, the possibility of production from sewage sludge, where $B_{12}$ analogues are known to occur in appreciable amounts, suggests caution in accepting compliance with present official monographs as an indication of adequate purity. Unless a more comprehensive examination can be made, and for small amounts of $B_{12}$ it is often uneconomic to do so, the integrity of the manufacturer must be relied on.

## Physical and chemical methods for vitamin $B_{12}$

### (*a*) Direct colorimetric and spectroscopic methods—

The simplest colorimetric method is that based on measurement of the red colour of $B_{12}$ in a suitable colorimeter provided with blue filters[30] or on a spectrophotometer at 550 m$\mu$. The method has obvious limitations, but may be used as a check on the concentration of solutions of known origin, particularly in the presence of colourless substances having ultra-violet absorption. Greater sensitivity may be obtained by measurement of the extinction at 361 m$\mu$ ($E^{1\%}_{1cm} = 207$), and this is generally suitable for aqueous solutions down to about 0·001 per cent. A measure of specificity is introduced by determining the ratios of the extinctions at 278, 361 and 550 m$\mu$ or at 341 and 376 m$\mu$, as proposed by Bruening, Hall and Kline.[33]

Whatever wavelengths are selected, the ratios should be the same within reasonable limits as those for pure $B_{12}$. Preferably a full absorption curve is recorded. Several methods have been proposed for increasing the specificity and sensitivity of the spectroscopic approach, particularly so that it can be applied to samples of biological origin, in which the $B_{12}$ may be present at extremely low concentrations and accompanied by analogues.

When treated with an excess of cyanide in alkaline solution, $B_{12}$ takes up one additional equivalent of cyanide to form the dicyanide complex; this is accompanied by a change from red to a purplish colour. Rudkin and Taylor[34] proposed a method based on this reaction and suitable for concentrations of at least 1 $\mu$g per ml. Excess of cyanide is added to the sample, and the pH of the mixture is adjusted to 9·5 to 10·0. After allowing the solution to stand for several hours, to complete the conversion of other cobalamins to $B_{12}$, sodium sulphate is added to give 20 per cent. w/v, and the solution is then extracted with benzyl alcohol. Chloroform is added to the combined benzyl alcohol extracts, and the $B_{12}$ is re-extracted into water. After dilution to volume, the extinction at 582 m$\mu$ is measured on a portion of the solution adjusted to pH 5 to 6 and on another portion containing alkali and cyanide. The difference in extinction, $\Delta E^{1\%}_{1cm} = 54$, is then calculated as $B_{12}$. Similar methods were recommended subsequently by Carneri[35] for liver extracts and for culture fluids by Janicki, Pawelkiewicz, Stawicki and Zodrow.[36] In the former method, $B_{12}$ as the dicyanide complex is extracted into benzyl alcohol and then re-extracted into water after the addition of chloroform. In the latter the $B_{12}$ is extracted with benzyl alcohol and then re-extracted into water, which after saturation with ammonium sulphate is extracted with acetone. After evaporation

of the acetone under reduced pressure, the $B_{12}$ content is determined as the dicyanide complex at 588 m$\mu$ ($E^{1\%}_{1cm} = 58{\cdot}2$). Another approach to the problem of obtaining spectroscopically pure $B_{12}$ from biological materials was proposed by Van Melle,[37] who suggested column chromatography on Amberlite XE97 carboxylic cation-exchange resin for the assay of liver extracts for injection. After preliminary treatment with cyanide at pH 7·5, the sample, containing 125 to 150 $\mu$g of $B_{12}$ and adjusted to pH 4, is passed through the resin, and impurities are removed by a series of solvent washes. The retained $B_{12}$ is then eluted with aqueous dioxan and determined by difference spectroscopy on the lines of Rudkin and Taylor's method.[34] Good agreement was obtained with results given by microbiological assay with *Lactobacillus leichmannii*, but the method has since[38] been found inadequate for distinguishing cobalamins from *pseudo* vitamin $B_{12}$ and "red pigments." For the determination of $B_{12}$ in cultures, Fisher[39] proposed extraction of the dried culture with hot benzyl alcohol in the presence of cyanide and water at pH 7 to 8. For moist materials, extraction with *n*-propanol was preferred. The $B_{12}$ is then determined by difference spectroscopy.

(*b*) INDIRECT SPECTROSCOPIC METHODS—

By subjecting $B_{12}$ to physical or chemical degradation, various products can be obtained somewhat more amenable to determination than $B_{12}$ itself. In general, methods based on such reactions are of limited value, since they will be as subject to interference by $B_{12}$ analogues and some degradation products as are the direct spectroscopic ones.

Fantes and Ireland,[40] and subsequently Heinrich,[41] proposed similar methods suitable for $B_{12}$ concentrates. These are based on hydrolysis of the $B_{12}$ with hot 5 or 10 *N* hydrochloric acid, esterification and extraction of the resulting red acids with heptyl or octyl alcohol and removal of impurities by dilution with light petroleum and washing with methanolic hydrochloric acid. The colour of the purified extract is then determined.

Exposure of $B_{12}$ to light causes release of cyanide, which can be recovered quantitatively if it is removed by aeration as it is formed. Boxer and Rickards[42,43,44,45] proposed methods based on the colorimetric determination of the cyanide released in this way. Any free cyanide in the sample is removed by preliminary aeration in the dark. Clearly these methods will distinguish $B_{12}$ from other cobalamins, but not from analogues in their cyano forms.

The same workers[46] sought to exploit the presence of 5:6-dimethylbenziminazole in the $B_{12}$ molecule. After hydrolysis with 0·1 *N* hydrochloric acid at 120° C, benzoylation and cleavage with concentrated sulphuric acid, the resulting 4:5-dimethyl-*o*-phenylenediamine is treated with acetylacetone for colorimetric or with alloxan for fluorimetric determination. The method is then sensitive to 10 $\mu$g of benziminazole.

(*c*) POLAROGRAPHY—

In 0·025 *M* phosphate buffer at pH 7·4, $B_{12}$ at concentrations of 0·005 to 0·020 $\mu$g per ml exhibits a well defined polarographic step at −1·53 volts against a saturated-calomel electrode.[30,47] The step is surmounted by a characteristic maximum, the height of which is influenced by pH and may be affected by other constituents of the solution. For these reasons, the method has received little attention.

(*d*) ISOTOPE-DILUTION METHODS—

Vitamin $B_{12}$ has been labelled isotopically with $^{14}C$, which can be introduced into the cyanide group by simple chemical exchange,[48] but the group is too labile for $B_{12}$ labelled in this way to be of value. The phosphorus and cobalt are much more firmly bound,[30] and no exchange can be induced. Though $^{32}P$ may be introduced by fermentation in the presence of labelled phosphate[49] and $^{60}Co$ formed by neutron activation, in either event only low specific activities are attainable.[50,51] Suitably labelled cobalt salts may be added to the fermentation medium, and by this means highly active Co-labelled $B_{12}$ may be made.[52] Of the possible isotopes $^{60}Co$ (half-life 5·2 years) and $^{58}Co$ (half-life 72 days) are generally the most useful; $B_{12}$ labelled in this way (now available from the Radiochemical Centre, Amersham) has proved of great value for analytical, biochemical and clinical investigations.[53]

Isotope-dilution assays are based on the principle that a small known amount of the radioactive substance to be determined (the "marker" or "tracer") is added to the sample, from which the pure substance is then isolated by any suitable means. From the activities and amounts of the marker and of the material isolated the amount of substance in the sample can be calculated. The method is of particular value for substances having no specific

properties that can be used analytically on crude samples or occurring together with closely related compounds from which they cannot be separated quantitatively. For $B_{12}$, isotope dilution provides an "absolute" method, which can be adapted to the assay of either actual or potential cyanocobalamin according as treatment with nitrite and cyanide is omitted or included.[54] Analogues and degradation products may interfere unless they are eliminated by rigorous purification. Although low yields in the purification can be tolerated, the need for taking enough sample to give sufficient pure $B_{12}$ both for counting (to determine dilution) and for spectroscopic analysis or microbiological assay (to determine the amount isolated) makes the method somewhat laborious.

Numerous purification steps applicable in isotope-dilution assays are described by Bacher, Boley and Shonk[55]; these steps may be combined in various ways for different types of samples. The minimum amount of total cobalamins required for assay is about 100 $\mu$g at a concentration of at least 0·1 $\mu$g per ml; a standard deviation of $\pm$4·3 per cent. is claimed. The assay of cobalamins in fermentation liquors serves as an example. A measured volume of sample containing a known amount of added marker is treated with nitrite and cyanide at pH 4 and is then boiled to convert all cobalamins to $B_{12}$ and to liberate any cobalamins bound to peptides. The mixture is clarified with zinc acetate at pH 8, and the filtrate is submitted to extractions with mixtures of cresol, butanol and carbon tetrachloride, so that a partly purified solution of the $B_{12}$ is obtained. Further purification on a mixed-bed ion-exchange column normally yields an eluate suitable for spectroscopic assay and for counting. If the final solution is not spectroscopically satisfactory, re-purification by the same or additional steps is necessary. The principles of this method, with in some instances the refinement of paper chromatography,[54] have also been applied to concentrates for oral use, feed supplements,[55] liver extracts, pharmaceutical preparations[56] and a variety of animal and vegetable materials.[57,58]

Bruening, Neuss, Numerof and Kline[38] in a collaborative study of an isotope-dilution method for $B_{12}$ in concentrates and liver preparations concluded that the method was specific for $B_{12}$, and it has received official recognition in the U.S. Pharmacopoeia, 15th Revision, First Supplement, 1956.

(*e*) REVERSED ISOTOPE-DILUTION ASSAYS—

The technique of reversed isotope dilution[54] is applicable to the assay of radio-$B_{12}$ in, for example, the fermentation liquors arising in the preparation of ($^{60}$Co) $B_{12}$. A known excess of ordinary $B_{12}$ is added to the sample, and pure $B_{12}$ is then isolated as described above. The activity of the isolated material and its amount permit calculation of the level of radio-$B_{12}$ in the sample on the lines of the normal isotope-dilution assay.

(*f*) OTHER PHYSICAL METHODS OF ASSAY—

When fluorimetric methods of assay can be applied, they usually have high sensitivity, and the development of commercial spectrofluorimeters, in which both the activating and emitted radiation can be recorded, has given to fluorimetric methods a measure of specificity of which the lack has hitherto been a serious limitation to their use. In a study of spectrofluorimetry, Duggan, Bowman, Brodie and Udenfriend[59] found that in phosphate buffer, pH 7, $B_{12}$ had an activation maximum at 275 m$\mu$ and a fluorescence maximum at 305 m$\mu$ and that a practical sensitivity of 0·003 $\mu$g of $B_{12}$ per ml was attainable.

These considerations suggest that spectrofluorimetry may be a much needed addition to the few satisfactory physico-chemical methods for $B_{12}$, particularly for coping with interference by analogues and other cobalamins, and should repay further study.

## METHODS FOR SEPARATING VITAMIN $B_{12}$ FROM CRUDE MATERIALS AND FROM ANALOGUES

Physico-chemical methods, with the exception of isotope dilution, are more or less non-specific for $B_{12}$; before they can be applied to materials of biological origin some preliminary concentration and purification of the $B_{12}$ is essential. Methods currently in use derived largely from those evolved in the early studies that led to the isolation of $B_{12}$; because $B_{12}$ has few chemical properties of any analytical value, the methods are based on selective adsorption or on some form of partition between immiscible solvents.

(*a*) ADSORPTION CHROMATOGRAPHY—

From what little has been published about the American work leading to the isolation of crystalline $B_{12}$ from liver, it is clear that chromatography of aqueous methanol solutions

on activated alumina played an important part[8] in the later stages of purification. As long ago as 1936 adsorption on charcoal had been used[60] for the initial concentration, and the method was employed in the early processes for commercial extraction of $B_{12}$ from fermentation liquors. Fantes, Page, Parker and Smith[30] also used charcoal, but found that better purification was attainable on columns of silica developed with suitable concentrations of aqueous ammonium sulphate solution; chromatography on bentonite formed one purification stage in other parallel work on the isolation of $B_{12}$. In general, however, such methods show low resolving power and so require rather large columns; they have largely been abandoned in favour of partition and resin absorption processes.

(*b*) PARTITION ANALYSIS—

Although based on the principle of partition between two immiscible or partly miscible solvents, in practice the processes employed fell into two distinct categories; in one, both phases are mobile (described simply as partition analysis or countercurrent distribution), and in the other, one phase is immobilised on a relatively inert support and the other phase is made to flow past it (column and paper chromatography).

The general theory of partition analysis has been considered by Heathcote and Duff,[61] who deal primarily with the separation of hydroxocobalamin and $B_{12}$. For simplicity and convenience of calculation the chosen ratio of phase volumes is generally unity, as in the 8-tube countercurrent distribution between benzyl alcohol and water proposed by Mader and Johl.[62] In this system, $B_{12}$ has a partition coefficient ($K = \frac{[\text{benzyl alcohol}]}{[\text{water}]} = 1{\cdot}2$) such that, after distribution is complete, the highest concentration of $B_{12}$ is attained in tube No. 4, the contents of which may then be assayed spectroscopically or microbiologically and the amount of $B_{12}$ easily calculated in relation to the theoretical fraction that should appear in that tube. Hydroxocobalamin ($K = 7{\cdot}7$), *pseudo* $B_{12}$, and non-cobalamin materials from fermentation broths and $B_{12}$ concentrates collect in the end tubes and are more or less completely separated from the $B_{12}$. A similar system, with phosphate buffer, pH 4, in place of water, is useful for the detection of traces of hydroxocobalamin in crystalline $B_{12}$. Nitritocobalamin is not separated from the $B_{12}$, and it is therefore preferable to include a preliminary treatment with sulphamic acid to convert it to hydroxocobalamin. After distribution, the contents of the first and fourth tubes are assayed spectroscopically, and the components are then calculated from simultaneous equations based on their known partition coefficients. Rosenblum and Woodbury[63] proposed a single distribution between benzyl alcohol and water before radioactivity measurement as a test for the identity of ($^{60}$Co) $B_{12}$ recovered in the assay of multivitamin preparations.

The many water-immiscible solvents proposed for the extraction, concentration and partial purification of $B_{12}$ from crude materials include phenol, cresol and other alkyl phenols, often diluted with chloroform, carbon tetrachloride or butanol. The separated organic phase is washed before re-extraction of $B_{12}$ into the aqueous phase, sometimes in the presence of benzalkonium chloride, which helps to retain impurities in the organic phase.[55] Probably the most efficient single purification step, however, is extraction of $B_{12}$ as the dicyano complex into benzyl alcohol from alkaline solution containing excess of cyanide and a high salt concentration.[8] McLaughlan, Rogers, Middleton and Campbell[57] recommended extraction in the presence of metabisulphite, instead of cyanide, as a means of eliminating *pseudo* $B_{12}$, factor A and deoxyribosides in a spectroscopic assay of $B_{12}$ in crude materials; however, by this method factor $B_{12}$ III is only partly removed.

(*c*) COLUMN AND PAPER PARTITION CHROMATOGRAPHY—

These two techniques played an important part in the isolation of $B_{12}$ and subsequently in the separation and recognition of the many $B_{12}$-like factors now known. In column chromatography the stationary aqueous phase may be water or buffer saturated with the organic phase and supported on silica, starch,[64] kieselguhr[51,65,66] or cellulose.[21,67] The original partition systems, generally based on butanol - water, have undergone little change. Smith *et al.*[51,65] carried out chromatography on acid-washed kieselguhr treated with 2 per cent. phosphate buffer (pH 7) and eluted with water-saturated *n*-butanol. In a modification of this system 15 per cent. w/v of phenol was added to the *n*-butanol. For moderately pure materials the course of development may be simply followed by the movement of the pink cobalt-containing compounds down the column. Chromatography on paper may also be

used for small-scale preparative purposes and as a final stage in the purification of $B_{12}$ in isotope-dilution assays. However, the main use of paper chromatography is in the separation of the many factors present in biological materials and often obtainable only in exceedingly small amounts. With 50 $\mu$g or so of material, visible spots are obtained. For smaller amounts the necessary sensitivity may be obtained by the bio-autograph technique considered in more detail below. Chromatography is usually carried out on Whatman No. 1, 4 or 3MM paper developed with *sec.*-butanol saturated with water.[51] The $B_{12}$ factors have a satisfactory range of $R_F$ values in this system, although the values are relatively low, and it is usually preferred to develop the chromatogram by the descending-solvent method for 16 to 18 hours in the dark, running the solvent off the bottom of the paper, which is serrated to promote even flow. $R_F$ values are then more conveniently expressed relative to that of $B_{12}$ taken as unity. A further refinement[15] is the addition of 1 per cent. w/v of either ammonia or acetic acid to the system. Somewhat different $R_F$ values are thereby obtainable for a given factor in the two systems (see Table I). Improved resolution of certain factors is claimed if the *sec.*-butanol - water solvent is first saturated with potassium perchlorate,[68] and the use of buffer-impregnated paper has been suggested.[69] As with paper electrophoresis, the addition of a trace of cyanide to the system ensures that the factors are maintained in the cyano form.

(*d*) ION-EXCHANGE METHODS—

The recovery of $B_{12}$ from a series of Amberlite ion-exchange resins was studied by Marsh and Kuzel.[56] They proposed this method as a means of separating $B_{12}$ from the other constituents of multivitamin preparations. The most satisfactory purification was obtained with columns of mixed cation and anion exchangers, particularly on an 8-cm bed of IRA-400, in the hydroxyl form, overlaid with a 7-cm bed of a 1 + 1 mixture of the same resin and IR-120 ($H^+$). The aqueous $B_{12}$ solution at pH 5 to 7 is passed through the column, and the appropriate pink fraction is collected for spectroscopic assay. Correction for loss on the column is necessary. Bacher, Boley and Shonk[55] subsequently proposed a modification of the procedure as a final purification step in an isotope-dilution assay, when quantitative recovery of the $B_{12}$ is unnecessary. On the commercial scale, use of Amberlite IRC-50 resin has largely displaced earlier methods for the purification of $B_{12}$ from fermentation liquors.

(*e*) PAPER ELECTROPHORESIS—

The general technique of electrophoresis on filter-paper has been the subject of a review by Parker.[70] This method, particularly as an adjunct to paper chromatography, provided valuable information in the studies that elucidated the structure of $B_{12}$.[71] It made possible analysis of the complex mixtures of basic, neutral and acid substances resulting from the acid hydrolysis of $B_{12}$. In 0·05 *N* phosphate buffer, pH 6·5, on Whatman No. 4 or 31 filter-paper, cobalt-containing pigments are separated with groups differing in their number of ionic charges, whereas neutral substances, including $B_{12}$ itself, remain as a group at the point of application.

It is usual to add a low concentration of cyanide to acid and neutral electrophoresis buffers, to maintain the compounds in their cyano forms and to avoid formation of additional zones from the hydroxo-derivatives. Alternatively, electrophoresis may be carried out in 0·1 *N* potassium cyanide (pH 10), the acidic dicyano-cobalamin then moving as a purple zone towards the anode. The most satisfactory separations, however, are generally attained in buffers of low pH and low ionic strength at a potential of 8 to 10 volts per cm.[15] In 0·5 to 2·0 *N* acetic acid, for example, factors $B_{12}$ III and *pseudo* $B_{12}$ and factor A, which cannot be separated chromatographically, may be resolved into separate zones. This method has been applied to the mixtures of $B_{12}$-like substances obtained from materials of biological origin[66,72] and, in combination with microbiological assay, for the determination of $B_{12}$ in liver extracts.[59] Paper electrophoresis may be combined with chromatography for two-dimensional separations[15] and has also been used for preparative purposes.[73]

## PHARMACEUTICAL PREPARATIONS CONTAINING VITAMIN $B_{12}$

INJECTION PRODUCTS—

Vitamin $B_{12}$, physiologically one of the most active substances known, may be administered orally or by injection. For the treatment of pernicious anaemia and related conditions 50 to 100 $\mu$g or more of $B_{12}$ in aqueous solution are administered. Intramuscular injection is preferable, since this avoids the uncertainties of absorption after oral administration.

Analysis of such simple preparations may be carried out, as in the official method, by calculating the extinction at 361 mμ as anhydrous cyanocobalamin. Care must be taken, however, to ensure the absence of any added bacteriostat that might invalidate the result. This may be checked by observing the ratios of the extinctions, as described for pure $B_{12}$, or by recording a full absorption curve on a suitable spectrophotometer. Partition methods have also been suggested as a means of ensuring that the $B_{12}$ used for preparing injections complies with the requirements for anhydrous cyanocobalamin content.[74] Occasionally, other members of the vitamin-B complex may be added. For the routine control of such preparations, methods for the separation of spectroscopically pure $B_{12}$ may be devised or variable reference solutions may be employed.[75] For occasional use one of the microbiological assays is simpler and generally satisfactory.

ORAL PREPARATIONS—

Oral preparations of $B_{12}$, chiefly tablets and liquids, are less restricted in scope than injection products. The American Drug Index (1958), for example, lists over 300 branded preparations in which $B_{12}$ is present with other vitamins, nutritional and mineral supplements, sedatives and anti-obesity drugs. The declared formulae are often of impressive length. Although providing an interesting, if time-consuming, task, a complete analysis of such products is rarely necessary. The $B_{12}$, often present only in microgram amounts, can usually be determined adequately by a suitable microbiological method, but some preliminary treatment, such as boiling under acid conditions in the presence of cyanide, may be necessary to ensure that the $B_{12}$ is available to the selected test organism under assay conditions. The amount of $B_{12}$ remaining may be often regarded as usefully indicating the stability of the preparation as a whole. All too frequently the deficiencies in $B_{12}$ found, particularly in samples from parts of the world where drug-control is not as strict as it might be, indicate clearly that the problem of formulating $B_{12}$ to ensure its subsequent stability has not been solved by the manufacturers concerned; at least, that is one possible explanation for some of the low results found.

## MICROBIOLOGICAL DETERMINATION OF $B_{12}$ IN NATURAL MATERIALS

Microbiological assays for $B_{12}$ originated from early observations that refined liver extracts contained a growth factor essential for certain micro-organisms. Shorb[12] found that *L. lactis* Dorner required the factor (LLD factor) in an amino acid *plus* tomato juice medium. The LLD content of refined liver extracts paralleled their haematopoietic activity. Skeggs, Huff, Wright and Bosshardt,[76] assaying animal protein factor (A.P.F.) by *L. leichmannii* and mouse growth, noted that the liver factor could replace A.P.F. in both assays. Hutner *et al.*,[77] investigating the nutritional requirements of the protozoon *Euglena gracilis*, found that refined liver extracts supplied an essential growth factor hitherto provided by crude casein.

Methods for the determination of $B_{12}$ by means of bacteria, protozoa, birds or mammals were developed concurrently with elucidation of the complex chemistry of the $B_{12}$ group of substances. Discrepancies in results for various assay methods were the first indication of the existence of natural $B_{12}$-like substances besides the cobalamins.[78,79] When these analogues of $B_{12}$ were isolated,[15,16,17,18,19,20] it was found that several could replace $B_{12}$ for both *Euglena* and *Lactobacilli*. This prompted further search for an organism that would show a specific $B_{12}$ requirement similar to that of birds and mammals. The chrysomonads *O. malhamensis*[80] and *Poteriochromonas stipitata*[81] appeared to satisfy this criterion.

The sensitivity of micro-organisms—see Table II—permits direct estimation of the extremely low $B_{12}$ content of some natural products, whereas prior concentration and purification are needed for any physico-chemical assay. An understanding of the limitations of the various test organisms and of the nature of the sample is, however, essential to ensure that the method of assay and the sample treatment selected are capable of giving a specific and quantitative result.

BOUND $B_{12}$ AND PRE-TREATMENT OF SAMPLES—

Vitamin $B_{12}$, cobalamins and $B_{12}$ analogues are wholly or partly bound to protein in materials such as liver, milk, serum, gut mucosa, faeces and fermentation broths. The $B_{12}$ in many of these complexes is not available to micro-organisms or animals, but in others it is utilised to different degrees.[82,83,84,85] Coenzyme forms of $B_{12}$ and of two analogues have

## TABLE I

### SOME PROPERTIES OF VITAMIN $B_{12}$, COBALAMINS AND NATURAL ANALOGUES

| Name | Base of nucleotide | References | Original sources | Absorption maxima, mμ | $E^{1cm}_{1\%}$ | $R_F$ values: *sec.*-Butanol - acetic acid - water | $R_F$ values: *sec.*-Butanol - ammonia - water | Ionophoretic mobility in 0·5 *N* acetic acid, $cm^2 \times V^{-1} \times sec.^{-1} \times 10^{-5}$ |
|---|---|---|---|---|---|---|---|---|
| Vitamin $B_{12}$ Cyanocobalamin | | | Liver, fermentation liquors, etc. | 278<br>361<br>520<br>550 | 115<br>207<br><br>63 | 0·25 | 0·30 | 0 |
| Vitamin $B_{12b}$ ($B_{12a}$) Hydroxocobalamin (neutral and acid) | 5:6-Dimethylbenzi-minazole | 14, 65, 168, 169, 170 | Liver and fermentation liquors (derived from $B_{12}$ by loss of –CN) | 270 to 277<br>352·5<br>530 | 137<br>150<br>56 | 0·11* | 0·08* | 4·3 |
| Aquocobalamin (alkaline) | | | | 272 to 278†<br>356†<br>418†<br>535† | 132†<br>127†<br>32†<br>59† | | | |
| Vitamin $B_{12c}$ Nitritocobalamin | | 14, 65 | Fermentation liquors (derived from $B_{12}$ by replacement of –CN with –NO) | 255 to 275<br>354<br>530 | 187 to 180<br>185<br>75 | 0·30* | 0·08* | 0 |
| *Pseudo* Vitamin $B_{12}$ | Adenine | 18, 66, 72 | Gut contents, faeces, pig and calf manure. Sewage sludge. | 278, 308, 320, 361, 518, 548 to 550 | 204 at 361 mμ | 0·11 | 0·085 | 1·5 |
| Factor A .. .. | 2-Methyladenine | 66, 72, 171 | Calf gut contents and manure. Sewage sludge | 280, 320, 361, 520, 548 | 204 at 361 mμ | 0·13 | 0·12 | 3·9 |
| Factor B .. .. | No nucleotide | 15, 16, 71 | Calf gut contents and manure. Sewage sludge | 276, 315, 355, 503, 530 | — | 0·50 | 0·45 | 5·0 |
| Factor C ($C_1$ and $C_2$) | ? Guanine | 15, 16, 170, 172, 173 | Calf gut contents and manure, fermentation broth. Sewage sludge | 273, 320, 356, 500, 530 | — | $C_1$ 0·02<br>$C_2$ 0·04 | 0·04<br>0·06 | −1·4<br>— |
| Factor D .. .. | Not known | 66 | Calf manure | — | — | — | — | Basic |
| Factor E .. .. | Not known | 66 | Pig and calf manure | 275, 360 | — | 0·35 | 0·40 | 0 |
| Factor F .. .. | ? 2-Methylmercaptoadenine | 16, 66 | Pig manure and chicken faeces | 361 | — | 0·21 | 0·17 | −0·3 |
| Factor G .. .. | Hypoxanthine | 66 | Pig and calf manure (de-amination of *pseudo* $B_{12}$) | 359, 526, 540 | — | Approx. 0·1 | — | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Factor H .. .. | 2-Methylhypoxanthine | 66 | Pig and calf manure (de-amination of factor A) | 358·5, 517, 540 | — | Approx. 0·1 | — | 0 |
| Factor I (Vitamin $B_{12}$ III) | 5-Hydroxybenzimidazole | 21, 22, 23, 66 | Sewage sludge, calf and pig manure | 295, 361, 518, 550 | 204 at 361 mμ | 0·13 | 0·14 | 0 |
| Factor J .. .. | ? No nucleotide | 174 | Sewage sludge | 353 | — | 0·31‡ | — | Basic |
| Factor K .. .. | Not known | 174 | Sewage sludge | 361 | — | 0·37‡ | — | 0 |
| Factor L .. .. | ? No nucleotide | 174 | Sewage sludge | — | — | 0·44‡ | — | Basic |
| Factor M .. .. | Not known | 174 | Sewage sludge | 361 | — | 0·31‡ | — | 0 |

* In the absence of cyanide. † At pH 10·0. ‡ Calculated from published values relative to $B_{12}$.

## TABLE II

### RESPONSE OF VARIOUS TEST ORGANISMS TO $B_{12}$, SOME ANALOGUES OF $B_{12}$ AND INTERFERING GROWTH FACTORS

| Test organism | Method and reference | Response range for $B_{12}$* | | Response to some analogues of $B_{12}$† | | | | | | | | | $B_{12}$-sparing activity‡ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Minimum, mμg per ml | Maximum, mμg per ml | Factor B | *Pseudo* $B_{12}$ | Factor A | Factor C | Factor E | Factor F | Factor G | Factor H | Factor I | Methionine | Desoxyribosides |
| *Ochromonas malhamensis* (Pringsheim) | Tube[80] | 0·01 | 0·16 | — | — | — | — | — | — | — | — | + | + | — |
| *Euglena gracilis* (*Z* strain) | Tube[147] | 0·00025 | 0·05 | — | + | + | + | | | | | | — | — |
| *Lactobacillus leichmannii* (A.T.C.C. 7830) | Tube (U.S.P., 15th revision) | 0·001 | 0·02 | — | + | + | + | — | | + | + | + | — | + |
| *Escherichia coli* mutant (N.C.1B. 8134) | Tube[139] | 0·04 | 0·25 | + | + | + | + | — | + | — | + | + | + | — |
| | Plate[138] | 5·0 | 500·0 | + | + | + | + | + | + | + | + | + | + | — |

* Refers to the final concentration in the assay tube.
† + denotes that analogue can replace $B_{12}$, but not necessarily on a weight basis; — denotes that analogue cannot replace $B_{12}$; no sign denotes no information.
‡ $B_{12}$-sparing activity only when present in large excess in relation to $B_{12}$.

recently been isolated by Barker and his co-workers[86,87,88,89] from bacterial fermentations and from rabbit liver. The coenzymes contain one molecule of adenine in place of cyanide and can be converted to $B_{12}$ or the corresponding analogue by heating with cyanide under acid conditions. In addition, this same treatment usually serves to release $B_{12}$ bound to protein and to convert other cobalamins to $B_{12}$.

*Liver and liver preparations*—A method for treating liver preparations is detailed in the report of the Analytical Methods Committee of the Society for Analytical Chemistry.[90] For each gram of sample, at pH 4·6 to 5·0, 0·5 to 5·0 mg of sodium cyanide in 1 per cent. aqueous solution are added. After the reaction has proceeded for 30 minutes at room temperature and 30 minutes in a boiling-water bath, the mixture is cooled, spun in a centrifuge and diluted. Treatment with takadiastase may be necessary if starch is present in the supernatant liquid. The final concentration of sodium cyanide in the assay medium should not exceed 10 $\mu$g per ml. This method is also satisfactory for "fish solubles," animal feeds and gut contents.

Coates *et al.*[79] use 50 ml of 1 per cent. sodium acetate buffer solution, pH 4·8, and 0·5 ml of 1 per cent. sodium cyanide solution for each 10 g of sample. The whole is heated in flowing steam for 30 minutes, cooled, and clarified by centrifugation. This treatment is satisfactory for assays with *L. leichmannii* or *E. coli*, but it should be noted that acetate ions are inhibitory to *O. malhamensis*,[80] the assay organism recommended by the Analytical Methods Committee.

*Milk*—Gregory[91] found that $B_{12}$ is not detectable in the ultra-filtrate of milk from many species and that some milks have a capacity for binding added $B_{12}$. To liberate the protein-bound $B_{12}$ in sows' or human milk, 1 ml of sample and 1 ml of 0·1 *M* sodium acetate buffer, pH 4·6, are warmed to 60° C in a water bath; 50 mg of papain and 1 drop of 1 per cent. w/v sodium cyanide solution are added. The mixture is held at 60° C for 1 hour and then steamed for 10 minutes to inactivate the enzyme. Good recovery of added $B_{12}$ was obtained by this treatment.

*Serum*—The blood sample should be allowed to clot. The separated serum may be stored at −20° C. For *E. gracilis* assays,[92,93] the bound $B_{12}$ may be liberated by diluting the serum with water and assay medium at pH 3·6 and heating the mixture at 100° C for 13 minutes. If a dilution factor of at least $\frac{1}{3}$ is employed, no precipitation of protein occurs. Uncombined $B_{12}$ may be determined by omitting the heat treatment or heating at only 56° C for 30 minutes. For the *L. leichmannii* assay described by Rosenthal and Sarett,[94] it is necessary to precipitate the serum protein first by heating with five volumes of 0·2 per cent. acetate buffer solution, pH 4·6, for 30 minutes at 100° C. An aliquot of the neutralised liquid is then taken for assay; this method has the advantage of eliminating the growth-stimulating properties of serum proteins. If the *O. malhamensis* method is to be used, the serum protein is first precipitated by heating at pH 5·0 without acetate buffer.[95]

*Urine*—The work of Ross[93] suggests that $B_{12}$ is not bound in urine, but care must be taken to avoid faecal contamination and subsequent bacterial growth. Baker, Sobotka, Pasher and Hutner,[96] comparing assay methods for the determination of $B_{12}$ in urine, diluted the samples in a pH 4·5 buffer and autoclaved for 30 minutes. Concentration of urine before assay is sometimes necessary. Davis and Chow[97] show that resting cells of *L. leichmannii* suspended in normal saline quantitatively adsorb $B_{12}$; this may be released for subsequent microbiological assay by heating the cells and adsorbed $B_{12}$ at 60° C for 1 hour. The technique is particularly useful for the determination of radioactive $B_{12}$ in urine (*e.g.*, after the administration of small doses of $B_{12}$ labelled with $^{60}Co$), when the radioactive material is adsorbed on the cells and the activity may be counted directly. The method gave better recovery of added radioactive $B_{12}$ than did conventional butanol extraction.

*Fermentation broths*—Fermentation broths normally require treatment to liberate the $B_{12}$ from the cells; for example, *Streptomyces griseus* fermentation broths may be satisfactorily treated by mixing 10 ml of broth with 10 ml of 0·01 *M* phosphate buffer, pH 2·5, and 1 to 2 g of kieselguhr, heating the mixture (which will be at about pH 4·5) for 15 minutes in a boiling-water bath, cooling, and adding 10 ml of potassium cyanide (16 mg per litre). After the mycelium has settled, an aliquot of the supernatant liquid is assayed.

Some bacterial fermentation broths have been found to require the more complex treatment considered above under isotope-dilution assays.[55] Other methods have recently been reviewed by Perlman.[98]

BIO-AUTOGRAPHS—

The distribution of $B_{12}$ and $B_{12}$ analogues in nature is now fairly well established. Animal tissues and body fluids normally contain only $B_{12}$ or cobalamins, but materials subject to bacterial fermentation, such as sewage sludge, faeces and gut contents, will probably contain many analogues in addition to $B_{12}$. Bio-autographs will readily detect the presence of such substances, which could invalidate a direct assay.[99,100] Even the most discriminating test organism, *O. malhamensis* (see Table II), will show a growth response to analogues containing substituted benziminazoles, *e.g.*, the naturally occurring factor I ($B_{12}$ III).

To obtain bio-autographs, papers loaded with 10-$\mu$l drops of suitably diluted samples and $B_{12}$ at 0·1 to 1·0 $\mu$g per ml are chromatographed in the usual way and, when dry, placed on nutrient agar seeded with an appropriate test organism, normally *E. coli* 113–3. After incubation, zones of exhibition are observed, corresponding to the positions of the factors on the developed chromatogram.

The growth factors methionine and desoxyribosides can be detected with *E. coli* and *L. leichmannii*, respectively.

Bio-autographs may be used for the quantitative determination of $B_{12}$ in materials containing factor I ($B_{12}$ III) or a large excess of other analogues that would invalidate all direct microbiological methods (see Table II and *O. malhamensis* method). Ford and Holdsworth[101] found that, when 0·13 per cent. of 2:3:5-triphenyl tetrazolium chloride was incorporated in the agar, the grown cells of *E. coli* contained a red pigment that increased the definition of the exhibition zones. A quantitative relationship was established by eluting the dye from excised growth zones with tetrahydrofuran. The colour density of the eluate was proportional to the log concentration of the $B_{12}$ applied to the chromatograms. Fantes and O'Callaghan[102] also used the bio-autograph technique for determining $B_{12}$ in the presence of an analogue. They established a log dose response curve for $B_{12}$ from the areas of the exhibition zones, as measured by their weight when cut from a photograph. This graph was used to determine the true $B_{12}$ content of unknowns developed on the same chromatogram.

Bio-autographs have been used for estimating penicillin in mixtures[103,104] and for determining the synergistic components of antibiotic E129.[105] With these techniques a methylene blue print is prepared, and the area of the inhibition zone is measured with a planimeter.

## MICROBIOLOGICAL-ASSAY METHODS WITH *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Escherichia coli*, *Euglena gracilis*, *Ochromonas malhamensis* AND OTHER MICRO-ORGANISMS

Both tube dilution and agar cup-plate techniques are used for the assay of $B_{12}$; each requires a basal medium deficient in $B_{12}$ but otherwise able to support dense growth of the test organism. The addition of sub-optimal levels of $B_{12}$ will then elicit a growth response proportional to the dose. For tube dilution methods graded doses of standard and samples are added to sterile broth in uniform tubes. The growth response after incubation is determined directly, by turbidimetric or colorimetric measurement, or indirectly by titration of acid formed, according to the nature of the test organism. For agar cup-plate assays graded doses of standard and sample are introduced into uniform cups cut in the agar medium. After incubation, circular zones of growth are observed around the cups containing $B_{12}$. The diameters of these exhibition zones should be proportional to the logs of the doses. The general principles of agar plate assays have been considered elsewhere.[106]

*Lactobacillus lactis* METHODS—

As previously mentioned, *L. lactis* Dorner = A.T.C.C. 8000 was the first bacterium found to have a growth requirement for $B_{12}$. Wright, Skeggs and Huff[107] demonstrated that it also has a requirement for thymidine. Cuthbertson and Smith described a plate assy for $B_{12}$ with this organism; thymidine at 5 $\mu$g per ml or more also gave exhibition zones, but these had characteristically indistinct edges and were readily distinguishable from those formed by $B_{12}$. Foster, Lally and Woodruff[108] also developed a plate assay; they claimed that the addition of sodium chloride (20 g per litre) eliminated the response to desoxyribosides, but that this possibly entailed loss of sensitivity. Other workers[109] have found that hypertonic solutions are toxic to *Lactobacilli* and that samples rich in salts may give erroneous results. Cuthbertson *et al.*[110,111] investigated the conditions in the

plate-assay method necessary for optimal response to $B_{12}$ and showed that control of aerobiosis is essential. Under anaerobic conditions $B_{12}$ was not required for growth.

Several attempts were made to establish turbidimetric assays with *L. lactis* Dorner,[112,113] but these methods were not reproducible and have been superseded by *L. leichmannii* methods.

*Lactobacillus leichmannii* METHODS—

*L. leichmannii*, like *L. lactis*, was known to have a nutritional requirement for thymidine desoxyriboside[114] and also has a requirement for $B_{12}$ when grown under strictly defined conditions.[76,115,116] Tube essays have been developed with *L. leichmannii* 313, A.T.C.C. 7830[117,118] and with *L. leichmannii* A.T.C.C. 4797.[116,118,119,120]

Turbidimetric measurements can usually be made after 20 to 24 hours, but titrimetric measurements require 72 hours' incubation. Constituents of the medium and its oxidation - reduction potential are both critical factors in eliciting a satisfactory dose response from these organisms.

Between 1949 and 1951 many workers developed satisfactory assay methods from the original observations. Stokstad *et al.*,[118,121] Skeggs *et al.*[120] and Thompson, Dietrich and Elvehjem[119] all developed media incorporating reducing agents and acid-hydrolysed casein in place of the enzymatic digest used previously. Stokstad selected thioglycollic acid, Thompson a mixture of ascorbic and fumaric acids, and Skeggs thiomalic acid as the best antioxidants for their respective basal media. The improved growth response to $B_{12}$ in the presence of the reducing agents was particularly marked for crude samples after autoclaving.

It was later realised that this was caused by protection of the heat-labile $B_{12a}$ ($B_{12b}$). Preliminary cyanide treatment of the sample or incorporation of cyanide in the medium converts other cobalamins to cyanocobalamin. These points in connection with *L. leichmannii* and *L. lactis* tube assays are discussed by Soars and Hendlin[122] and by Cooperman, Drucker and Tabenkin.[123]

Lees and Tootill[124] and Emery, Lees and Tootill[125] investigated conditions affecting the growth response to $B_{12}$ and found the $E_h$ of the medium to be critical, showing a mathematical relationship between the response of *L. leichmannii* and the depth of medium in the tube.

The official method in the U.S. Pharmacopoeia, 14th Revision, requires a medium incorporating ascorbic acid, as reducing agent, and tomato juice, which may provide a growth factor or additional reducing substances. Tomato juice is a variable entity, and certain batches may prove unsuitable. Emery *et al.*[125] found Crest brand (New Zealand) to be reliable, but other workers[120,121] have preferred to eliminate it from the medium.

The U.S. Pharmacopoeia method has been evaluated by Campbell *et al.*[126] A (3 + 3) design was used to assay the $B_{12}$ contents of two liver extracts and two concentrates from fermentation sources on five consecutive days. The levels of 0·02, 0·04 and 0·08 m$\mu$g of $B_{12}$ per 10 ml of medium were selected for the standard, eight replicate tubes of each dose of standard and three replicates tubes of each dose of appropriately diluted sample being used. All tubes were randomised before adding the basal medium and during autoclaving, incubation and titration, since it had been shown that position during these operations influenced the response. Titrations were made with bromothymol blue as indicator. The method was found to be precise and reproducible. The linear relationship between log dose and log response was valid and not influenced by the type of product tested. The statistical methods used are fully described. Using the assay over a period of many months, the authors found the limits of error to vary from $\pm 5$ to $\pm 10$ per cent. ($P = 0{\cdot}05$), but the method will sometimes yield atypical responses for unknown reasons.

The log/log relationship may also be applied to a 16- to 20-hour turbidimetric method with the U.S. Pharmacopoeia medium modified by omitting the tomato juice and reducing the concentration of glucose by 25 per cent. This application is also discussed by Wood.[127] The U.S. Pharmacopoeia method and two modifications of it were compared with *E. coli* mutant and *L. leichmannii* plate assays for the determination of $B_{12}$ in liver extracts.[128]

More sensitive methods with *L. leichmannii* have been described. Hoff-Jørgensen[129] developed a turbidimetric assay with A.T.C.C. 7830; he employed a standard range of 2·5 to 20·0 $\mu\mu$g of $B_{12}$ per ml of broth and so approached the sensitivity of the *E. gracilis* assay considered below. One advantage of increased sensitivity is that greater dilution of samples minimises interference by desoxyribosides. This improvement was achieved by replacing

$Na^+$ by $K^+$ and $NH_4^+$ and by the use of small volumes of medium in narrow tubes (100 mm × 8 mm internal diameter). Tomato juice was omitted from the medium, but thioglycollic and ascorbic acids were incorporated as reducing agents, and the medium contained potassium cyanide. With triplicate tubes of each of the six standard doses and four doses of sample, the standard deviation of repeated independent assays of several crude materials was found to be about 5 per cent.

Using *L. leichmannii* A.T.C.C. 4797 Rosenthal and Sarett[94] developed a sensitive titrimetric assay for determining $B_{12}$ in serum. They employed a modification of Thompson's medium[119] developed by Register and Sarett,[130] in which the ascorbic acid is added after the adjustment of the pH to 7·0. The standard range of 40 to 100 $\mu\mu$g of $B_{12}$ per tube permits sera to be diluted (1 + 10) during pre-treatment (see below).

Girdwood[131] also developed an assay sufficiently sensitive for sera. He further modified Thompson's medium by replacing the ascorbic and fumaric acids with thioglycollic acid. He determined the response turbidimetrically after overnight incubation.

Noer,[132] investigating the effects of omitting single constituents of several media used for *L. leichmannii*, found that several may be regarded as non-essential for a satisfactory growth response to $B_{12}$, but he has not been able to formulate a strictly synthetic medium.

*Uses and limitations of the* L. leichmannii *assay*—Of the microbiological assays the *L. leichmannii* tube method is probably the one most susceptible to unpredictable variations; hence the many published modifications. The medium is expensive and laborious to prepare. The method was widely used for several years when it was thought to be specific for $B_{12}$, but it is now known that analogues containing purines in the nucleotide can replace $B_{12}$ as growth factors for *Lactobacilli*. The modifications of the method with high sensitivity[94,130,132] provide a method for determining the $B_{12}$ content of sera and body fluids when the absence of $B_{12}$ analogues can be assumed. The short incubation period of 16 to 20 hours can be a considerable advantage over the 5 to 6 days needed for the *E. gracilis* assay.

*Escherichia coli* MUTANT PLATE AND TUBE ASSAY—

*E. coli* does not have a natural requirement for $B_{12}$, simple synthetic media being adequate to supply the nutritional needs of wild strains. In the hope of providing the basis for a simple microbiological assay for $B_{12}$, Davis and Mignioli[133] used the penicillin technique of Davis and Lederberg[134] to produce mutants of *E. coli* with an induced requirement for $B_{12}$. The isolate 113–3, A.T.C.C. 10799, A.T.C.C. 11105 and N.C.I.B. 8134 requires $B_{12}$ or methionine; it has proved a stable mutant and was found to give a graded dose response to $B_{12}$ (0·005 to 5·0 $\mu$g per ml) when grown in simple synthetic media in agar cup-plate assays.[135,136] The method was developed for use with large assay plates.[137] The addition to the agar of asparagine (4 g per litre) and of the amino acids used by Burkholder[138] at 10 mg per litre increases the density of the growth within the zones of exhibition. Zones obtained from methionine (at 1 mg per ml) are less dense and more diffuse than those obtained from much lower levels of $B_{12}$. The quantitative and qualitative effects of a number of possible interfering substances have been investigated.[139]

Burkholder[138] described the development of a turbidimetric assay with *E. coli* 113–3. By incubating assay tubes on a shaker, half-maximum level of growth was found at 0·12 m$\mu$g of $B_{12}$ per ml. Turbidimetric methods with simple synthetic media have been described,[140,141] but they are not suitable for the determination of $B_{12}$ in samples rich in amino acids. To assay sera, Grossowicz, Arnovitch and Rachmilewitz[142] supplemented the basal medium with methionine-free acid-hydrolysed caesin.

*Advantages and limitations of the* E. coli *mutant methods*—Factor B, which is $B_{12}$ without the nucleotide, and any of the authenticated analogues (containing either purines or benziminazoles in the nucleotide) but not desoxyribosides can replace the $B_{12}$-methionine requirement of the *E. coli* mutant 113–3. It is therefore the best test organism for bio-autographs.

The moderately sensitive *E. coli* mutant assays are economical of time and material, but discrimination in usage is essential.

*Euglena gracilis* METHODS—

Hutner *et al.*[77] found that $B_{12}$ and thiamine were essential for the growth of the algal flagellate *E. gracilis* var. *bacillaris*. It showed a growth response proportional to concentrations of 0·0015 to 0·15 m$\mu$g per ml of A.P.A. ($B_{12}$) in a simple basal medium containing salts of citric, butyric and glutamic acids, trace metals and thiamine chloride.

The assay method was developed by Ross[93] and by Robbins *et al.*[143,144] The former described in detail the *E. gracilis* method used to assay $B_{12}$ in serum and body fluids. The medium was slightly modified and incorporated a higher level of thiamine. Incubation of illuminated assay tubes for eight days was necessary for an optimal dose-response curve between levels of 1·25 to 50 $\mu\mu$g of $B_{12}$ per ml.

Heinrich and Lahann[145] evaluated various methods, such as cell count, turbidity and alkali titrations, for measuring the response of *E. gracilis* var. *bacillaris* to $B_{12}$ in a simple basal medium. Spectrophotometric determination of an extract of the chlorophyll formed gave greater sensitivity (0·001 $\mu\mu$g of $B_{12}$) and reproducibility.

Hutner, Bach and Ross[146] described an improved method with *E. gracilis Z* strain and an enriched medium. A denser growth response was obtained from both *Z* and *bacillaris* strains when sucrose and a dicarboxylic acid, together with sources rich in nitrogen (aspartic acid and ammonium ions), were incorporated in the basal medium. Under these conditions, the pH did not rise appreciably above the initial value of 3·6 during the growth of the culture, and adequately diluted sera showed no precipitation during the tests. The increased growth response to doses of 0·25 to 50 $\mu\mu$g of $B_{12}$ per ml was more marked from *E. gracilis Z* strain, which is therefore preferred for assays. Washing the inoculum increased the sensitivity and accuracy of assays, probably by removing a $B_{12}$-binding factor from the inoculum culture fluid. Incubation in an evenly illuminated water bath for 5 to 6 days was optimal. In a series of comparative assays, mean results from the old and new methods agreed to within 10 per cent. Recovery of $B_{12}$ added to serum and urine was good, but even with the improved method some variation was experienced within assay batches.[147]

Cooper[148] made a comparison of the *E. gracilis* var. *bacillaris* and *E. gracilis Z* strain methods for the assay of $B_{12}$ in serum. Both procedures were modified by the use of diluted but unwashed inocula. The serum $B_{12}$ values obtained from the *Z* strain method were consistently lower, though proportional to, those from the *bacillaris* strain, indicating non-specific growth response to serum protein by the latter.

Ford[80] obtained satisfactory results with *E. gracilis* incubated in the dark in the medium normally used for *O. malhamensis*.

The addition of cyanide to medium and samples does not appear to be necessary in *E. gracilis* assay methods. Hutner, Bach and Ross[146] suggest that, as light tends to remove CN from the $B_{12}$ molecule, *Euglena* utilises hydroxocobalamin.

Robbins, Hervey and Stebbins[149] developed a filter-paper disc assay, with standards of 0·001 to 0·1 $\mu$g of $B_{12}$ per ml on agar plates seeded with *E. gracilis* var. *bacillaris*, but 7 days' incubation in an illuminated temperature-controlled cabinet was necessary.

*Advantages and limitations of* E. gracilis *methods*—The great sensitivity of the *E. gracilis* tube method constitutes its main advantage. *E. gracilis*, unlike bacteria, is insensitive to antibiotics in, for instance, sera, and sulphonamide inhibition[150] can be overcome by adding *p*-aminobenzoic acid.

On the other hand, the long incubation period and the response of *Euglena* to some of the $B_{12}$ analogues are in its disfavour. This non-specificity is not a serious drawback for the assay of body fluids, in which $B_{12}$ analogues are normally not found.

*Ochromonas malhamensis* METHODS—

In their general survey of the use of protozoa in analysis, Hamilton, Hutner and Provasoli[151,152] drew attention to the unique position of certain photosynthetic chrysomonads, which are also phagotrophic, being able to utilise intact proteins. Since in a chemically defined medium their nutritional needs included $B_{12}$, thiamine, biotin and histidine, it was postulated that these micro-organisms might have a specific $B_{12}$ requirement similar to that of birds and mammals. The growth response to $B_{12}$ and thiamine was linear for a wide range of concentrations. The $B_{12}$ required could be spared, but not completely replaced by methionine.

From these observations Ford[80] developed a method suitable for the determination of $B_{12}$ in crude materials. To eliminate the growth stimulus sometimes observed from such samples and to obtain optimal responses in the dark, vitamin-free acid-hydrolysed casein was added to an enriched medium.

The test organism *Ochromonas malhamensis* (Pringsheim isolate) was maintained by transfer at 5-day intervals in a basal medium supplemented with 0·2 m$\mu$g of $B_{12}$ per ml incubated

at 27° C under a "Striplite" tungsten-filament lamp. One drop per assay tube of this culture was used as inoculum. Assay tubes were shaken at 28° to 30° C in darkness for 72 hours. A good dose-response curve was obtained from concentrations of 0·01 to 0·16 mμg of $B_{12}$ per ml.

The activities of several naturally occurring analogues were determined for *O. malhamensis*, *E. gracilis*, *L. leichmannii* and *E. coli*. Neither factors A, B, C nor *pseudo* vitamin $B_{12}$ could replace the $B_{12}$ required by *O. malhamensis*. All were utilised by *E. coli* and all except factor B by *L. leichmannii* and *E. gracilis*. The activities of the analogues expressed as $B_{12}$ varied according to the method or medium employed; moreover, unless two different substances, such as $B_{12}$ and factor B, show parallel dose-response curves, it is not legitimate to express the activity of one in terms of the other.

Ford compared the $B_{12}$ activity of some natural materials by the *O. malhamensis* and *E. coli* methods. High $B_{12}$ "values" from the *E. coli* method were found for samples known to contain analogues, whereas values by the two methods were in good agreement for samples known to contain mainly $B_{12}$. Fairly good recovery of added $B_{12}$ was obtained for all samples by both methods. This would normally be indicative of the validity of the assay and so illustrates the uselessness of such criteria when applied to non-specific methods.

Ford[153] found that the relation between the rate of growth and the concentration of cyanocobalamin is described by an equation in the form of an adsorption isotherm. The clinically inactive analogues were taken up by *O. malhamensis* to about the same degree as was cyanocobalamin; they inhibited competitively the growth response to cyanocobalamin, apparently by blocking a cell mechanism for binding the vitamin, but, since at least a 100-fold excess of *pseudo* vitamin $B_{12}$ was necessary for this interference, it is unlikely that, in general, determinations by the *O. malhamensis* method would give misleadingly low results.

*Advantages and limitations of the* O. malhamensis *method—O. malhamensis* shows to the $B_{12}$ vitamins a selective response similar to that of birds and mammals. Only factor I ($B_{12}$ III) of the known naturally occurring analogues can replace the $B_{12}$ required by this organism. Likewise, it is the only natural analogue found to be effective against pernicious anaemia in relapse.[154]

This organism provides the most selective microbiological method available for direct $B_{12}$ determination, and its use has therefore been recommended by the Analytical Methods Committee.[90] *O. malhamensis* responds to small doses of $B_{12}$; although unfamiliar to most bacteriologists, it is easily maintained in pure culture.

ASSAY METHODS WITH OTHER MICRO-ORGANISMS—

A method of $B_{12}$ determination with *Poteriochromonas stipitata* was described by Barber *et al.*[81] This chrysomonad shows a selective response to $B_{12}$ similar to that of *O. malhamensis*.

The use of other potential assay organisms, namely "Lochead 38," *Bacillus stearothermophilus* and *Phormidium persicinum*, has been reviewed by Ford and Hutner.[155]

## ASSAYS WITH HIGHER ANIMALS

It was observed during the second World War that laboratory animals and chickens fed on wholly vegetable diets failed to grow satisfactorily and that their young had an unusually high mortality. These effects persisted when the diets were supplemented with all the vitamins and essential nutrients then known, but could be prevented by the addition of animal protein. A search for the "animal protein factor" was attended by considerable difficulties over the development of an assay for the missing factor, since most young appeared to have considerable reserve of the factor accumulated during lactation or transmitted in the egg. Initially the young of depleted parents were required for assays of the factor, but it was later found that the rate of depletion could be accelerated by subjecting the animals to a "stress factor,"[156] such as could be induced by the excessive feeding of thyroid or iodinated casein combined with a high fat or other unbalanced diet.

In 1947, Erschoff[156,157] and Betheil, Wiebelhaus and Lardy[158] found that satisfactory growth of rats treated in this way could be restored by feeding whole liver; earlier, Rubin and Bird[159] had shown that dried cow manure provided the missing factor for chicks. After the isolation of $B_{12}$ from liver had been achieved, Ott, Rickes and Wood[160] and Nichol *et al.*[161] found that addition of $B_{12}$ to a deficient diet counteracted the thyrotoxic effect of iodinated casein and could replace completely the animal protein factor activity of condensed "fish solubles" and of injectable liver preparations. Erschoff[162] also observed that depletion of

$B_{12}$ in rats could be accelerated by a high-lactose diet, and Cuthbertson and Thornton[163] subsequently developed an assay based on this observation.

Apart from the necessity for obtaining test animals with adequate $B_{12}$ depletion, other difficulties beset assays of this type. Considerable quantities of $B_{12}$-like substances are synthesised in the gut, from which direct absorption is possible. The animals must not have access to their own droppings. Some substances, particularly methionine, appear to have a $B_{12}$-sparing effect, and the presence of any antibiotic in the diet may have a further disturbing influence. Although they are costly, time-consuming and subject to large errors, animal assays have a great advantage over microbiological methods for the assay of natural products, in that they measure only cobalamins. The one known exception is $B_{12}$ III, which, although claimed to be effective clinically in man, has only about one-twentieth of the activity of $B_{12}$ in chicks. Other factors do not interfere, provided that no antagonism to or potentiation of the cobalamins occurs.

For animal tissues, in which $B_{12}$ activity is almost entirely due to cobalamins, good agreement between microbiological and animal methods is attained, but for products of microbiological origin, in which other factors may predominate, the discrepancies found will depend on the micro-organism selected for assay and can be large. By a suitable combination of the physical and microbiological methods now available for $B_{12}$, it is generally possible to identify the $B_{12}$-like compounds present in any given material and to determine approximately the content of each. All the same, care must be exercised in any attempt to interpret the results in terms of "$B_{12}$ activity" for a particular animal species. Not only do the various factors have different activities for different organisms, both higher and lower, but the form in which the $B_{12}$-activity is bound may govern its availability to a particular species. With the introduction of commercial animal-feed concentrates containing $B_{12}$, which may be derived from a variety of materials and added in relatively crude form, a claim for $B_{12}$-activity for a specified animal species is best supported by reference to the results of adequate feeding experiments with that species.

A great many varied biochemical, nutritional and metabolic studies on $B_{12}$-like compounds have been made with animals; these and other applications have been reviewed by Coates *et al.*[164,165] and by Johnson,[166] who give many additional references.

## Clinical assays

The effect of administering $B_{12}$ in cases of anaemia associated with deficiency of the vitamin is two-fold. It results in an increase in the percentage of blood reticulocytes, lasting for about 5 days, and in a slow continuous rise in the total number of red cells until normal values are reached. Vitamin $B_{12}$ is effective both by injection and orally, but much smaller doses are required for a given response by injection, and this is the route usually preferred in the treatment of anaemias. Absorption of $B_{12}$ after oral administration is less certain, even with the necessarily higher dosage, particularly in cases associated with insufficiency of intrinsic factor, normally secreted in adequate amounts by the stomach mucosa and upon which proper absorption appears to depend.

The activity of materials containing $B_{12}$ may be estimated by measuring the increase in red blood cells and the volume of packed cells in cases of pernicious anaemia in relapse 15 days after the injection of a single dose equivalent to 10 to 160 $\mu$g of $B_{12}$. Over this range the effect is proportional to the logarithm of the dose, but it varies from patient to patient and depends to some extent on the initial red-cell count. As a method of assay, clinical response is cumbersome and inaccurate; nevertheless, before the development of microbiological methods it provided the only guide during the early work on the isolation of $B_{12}$ from liver. Of the natural $B_{12}$-like substances, only the cobalamins and $B_{12}$ III (factor I) have the haematological activity typical of $B_{12}$ itself. However, the many unnatural analogues that can be made biosynthetically require careful clinical evaluation, as also do those compounds now known to have antagonism to $B_{12}$.

Many aspects of the physiological effects and of the therapeutic uses of $B_{12}$ have been covered by Ungley[167] and by the papers presented at the Hamburg Symposium (1956).[4]

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# The Presumptive Detection of Preservatives in Canned Cured-meat Products by a Simple Yeast-growth Test

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A simplified test for detecting preservatives in processed cured-meat products, such as canned ham, is described. Two portions of the product are separately homogenised in a mixer with double-strength agar, and the pH values of the mixtures are adjusted to 3·6 and 7·0. The plates are then inoculated in streaks with a sodium chloride-tolerant yeast (*Candida brumptii*) and incubated for about 24 hours at 30° C. Absence of extensive growth of the test strain on the food - agar mixture indicates the presence of added antimicrobial agents, provided that a blank experiment carried out with the same type of food known to be free from added preservatives exhibits copious development of the yeast.

The proposed test is generally more sensitive than the fermentation test previously described by Mossel and is far more simple. Natural or authorised added constituents of cured-meat products, including phosphates, do not give rise to falsely positive results for preservatives.

A few years ago a test was described for detecting preservatives in meat products, etc.; an acid and an alkaline extract of the food product were tested for the presence of antimicrobial agents by adding dextrose and sufficient cells of *Saccharomyces cerevisiae* to ensure, for unpreserved samples, copious fermentation within 48 hours' incubation at 25° C.[1] This test was entirely satisfactory in that it was sufficiently sensitive for the purpose and never led to falsely positive results. However, the technique was found to be somewhat cumbersome for use in smaller laboratories, as rather elaborate preparation of the extracts was necessary before the test was begun. When sufficient experience had been obtained with a recently developed simple growth test for detecting antibacterial antibiotics in foods, we therefore attempted to apply the same principle to test for preservatives in foods. In the former test the sample is homogenised with double-strength agar and is then inoculated in streaks with the bacteria used for the routine detection of antibiotics.[2]

A prerequisite for the use of this principle in detecting preservatives in meat products is that the test organism used must be tolerant to curing salts in the concentrations present in such products and must also be sufficiently sensitive to detect the preservatives, which are occasionally used without authorisation. Several test organisms regularly used in this Institute for the tentative characterisation of antimicrobial agents detected in foods were tested for suitability. These organisms were *Staphylococcus aureus, Streptococcus cremoris, Streptococcus faecalis, Lactobacillus plantarum, Lactobacillus casei, Bacillus cereus, Clostridium perfringens, Pseudomonas aeruginosa, Pseudomonas fluorescens, Acetobacter aceti, Serratia marcescens, Escherichia coli, Aerobacter aerogenes, Salmonella typhimurium, Proteus vulgaris, Saccharomyces cerevisiae, Candida brumptii, Neurospora sitophila, Aspergillus niger, Aspergillus oryzae, Fusarium lini, Mucor racemosus* and *Penicillin expansum*; *C. brumptii*[3] appeared to be the most sensitive of those organisms that developed copiously on mixtures of meat products and agar.

In order to detect hexamine and acid preservatives, such as benzoic and sorbic acids,[4] the pH had to be fixed at as low a value as could be consistently tolerated by the test strain; this value was found to be 3·6. A parallel test[4] at pH 7·0 appeared to be necessary to detect the presence of boric acid, another occasionally used preservative.

## Method

### Substrate—

Prepare an agar containing 2 g of dextrose, 6 g of meat-extract paste, 10 g of powdered yeast extract and 30 g each of tryptone, dehydrated peptonised milk and agar in 1 litre of distilled water, and dissolve the ingredients by heating. If prepared for immediate use, this medium need not be sterilised, as the products to be tested will not usually be sterile.

By using a motor-driven mixer, homogenise an approximately 50-g sample of the meat product with 50 ml of the agar, heated (or cooled) to 60° C, and with sufficient of a sterile 10 per cent. solution of sodium hydroxide to bring the pH of the mixture to 7·0 ± 0·2. Similarly homogenise a second 50-g sample with 50 ml of the agar and sufficient of a sterile 25 per cent. solution of tartaric acid to bring the pH to 3·6 ± 0·1.

Rapidly pour the viscous mixtures so obtained into Petri dishes, allow to solidify, and after solidification invert the layers of agar so that the smooth surfaces are uppermost. Dry the uncovered plates for about 1 hour at approximately 35° C.

INOCULUM—

Prepare a subculture of *C. brumptii* by painting a streak on tubes containing an agar prepared from 1 litre of distilled water, 5 g of powdered yeast extract, 20 g of dextrose, 60 g of sodium chloride and 15 g of agar and incubating for about 24 hours at 30° C. Emulsify a loopful of this subculture in sterile saline to obtain a density of the order of $10^6$ viable cells per ml.

INOCULATION AND INCUBATION—

By using an inoculating needle about 3 mm in diameter, paint on the prepared plates two streaks, each about 2 cm long, in the shape of a V. Cover the plates with lids containing discs of filter-paper about 8 cm in diameter and moistened with anhydrous glycerol.[2] Incubate at 30° ± 2° C for about 24 hours, and check the growth on the streaks with the naked eye.

BLANK TEST—

Carry out the procedure described above with a sample of exactly the same type of meat product, but one known to be free from added preservatives. If necessary, adjust the concentrations of curing salts in the blank to about the same levels as those in the test sample.

## DISCUSSION OF RESULTS

The results found for a few preservatives and one antifungal antibiotic (pimaricin[5]) are shown in Table I. For comparison, the levels of detection of the same antimicrobial agents found by the previously described fermentation test[1] are also shown. The hams to which these preservatives were added were of standard Dutch type, having pH values between 5·5 and 6·5 and containing less than 6 per cent. of sodium chloride, less than 0·2 per cent. of potassium nitrate and less than 0·02 per cent. of sodium nitrite. The level of detection is defined as the concentration of antimicrobial agent in the meat product that gives rise to no growth or at best to a few punctiform colonies on plates inoculated with *C. brumptii*, copious development of the yeast being observed in a blank experiment. The results in Table I show that the proposed test is generally more sensitive than the fermentation test.

### TABLE I

### LEVELS OF DETECTION OF PRESERVATIVES IN HAM

*C. brumptii* was used in the proposed test and *S. cerevisiae* in the fermentation test

| Antimicrobial agent | Level of detection by— proposed test, % | fermentation test, % |
|---|---|---|
| Boric acid .. .. .. | $5 \times 10^{-1}$ | $5 \times 10^{-1}$ |
| Hexamethylenetetramine .. | $5 \times 10^{-2}$ | $1 \times 10^{-1}$ |
| Benzoic acid .. .. .. | $5 \times 10^{-2}$ | $1 \times 10^{-1}$ |
| Ethyl monobromoacetate .. | $5 \times 10^{-4}$ | $1 \times 10^{-3}$ |
| Sorbic acid .. .. .. | $2 \times 10^{-2}$ | $1 \times 10^{-1}$ |
| Pimaricin .. .. .. | $5 \times 10^{-3}$ | $2 \times 10^{-3}$ |

It was ascertained that exceptionally high concentrations of meat-curing salts—0·2 per cent. of potassium nitrate and 0·02 per cent. of sodium nitrite—did not give rise to positive results for preservatives by causing inhibition of growth under the specified conditions. However, the presence of some naturally occurring substances might wrongly suggest the presence of added antimicrobial agents. For example, lactic acid[6] or some unknown product formed from constituents of muscle during the curing process might be sufficiently antimicrobial to inhibit growth of *C. brumptii*. To investigate this possibility, about twenty

samples of ham, some bought in the open market and some prepared in the Institute's laboratory of meat technology, were studied. Tests carried out with these samples appeared to be exact duplicates of each other; hence it is unlikely that natural variation between individual samples will cause interference with the test.

The use of various phosphates as additives to cured-meat products has recently been suggested.[7] Five brands of such phosphates were added to ground ham in concentrations up to 0·3 per cent., calculated as $P_2O_5$. None of these phosphates inhibited or even retarded the growth of *C. brumptii* under the conditions of the test.

To summarise, the proposed test is more sensitive and far more simple than the fermentation test,[1] as well as being equally reliable; its use is therefore recommended.

We thank our colleague, Mr. B. Krol, M.Sc., for preparing hams and for various valuable suggestions.

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# An Examination of the Occurrence of Honeydew in Honey

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Honey containing honeydew, the sweet and sticky excretion of certain leaf-sucking insects, is not a safe food for bees; a simple means of distinguishing honeydew from floral honey is described.

Forty-two honeys were analysed for moisture, colloid, nitrogen, dextrin, reducing sugars, free acidity and ash, and values for specific rotation and pH were measured. The experimental results were subjected to discriminatory analysis, and a linear discriminant function was evolved, based on the results for ash, pH and reducing sugars; the numerical value of this function for any sample serves to classify the sample as floral or honeydew in origin. Sixteen of the samples tested were found to contain honeydew.

Plants normally produce sufficient nectar to supply the bee colony with enough honey to ensure its survival, but in periods of prolonged drought the flow of nectar greatly diminishes, and the bees must augment the supply of honey from supplementary sources of sugar. Under such conditions, they will often collect honeydew, a sweet and sticky fluid excreted on foliage by leaf-sucking insects, mainly aphids and scale insects. Several workers have analysed honeydew, and its composition has been found to vary greatly with the plant and insect producing it. Schoofs,[1] Gontarski[2] and Mawson[3] found sucrose, glucose, fructose, water and dextrin to be the main constituents, but Tanret[4] and Nottbohm and Lucius[5] found that some samples contained large amounts of the trisaccharide melezitose. Smaller amounts of mineral ash were found by Büttner[6] and Ermin,[7] and various acids, amino acids and phosphates by Gray and Fraenkel,[8] Gray,[9] Mittler[10] and Fuchs and Pöhm[11]; several complex sugars were found by Wolf and Ewart.[12]

The collection of honeydew is a nuisance to beekeepers, since the honey then produced is often too dark and rank-flavoured for sale, and, more important, bees feeding on it are greatly weakened or killed. According to Temnov,[13] the harmful constituents appear to be in the mineral matter, and Konoplev[14] has shown that the high pH produced by the buffer action of these minerals may be harmful to bees and has described a method of counteracting this effect by artificially decreasing the pH of the honey.

## Table I

### Values found for various honeys

| Sample No. | Type of honey* | Locality | Suggested source | Approximate date of extraction | Colour | State | Flavour | pH | Ash on dry material, % | Dextrin on dry material, % | Specific rotation, $\alpha_D^{20}$ degrees | Colloid on dry material, % | Moisture content, % | Reducing sugars on dry material, % | Nitrogen on dry material, % | Free acidity (titre of 0·1 *N* NaOH per 100 g), ml | Value of discriminant function (*X*) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1E† | F | Roch, Pembrokeshire | — | 1958 | Yellowish cream | Medium granulation | Very sweet | 3·65 | 0·13 | 1·94 | −9·29 | 0·44 | 19·7 | 90·6 | 0·08 | 40 | 92·79 |
| 18D† | F | Kilgetty, Pembrokeshire | — | 1957 | Creamy white | Very fine granulation | Very sweet | 3·81 | 0·12 | 2·01 | −5·57 | 0·29 | 16·0 | 87·5 | 0·05 | 26 | 87·32 |
| 3E | F | Spittal, Pembrokeshire | — | 1958 | Yellowish cream | Medium granulation | Bittersweet | 3·82 | 0·17 | 2·04 | −8·13 | 0·24 | 22·3 | 94·3 | 0·07 | 38 | 95·98 |
| 19D† | F | Roch, Pembrokeshire | — | 1957 | Cream | Fine granulation | Very sweet (fruity) | 3·82 | 0·12 | 2·56 | −10·43 | 0·28 | 18·0 | 88·1 | 0·07 | 28 | 88·06 |
| 6D | F | St. Ishmaels, Haverford West, Pembrokeshire | Mustard and ivy | August, 1957 | Light cream | Coarse granulation | Very sweet | 3·87 | 0·13 | 3·81 | −8·89 | 0·43 | 20·6 | — | — | — | — |
| 4D† | F | Tenby, Pembrokeshire | — | 1957 | Creamy white | Very coarse granulation | Sweet | 3·90 | 0·15 | 2·13 | −9·04 | 0·23 | 17·9 | 94·9 | 0·13 | 20 | 96·39 |
| 63C | F | House compound, Kandy, Ceylon | — | 1956 | Dull cream | Beginning to granulate; very fine granules | Citrus fruit | 3·93 | 0·41 | 2·71 | −12·48 | 0·05 | 17·8 | — | — | — | — |
| 8D | F | Reading, Berkshire | Waste marshmallow | October, 1957 | Pink | Medium granulation | Strawberry | 3·95 | 0·33 | 5·22 | −2·02 | 0·56 | 18·0 | 87·8 | 0·18 | 41 | 83·99 |
| 20D | F | Cornell University, New York State, U.S.A. | Golden rod (fall flower) | October, 1956 | Orange | Coarse granulation | Strong | 3·95 | 0·38 | 2·74 | −5·12 | 0·75 | 21·7 | 90·6 | 0·15 | 64 | 87·23 |
| 36D† | F | Tauranga, New Zealand | Rewarewa (*Knightia excelsa*) | 1957 | Brownish amber | Liquid | Strong (sweet) | 4·00 | 0·69 | 2·18 | −6·96 | 0·62 | 16·0 | 87·4 | 0·10 | 56 | 78·61 |
| 35D† | F | Wellington, New Zealand | Manuka (*Leptospermum scoparium*) | 1957 | Dark orange | Very coarse granulation | Objectionable (burnt sugar) | 4·12 | 0·34 | 2·38 | −20·38 | 0·27 | 18·6 | 90·7 | 0·06 | 36 | 86·45 |
| 23D† | F | Richville, Minnesota, U.S.A. | Buckwheat | 1956 | Dark orange | Coarse granulation | Caramel | 4·13 | 0·49 | 2·28 | −4·26 | 0·76 | 18·4 | 89·5 | 0·09 | 56 | 82·88 |
| 62C | F | Botanic Gardens, Peradeniya, Ceylon | — | 1956 | Yellowish cream | Beginning to granulate; coarse granules | Sickly sweet | 4·17 | 0·45 | 2·02 | −12·80 | 0·52 | 17·3 | — | — | — | — |
| 9D† | F | Gravesend, Kent | Near sugar dump | 1957 | Buff | Coarse granulation | Bittersweet | 4·19 | 0·34 | 1·98 | −4·59 | 0·32 | 16·8 | 88·9 | 0·16 | 32 | 83·39 |
| 31D | F | New York State, U.S.A. | Buckwheat | 1956 | Buff | Beginning to granulate; fine granules | Caramel | 4·24 | 0·28 | 2·24 | −12·48 | 0·78 | 18·2 | 87·6 | 0·14 | 46 | 81·92 |
| 32D† | F | New York State, U.S.A. | Buckwheat | 1957 | Dark orange | Coarse granulation | Caramel | 4·24 | 0·31 | 1·83 | −9·39 | 0·73 | 19·1 | 90·2 | 0·17 | 44 | 85·13 |
| 3D | F | Dublin, Eire | Honeydew | September, 1957 | Black with greenish tint | Liquid | Fairly sweet | 4·26 | 0·23 | 2·82 | −2·24 | 0·39 | 15·8 | — | — | — | — |
| 34D† | F | Wellington, New Zealand | Rata (*Metrosideros umbellata*) | 1957 | Creamy white | Very fine granulation | Very sweet | 4·27 | 0·44 | 1·80 | −10·02 | 0·44 | 16·8 | 92·0 | 0·16 | 40 | 85·77 |
| 13D | F | Bristol, Gloucestershire | Honeydew or molasses store | 1951 | Dark brown | Very coarse granulation | Objectionable (burnt sugar) | 4·28 | 0·69 | 1·95 | −7·16 | 0·42 | 18·4 | 85·5 | 0·13 | 58 | 73·67 |
| 2E | F | Valley Narbeth, Pembrokeshire | — | 1958 | Light amber | Medium granulation | Very sweet | 4·35 | 0·46 | 2·36 | −6·85 | 0·47 | 19·0 | 91·1 | 0·10 | 30 | 83·62 |
| 5D | F | St. Ishmaels, Haverford West, Pembrokeshire | Sycamore | 1954 | Light amber | Beginning to granulate; coarse granules | Heather | 4·37 | 0·79 | 1·85 | −5·58 | 1·51 | 17·3 | — | — | — | — |
| 2D | F | Hawick, Roxburghshire | Spruce and sycamore | August, 1957 | Dark cream | Medium granulation | Very sweet | 4·38 | 0·22 | 1·99 | −9·08 | 0·31 | 17·1 | 94·4 | 0·08 | 30 | 90·85 |
| 14D | F | Greenock, Renfrewshire | Conifers | August, 1957 | Buff | Fine granulation | Very sweet | 4·38 | 0·51 | 4·20 | −5·93 | 0·38 | 18·6 | 87·1 | 0·10 | 42 | 77·25 |
| 11D‡ | H | Palmers Green, Middlesex | Honeydew | 1949 | Dark brown | Beginning to granulate; coarse granules | Objectionable | 4·42 | 1·01 | 3·32 | −5·14 | 0·21 | 19·9 | 81·4 | 0·17 | 58 | 62·94 |
| 15D | H | Greenock, Renfrewshire | Heather and molasses | September, 1957 | Buff | Very fine granulation | Very sweet | 4·48 | 0·59 | 4·01 | −5·97 | 0·86 | 17·6 | — | — | — | — |
| 27D | H | Corbally, Limerick | Lime and oak | 1957 | Cream | Fine granulation | Very sweet | 4·49 | 0·27 | 3·20 | +3·15 | 0·49 | 12·6 | — | — | — | — |
| 21D‡ | H | White River Junction, Vermont, U.S.A. | Honeydew | August, 1956 | Brown | Liquid | Objectionable (sweet) | 4·50 | 0·63 | 4·54 | +4·79 | 0·75 | 17·7 | 77·3 | 0·08 | 56 | 61·29 |
| 10D | H | Brasted, Westerham, Kent | Oak | October, 1957 | Dark cream | Very fine granulation | Fairly sweet | 4·53 | 0·40 | 4·10 | −3·14 | 1·68 | 15·3 | — | — | — | — |
| 33D | F | Perthshire | — | 1957 | Khaki | Beginning to granulate; fine granules | Heather | 4·60 | 0·40 | 1·70 | −7·91 | 0·20 | 15·7 | 85·0 | 0·08 | 36 | 73·88 |
| 24D‡ | H | Laurel, Garret County, Maryland, U.S.A. | Buckwheat | August, 1956 | Dark brown | Liquid | Objectionable (burnt sugar) | 4·62 | 0·85 | 3·57 | +2·63 | 0·59 | 18·3 | 79·6 | 0·11 | 52 | 60·77 |
| 12D‡ | H | Watford, Hertfordshire | — | 1949 | Dark brown | Liquid | Treacle | 4·63 | 1·02 | 5·20 | +7·30 | 0·22 | 18·3 | 77·4 | 0·16 | 58 | 55·57 |
| 25D | F | Catskill Mountains, New York State, U.S.A. | Wild thyme | 1957 | Yellowish cream | Very fine granulation | Very sweet | 4·68 | 0·50 | 2·62 | −9·59 | 0·49 | 18·0 | 89·3 | 0·07 | 42 | 77·91 |
| 22D‡ | H | Hamilton City, California, U.S.A. | Oak honeydew | October, 1956 | Brown | Liquid | Very sweet | 4·69 | 0·79 | 2·94 | −8·89 | 0·89 | 14·5 | 80·5 | 0·21 | 78 | 62·16 |
| 29D | H | Manton, Sierra Nevada Mts, California, U.S.A. | Incense cedar | September, 1957 | Dark orange | Fine granulation | Slightly objectionable (bitter) | 4·70 | 1·04 | 17·78 | +23·82 | 0·45 | 15·6 | 69·1 | 0·07 | 76 | 43·32 |
| 26D | H | Catskill Mountains, New York State, U.S.A. | Wild thyme | 1957 | Yellowish cream | Very fine granulation | Very sweet | 4·80 | 0·67 | 2·83 | −10·36 | 0·48 | 18·0 | 87·7 | 0·07 | 40 | 72·63 |
| 1D | F | Inverness, Scotland | Heather | 1957 | Deep orange | Liquid | Heather | 4·85 | 0·93 | 3·12 | −11·68 | 4·28 | 16·4 | — | — | — | — |
| 28D‡ | H | Cheltenham, Gloucestershire | Lime | 1956 | Greenish brown | Coarse granulation; fermenting | Sweet | 5·03 | 0·86 | 4·08 | −4·31 | 0·48 | 19·1 | 84·4 | 0·13 | 46 | 63·84 |
| 16D‡ | H | Switzerland | Fir (*Abies alba*) | 1957 | Brownish amber | Fine granulation | Fairly sweet | 5·14 | 0·94 | 6·38 | +14·00 | 0·12 | 14·1 | 73·1 | 0·06 | 42 | 46·40 |
| 17D‡ | H | South Switzerland | Sweet chestnut (*Castanea sativa*) | 1957 | Amber | Liquid | Very objectionable (bitter) | 5·15 | 1·28 | 2·71 | −3·99 | 0·48 | 15·7 | 81·0 | 0·12 | 38 | 53·02 |
| 7D‡ | H | Old Windsor, Berkshire | Near river | 1957 | Khaki | Fine granulation | Bittersweet | 5·21 | 0·93 | 3·59 | −4·08 | 0·51 | 17·2 | 82·1 | 0·16 | 60 | 58·33 |
| 30D‡ | H | Corning, Sacramento Valley, California, U.S.A. | Oak gall honeydew | September, 1957 | Dark amber | Liquid | Very sweet | 5·24 | 0·95 | 2·91 | −6·03 | 0·62 | 15·8 | 77·8 | 0·23 | 80 | 51·91 |
| 64C | H | Hakgala gardens, N'Eliya, Ceylon | — | 1956 | Orange | Coarse granulation | Fairly sweet (sickly) | 5·59 | 1·27 | 2·79 | −7·44 | 0·10 | 17·6 | — | — | — | — |

* Floral honey = F; honeydew honey = H.
† Sample of floral honey used in deriving equation for discriminant function (see Appendix, p. 415).
‡ Sample of honeydew honey used in deriving equation for discriminant function (see Appendix, p. 415).

Since the obvious method of preventing harm to the bees is to remove honeydew honey from the hive, its identification is important. As well as by its dark colour and rank flavour, it can be distinguished from floral honey by its higher contents of ash, dextrin,[15] sucrose[16] and reducing sugars,[17] its higher pH[14,18] and free acidity[18] and its dextro-rotation, compared with the laevo-rotation of floral honey.[19,20] In this paper the examination of the relative values of these properties for detecting honeydew in honey is described.

## Methods

### Ash, colloid, pH and free acidity—

The cleaning of samples and determinations of sulphated ash, colloid, pH of a 10 per cent. solution and free acidity by titration against 0·1 *N* sodium hydroxide were carried out as described previously.[21]

### Nitrogen—

Nitrogen was determined by a modification[21,22] of Hitchcock and Belden's[23] semi-micro Kjeldahl method.

### Reducing sugars—

Lane and Eynon's volumetric method,[24] involving reduction of Fehling's solution and the use of methylene blue as internal indicator, was used.

### Moisture—

Although Wedmore[25] has shown that the refractive-index method when applied to buckwheat and honeydew honeys can give moisture contents up to 0·5 per cent. low, he also notes that the standard A.O.A.C. vacuum-drying method used by Chataway[26] can give errors up to ±1 per cent. unless the analyst has special experience with honey. The refractive-index method used by Mitchell, Donald and Kelso[21] was therefore used, and the values of refractive index were converted to moisture contents by using corrected forms of Chataway's tables.[25]

### Specific rotation—

Approximately 10 g of honey were weighed accurately and dissolved in 50 ml of distilled water. The solution was made up to 100 ml with distilled water in a calibrated flask, and mutarotation was brought to completion by setting aside for 24 hours. The solution was then clarified by means of a diatomaceous-earth filter aid and a Whatman No. 42 filter-paper, and the polarisation was measured in a 200-mm tube at 20° C, a Hilger and Watts triple-field polarimeter with sodium-vapour lamp illumination being used.

### Dextrin—

The method used was based on those described by Browne and Zerban for determining dextrin in honey[27] and by Ruff and Withrow for dextrin in cane sugar.[28]

Approximately 5 g of honey were weighed accurately into a 250-ml beaker and dissolved in 5 ml of distilled water. The solution was acidified with 0·5 ml of concentrated hydrochloric acid, and 50 ml of absolute ethanol were added, dropwise, from a burette, with continuous and vigorous stirring; the precipitate was allowed to settle overnight. A further 10 ml of absolute ethanol were added to test for completeness of precipitation, and the precipitate was then separated on a Whatman No. 42 filter-paper and washed with five 20-ml portions of absolute ethanol. The filter-paper was dried to constant weight in a vacuum oven at 40° C in a vacuum of 29 inches of mercury. The filter-paper was then washed with three 20-ml portions of boiling distilled water and dried to constant weight as before, the difference in weight being taken as the amount of dextrin in the sample. (Possible occlusions of sugar and ash in the precipitate[27] were neglected, as the test was only intended to compare results for honeydew and floral honeys.)

## Results

The results of the various tests, arranged in order of increasing pH, are shown in Table I; when no value is given, the determination could not be made because of the smallness of the sample. Table II shows the ranges of values obtained in some of the tests.

Moisture contents ranged from 12·6 to 22·3 per cent. (average 17·5 per cent.) and gave no indication of the source of the honey.

Colloid contents also gave little indication of the type of honey, but it was noted that, although the colour of the floral honeys deepened as the colloid content increased, there was no corresponding change in colour for honeydew honeys. For samples having the same colloid content, honeydew honeys were much darker, possibly indicating that less of their colouring matter was present in a colloidal state.

TABLE II

RANGES OF VALUES FOR FLORAL AND HONEYDEW HONEYS

| | Floral honey | | | Honeydew honey | | |
|---|---|---|---|---|---|---|
| | Minimum | Maximum | Average | Minimum | Maximum | Average |
| Colloid content, % .. .. | 0·05 | 4·28 | 0·62 | 0·10 | 1·68 | 0·56 |
| Nitrogen content, % .. .. | 0·05 | 0·18 | 0·11 | 0·07 | 0·22 | 0·13 |
| Dextrin content, % .. .. | 1·70 | 5·22 | 2·57 | 2·71 | 17·78 | 4·62 |
| Specific rotation, degrees .. | −20·38 | −2·02 | −8·35 | −10·36 | +23·82 | −0·17 |
| Free acidity, ml of 0·1 *N* NaOH per 100 g .. .. .. | 20 | 64 | 40 | 38 | 80 | 57 |
| Reducing-sugar content, % .. | 85·0 | 94·9 | 89·6 | 69·1 | 87·7 | 79·3 |
| Ash content, % .. .. | 0·12 | 0·93 | 0·40 | 0·27 | 1·28 | 0·84 |
| pH .. .. .. .. | 3·65 | 4·85 | 4·13 | 4·42 | 5·59 | 4·83 |

Nitrogen contents showed too little variation to allow good differentiation between the two types of honey.

The dextrin contents found for honeydew honeys, greater than those of the floral honeys, are in agreement with Johnson, Wright and Chapman's results.[15] (This test was of limited use in detecting honeydew, because of the overlap in the ranges of values.) The high dextrin contents of a few of the floral honeys, which may lead to their mis-classification as honeydew honeys, may have been due to precipitation by the ethanol of substances other than dextrin, present owing to adulteration of the honey by the bees having collected molasses, jam, etc.

Although the free acidity of the honeydew honeys was found to be greater than that of the floral honeys, the difference in free acidity between the two types of honey was much less than that reported by Chistov,[18] who found that the free acidity of honeydew honey was 3·3 times that of floral honey.

Specific rotation was of limited application in detecting honeydew in honey, since, although all the dextro-rotatory honeys were found to contain honeydew, all the honeydew honeys were not dextro-rotatory, and a laevo-rotatory honey might or might not contain honeydew.

The reducing-sugar contents of the honeydew honeys were markedly lower than those of the floral honeys; this agrees with results previously found for some American honeys.[17]

The higher pH values of the honeydew honeys, as compared with those found for the floral honeys, were consistent with the ranges of values found by Temnov[13] and Chistov[18] for some Russian honeys.

The ash contents of the honeydew honeys were appreciably higher than those of the floral honeys, the ranges of values found for both types being rather higher than those found by previous investigators.[15,16] There appeared also to be an over-all deepening in colour as the ash content increased, irrespective of the source of the honey, an effect previously noted by Schuette and Remy.[29]

Since it was apparent that neither qualitative tests, such as colour, state or flavour, nor any of the quantitative tests described above, because of overlapping in the ranges of values for the two types of honey, could differentiate between floral and honeydew honey with certainty, the results were subjected to the statistical process of discriminatory analysis.[30] The general object of such an analysis is to find the rule for assigning individual samples to their correct groups, so as to make as few mistakes as possible over a large number of samples.

For our purpose, a linear function, $X$ (the discriminant function), of the nine quantitative analytical characteristics shown in Table I was sought, the condition being that the function should provide as efficient a criterion as possible for distinguishing between floral and honeydew honeys. It is usually found in such instances that only two or three of the analytical characteristics need be combined to form the discriminant function, extension beyond this

number merely increasing the complexity of the function without correspondingly increasing its discriminatory power. Mathematical investigation showed that an efficient discriminant function was given by the equation—

$$X = -8{\cdot}3x_1 - 12{\cdot}3x_2 + 1{\cdot}4x_3,$$

in which $x_1$ is the pH, $x_2$ is the percentage of ash and $x_3$ is the percentage of reducing sugars (for the derivation of this equation, see Appendix below). The mean values of $X$ for authentic floral honeys and honeydew honeys are 86·7 and 57·6, respectively.

In practice, therefore, any honey found to have a value of $X$ greater than 73·1 (see Appendix) may be classified as floral, and a honey having a value less than 73·1 may be classified as honeydew; the lower the value of $X$, the greater the amount of honeydew likely to be present in the sample. Confirmatory evidence, which may be used in detecting honeydew in honeys having values of $X$ close to 73·1, is the possession of a dark colour, an unpleasant flavour, dextro-rotation, a high dextrin content or a high value for free acidity. The high colloid content of heather honey may be used to distinguish it from honeydew honey.

We gratefully acknowledge the valuable help given by many beekeepers, who supplied the samples of honey, and in particular by Dr. E. P. Jeffree, Aberdeen University, who arranged for the collection of samples. We also thank Mr. C. M. Fullarton for practical assistance.

## APPENDIX

### Derivation of discriminant function

Ten samples each of honeydew and floral honey ($N_H$ and $N_F$, respectively) were chosen at random. The mean value of each variate was calculated for each group, the units being those stated in Table I.

| Variate | Value for floral honey (A) | Value for honeydew honey (B) | Value of (A–B) |
|---|---|---|---|
| $x_1$ | 4·013 | 4·863 | −0·850 |
| $x_2$ | 0·313 | 0·926 | −0·613 |
| $x_3$ | 89·98 | 79·46 | 10·52 |

The matrix of the sum of the squares and products about the means was—

| | $x_1$ | $x_2$ | $x_3$ |
|---|---|---|---|
| $x_1$ | 0·073223 | 0·022885 | 0·028100 |
| $x_2$ | — | 0·032436 | −0·018278 |
| $x_3$ | — | — | 7·650000 |

where the number in the $i$th column of the $j$th row is—

$$_{H,F}\Sigma \frac{(x_i - \bar{x}_i)(x_j - \bar{x}_j)}{N_H + N_F - 2}.$$

The inverse of the above matrix is—

| | $x_1$ | $x_2$ | $x_3$ |
|---|---|---|---|
| $x_1$ | 17·5881 | −12·4623 | −0·0944 |
| $x_2$ | — | 39·7019 | 0·1406 |
| $x_3$ | — | — | 0·1314 |

The discriminant function, $X$, is given by the expression—

$$X = \sum_{i=1}^{p} l_i x_i,$$

in which $p$ is the number of variates and the coefficient $l_i$ is given by—

$$l_i = \sum_{j=1}^{p} a^{ij}(\bar{x}_{Fj} - \bar{x}_{Hj}),$$

where $a^{ij}$ is the number in the $i$th row of the $j$th column of the inverse matrix and $(\bar{x}_{Fj} - \bar{x}_{Hj})$ is the difference between the mean values of $x_j$ for floral and honeydew honey.

Evaluation of these coefficients gave—

$$l_1 = -8{\cdot}3036$$
$$l_2 = -12{\cdot}2652$$
$$l_3 = +1{\cdot}3763$$

The value of the discriminant function is therefore given by—

$$X = -8{\cdot}3036x_1 - 12{\cdot}2652x_2 + 1{\cdot}3763x_3.$$

Substitution of the mean values of $x_1$, $x_2$, and $x_3$ in this equation gives—

$$\bar{X}_H = 57{\cdot}6228$$
$$\bar{X}_F = 86{\cdot}6782$$

The difference between these values is 29·0554.

To find the significance of this difference, the F test was used, F being given by the expression—

$$F = \frac{N_H N_F (N_H + N_F - p - 1)}{(N_H + N_F)(N_H + N_F - 2)} \times \frac{V}{p}$$

in which V is the variance of $X$, *i.e.*, $(\bar{X}_F - \bar{X}_H)$. The value of F is therefore—

$$\frac{10 \times 10 (10 + 10 - 3 - 1)}{(10 + 10)(10 + 10 - 2)} \times \frac{29{\cdot}0554}{3} = 43{\cdot}045.$$

The high value of F means that the discriminant function is likely to be effective and that there is a negligible chance of mis-classification.

The standard deviations of $X_H$ and $X_F$ were calculated, and a point was found that was an equal number of standard deviations from each of the means; this number was 73·095.

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# A Method for the Quantitative Separation of Fatty Acids from Unsaponifiable Matter

By H. G. SAMMONS and SHEILA M. WIGGS

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Fatty acids have been separated from unsaponifiable matter by adsorption on alumina from solution in light petroleum. The acids are quantitatively removed by conversion *in situ* to their methyl esters followed by elution with diethyl ether. The recovery of both unsaponifiable matter and fatty acids, as methyl esters, is greater than 90 per cent.

Before the methyl esters of fatty acids are subjected to vapour-phase chromatography, the acids must first be separated from contaminants, such as unsaponifiable matter. Separation is usually carried out by shaking an aqueous alcoholic solution of the acids, as sodium or potassium salts, several times with diethyl ether[1] or light petroleum.[2] Pikaar and Nijhof[3] used light petroleum instead of diethyl ether for the micro-determination of fatty acids in blood serum. However, in either solvent, especially for faecal extracts, we found that soap emulsions were often formed; these emulsions could be broken only with difficulty and caused some losses.

In 1949, van de Kamer, Huinink and Weijers[4] described a method for determining fatty acids in faeces, in which they extracted the saponifying medium, after acidification, with light petroleum. This method is ideal for determining total fatty acids by titration, but is unsuitable for the direct preparation of fatty acids, as much of the unsaponifiable material is extracted at the same time as the acids. Sylvester, Ainsworth and Hughes[5] described a method for determining unsaponifiable matter in fats by adsorbing the fatty acids on alumina from solution in light petroleum. Clearly, if a method could be found to remove the fatty acids quantitatively from the alumina, then the two procedures previously described could be combined to give a simple method for preparing pure fatty acids from almost any source. No solvent was found to be suitable for this purpose, but it was finally achieved by forming the methyl esters *in situ* on the alumina and then eluting them from it with diethyl ether.

## Method

Reagents—

*Alcoholic potassium hydroxide solution*—A 5 per cent. w/v solution of potassium hydroxide containing 0·4 per cent. of amyl alcohol.

*Hydrochloric acid, diluted* (2 + 1)—Analytical-reagent grade.

*Light petroleum, boiling range* 40° *to* 60° C—Analytical-reagent grade.

*Diethyl ether*—Analytical-reagent grade.

*Alumina*—Chromatographic grade (obtainable from the British Drug Houses Ltd.).

*Hydrogen chloride solution,* 1 *per cent., methanolic.*

Procedure—

Saponify an aliquot of an homogenate of faeces or food or a specimen of serum by heating gently under reflux for at least 60 minutes with 4·5 volumes of alcoholic potassium hydroxide solution on a bath of boiling water. (Faecal specimens consisted of a 24-hour collection diluted to 1 litre with distilled water and then homogenised.) Cool, add 1·5 volumes of diluted hydrochloric acid (2 + 1), and extract liberated fatty acids and unsaponifiable matter into an equal volume of light petroleum by shaking in a separating funnel. Repeat the extraction twice, and combine the light petroleum extracts. Evaporate to dryness at below 50° C under reduced pressure, and extract the residue with fresh solvent. Filter, and evaporate the light petroleum to small volume under reduced pressure. Add approximately 1 g of alumina for each 25 mg of fatty acid present, shake the mixture for a few minutes, preferably mechanically, and decant the supernatant liquid through a filter. Extract the alumina four times with diethyl ether, filtering and combining the extracts. Remove the solvent from this mixture by evaporation to give the unsaponifiable matter, which can be weighed and analysed.

Allow the ether to evaporate from the alumina, cover with methanolic hydrogen chloride solution, and set aside overnight in a glass- or plastic-stoppered flask at 37° C. (Heating under reflux, although more rapid, causes considerable bumping.) Remove the methanolic solution by filtration, wash the alumina with ether, and remove combined solvent and hydrochloric acid by evaporation under reduced pressure at low temperature. Carefully extract the residue, which contains some aluminium chloride, with light petroleum, and filter the mixture. Partially remove the solvent, add a small amount of anhydrous sodium sulphate to remove traces of water, filter, remove solvent, and weigh the methyl esters.

## Results

Slight manipulative losses appear to be inevitable, and recovery by the proposed method is normally between 90 and 95 per cent. For laboratory-reagent grade oleic acid, the results were—

| | | |
|---|---|---|
| Weight of sample, g .. .. .. .. | 0·0874 | 0·6877 |
| Weight of methyl ester recovered, g .. .. | 0·0843 | 0·6880 |
| Recovery, as fatty acid, % .. .. .. | 92·0 | 93·0 |

Samples of faecal lipids were prepared as indicated under "Procedure." Before treatment with alumina, the light petroleum extracts were evaporated to dryness and weighed; the results of a series of recovery experiments are shown in Table I.

### Table I

### Recovery of faecal lipids by proposed method

| Weight of sample, g | Weight of methyl esters recovered, g | Weight of fatty acids equivalent to recovered methyl esters, g | Weight of unsaponifiable matter recovered, g | Total recovery, % |
|---|---|---|---|---|
| 0·3075 | 0·2334 | 0·222 | 0·0586 | 92 |
| 0·2754 | 0·2256 | 0·215 | 0·0333 | 91 |
| 0·5638 | 0·2507 | 0·238 | 0·3233 | 98 |
| 0·6446 | 0·2971 | 0·283 | 0·3205 | 94 |
| 0·5818 | 0·4050 | 0·387 | 0·1545 | 92 |
| 0·2694 | 0·1687 | 0·161 | 0·0908 | 94 |
| 0·2025 | 0·1340 | 0·128 | 0·0576 | 91 |

## Discussion of the method

The proposed method has been applied to the preparation of the methyl esters of the fatty acids present in faeces, foods and serum in a form suitable for analysis by vapour-phase chromatography. Although the entire procedure extends over 2 days, the working time from, say, the preparation of a faecal homogenate to placing the sample on the chromatographic column is less than 5 hours. There appears to be a relationship between the amount of aluminium chloride produced during the formation of the methyl esters and the amount of fatty acids present, but this relationship has not been fully explored. Little or no aluminium chloride is formed if fatty acids are absent. The method has been successfully applied to the analysis of small volumes of serum containing only a few milligrams of fatty acid.

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# The Isolation and Determination of Volatile Compounds by Adsorption on Charcoal

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The use of adsorption on and desorption from charcoal in the analysis of volatile compounds from foods has been investigated. The apparatus and methods used are described, and results for some alcohols and esters with low boiling-points are reported. No chemical changes took place in the compounds studied, and complete recovery was obtainable.

Adsorption on charcoal from a stream of gas and subsequent desorption is used in the enrichment, isolation and determination of volatile compounds from foods.[1,2,3] However, no systematic investigation of the completeness of desorption or the occurrence of breakdown products in the desorbed compounds has been reported. The work described here was undertaken in order to obtain more information on these matters and to assess the possibilities and limitations of adsorption on charcoal as a means of investigating volatile compounds in foods.

The vapour-displacement technique was used for desorption, as high concentrations of desorbed compounds could be obtained by this method. The procedures and apparatus used in displacement chromatography have been adequately described by Claesson[4] and James and Phillips.[5]

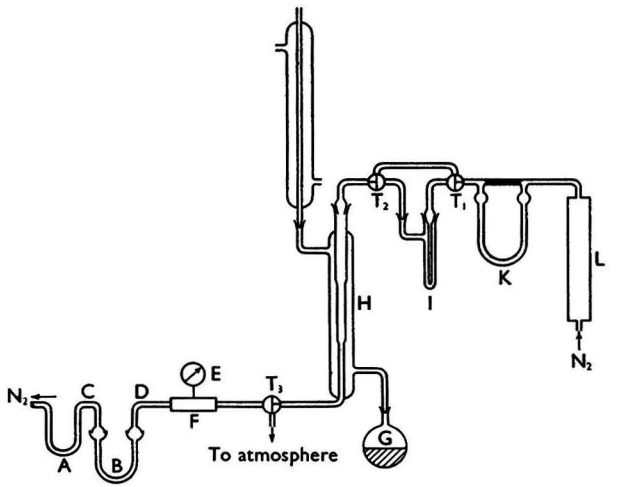


Fig. 1. Apparatus for vapour-phase displacement chromatography

## Method

Apparatus—

Displacement chromatography was carried out with the apparatus shown diagrammatically in Fig. 1; this apparatus is essentially the same as that used by James and Phillips.[5]

The main difference consists in the three-way stopcocks, $T_1$, $T_2$ and $T_3$. The stopcocks $T_1$ and $T_2$ were inserted in the gas stream in order to direct the gas into the top of the column either with or without loading it with the displacer vapour.

By means of $T_3$ the gas stream could be directed into either the atmosphere or the katharometer, F.

The gas leaving the katharometer could be condensed in a cool trap, B, which was kept protected from atmospheric moisture by a U-tube, A, filled with molecular sieve 4A (obtained from the Union Carbide and Carbon Corporation, New York).

The katharometer consisted of a single-element platinum-wire conductivity cell having a resistance of 18 ohms. The wire was heated by a 6-volt accumulator and formed part of a Wheatstone bridge circuit. The out-of-balance of the bridge was indicated by a spotlight galvanometer having a sensitivity of about $10^{-8}$ amp per mm at a distance of 1 metre.

The saturator, I, was constructed as described by Gage.[6]

REAGENTS—

The alcohols, esters and dioxan used were of laboratory- or analytical-reagent grade obtained from well known suppliers.

*Activated charcoal*—Norit ACS grade, obtained from Norit, N.V., Amsterdam, The Netherlands, was used; according to specification, the surface area was 1170 sq. metres per g, as determined by the BET method from the benzene isotherm at 20° C.

PROCEDURE—

Before each experiment, adsorption tube H was about two-thirds filled with 2 g of activated chacoal. The compound being investigated was then placed on the top of the column as described below.

A small glass tube was filled with 0·5 g of charcoal, and the total weight of tube and adsorbent was noted. The tube was then connected to a small gas-washing bottle containing a few millilitres of the liquid sample, and air, saturated with the vapour of the sample by passage through the wash-bottle, was aspirated through it. When the sample of charcoal was saturated with the vapour, the tube was disconnected and re-weighed. The amount of compound adsorbed, usually between 70 and 100 mg, was determined from the difference between the two weights. The contents of the tube were placed on the top of the column, and the column was immediately closed.

The flow of nitrogen was set at about 20 ml per minute, and the temperature of the column was rapidly brought to 100° C by boiling water in flask G. The stream of gas was vented to atmosphere through $T_3$ as long as water vapour condensed in the capillary connecting the column with $T_3$. (The column of charcoal was usually dry within 15 to 20 minutes.) When no more droplets were visible in the capillary, $T_3$ was turned so that the stream of gas passed into the katharometer. By turning stopcocks $T_1$ and $T_2$ to the appropriate positions, the nitrogen bubbling through the saturator at I was then saturated with dioxan, and displacement was begun. After 10 to 15 minutes, a trap consisting of a U-tube, 4 mm internal diameter and cooled in a mixture of dry ice and acetone, was connected by means of ball-joints to the katharometer and the drying tube at D and C, respectively.

Desorption, which under these conditions took about 40 minutes, was followed by means of the galvanometer. When true displacement occurred, two deflections of the galvanometer were observed. The first deflection indicated the entrance of the displaced compound into the katharometer, and the second was caused by break-through of the displacing dioxan. The trap was disconnected 3 to 5 minutes after the second deflection, and 0·1 ml of dioxan was added to its contents. Both ends were then closed by ball-joint stoppers, and the trap was allowed to attain room temperature. Its contents were then thoroughly mixed, and their volume was measured by aspiration into a calibrated 0·5-ml syringe.

A 5-$\mu$l sample of the mixture was analysed by vapour-phase chromatography, a 3-metre column of Carbowax 1540 at 70° C being used; the results were plotted on a 1-mV full-scale automatic recorder. When the chromatogram was complete, the ratio of the height of the peak for dioxan to the height of the peak for the desorbed compound was calculated. This ratio was then compared with those obtained from chromatograms of mixtures containing known amounts of dioxan and the compound being investigated, and the ratio of desorption of the compound was deduced. (The relative error in determining the peak-height ratios for mixtures of known composition was of the order of 4 per cent.)

## Discussion of the method

The adsorption and desorption of some aliphatic alcohols and esters having fairly low boiling-points were studied by the proposed method; the results are shown in Table I.

### Table I

### Recovery of aliphatic compounds after desorption

| Compound | Number of tests ($n$) | Mean recovery from $n$ tests, % | Standard deviation of a single observation |
|---|---|---|---|
| Methyl formate .. .. .. | 4 | 4·4 | 3·58 |
| Ethyl formate .. .. .. | 4 | 90·8 | 9·64 |
| *iso*Propyl formate .. .. | 5 | 49·2 | 2·87 |
| *n*-Propyl formate .. .. | 4 | 79·0 | 8·22 |
| Ethyl acetate .. .. .. | 3 | 85·5 | 9·04 |
| Methanol .. .. .. | 3 | 1·3 | 0·60 |
| Ethanol .. .. .. .. | 3 | 22·5 | 3·48 |
| *iso*Propyl alcohol .. .. | 4 | 88·8 | 3·97 |
| *n*-Propyl alcohol .. .. | 4 | 83·9 | 18·1 |

No indications of breakdown products liberated during desorption were observed on the final chromatograms of any of the compounds studied. Small shoulders on the fronts of the alcohol peaks were attributed to the formation of the corresponding formate esters on the Carbowax column.[7] It was noticed that formate was also formed from the standard alcohol - dioxan samples, *i.e.*, there was no interference with the determination.

Table I shows that the recoveries of methanol and methyl formate were extremely low. With these samples, no separate galvanometer deflections during displacement were observed before the deflection produced by dioxan, and the low recoveries were considered to be caused by losses during elution with nitrogen in the drying period. For methyl formate, this explanation was shown to be correct by the experiment described below.

The same apparatus was used as in the proposed method, but between $T_3$ and D, instead of passing through the cooled trap, B, the gas stream was bubbled through an accurately measured excess of standard sodium hydroxide solution cooled in ice - water mixture. The column was loaded with charcoal saturated with methyl formate or ethyl acetate, and the stream of nitrogen was passed through the first of two gas-washing bottles during the drying period; during displacement by dioxan, $T_3$ was turned so that the gas stream passed through the second gas-washing bottle. After the ester had been hydrolysed, the excess of sodium hydroxide solution in each bottle was determined by titration against hydrochloric acid. The results are shown in Table II, from which it can be seen that the recovery of methyl formate was nearly 100 per cent.

### Table II

### Recovery of methyl formate and ethyl acetate

| Sample | Recovery during drying period, % | Recovery during displacement period, % | Total recovery, % |
|---|---|---|---|
| Methyl formate .. .. | 92·0 | 4·5 | 96·5 |
| | 81·3 | 9·8 | 91·1 |
| | 90·1 | 8·0 | 98·1 |
| Ethyl acetate .. .. | Nil | 100·0 | 100·0 |
| | | 98·7 | 98·7 |
| | | 100·5 | 100·5 |

The loss of methyl formate when condensation was used for trapping the desorbed compound was therefore mainly caused by elution during the drying period. The results for ethyl acetate indicate that this compound was completely desorbed from the charcoal. The only reasonable explanation for the 15 per cent. loss in the first series of experiments is that the trapping mechanism was less efficient than collection in sodium hydroxide solution. In flavour research, therefore, careful attention should be given to the trapping mechanism.

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# Differential Electrolytic Potentiometry

## Part III.* An Examination of the Variables of the Method Applied to Inactive Reductants

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By using the determination of hydrazine with potassium bromate as a test reaction, a study has been made of the electrical variables in the differential electrolytic potentiometry of irreversible reductants, *i.e.*, applications in which the electrolytic anode is inactive, and experimental design criteria have been formulated. The effects have been examined of (*a*) isolating individual electrolysis electrodes, (*b*) varying the electrolysis current with fixed electrode area, (*c*) reducing the size of the electrodes to the micro and ultra-micro scales, (*d*) varying the electrode area at fixed current and (*e*) variation in source potential and stabilising series resistance of the electrolysis current. All these factors exert considerable influence on the characteristics of the titration curves, and the differential effect depends on functions of the anode and cathode current densities and inversely on a function, probably the square root, of the ballast load (product of the source voltage and the ballast resistance). There is a maximum tolerable cathode current density (dependent also on anode current density) and a minimum ballast load, above which the titration-curve forms become distorted. Accurate results may still be extracted outside these limits, but it is deemed best to work within them.

THE applications of differential electrolytic potentiometry to redox titrimetry on the macro[1] and ultra-micro scales[2] have been surveyed, and some observations on the nature and mechanism of the method have been made.[1] The method consists essentially in passing a minute heavily stabilised electrolysis current across a pair of stationary electrodes immersed in the stirred titration solution and measuring the resultant potential set up across the electrodes. It has already been noted that these electrodes behave independently of each other; that, in comparison with the behaviour of an indicator electrode, the potential of the electrolysis anode leads and the potential of the electrolysis cathode lags behind the potential of the indicator electrode; and that, if one of the chemical species is not reversibly electrolysed, the appropriate electrode maintains a more or less steady potential, but the other shows a magnified change in potential.

A single reaction was selected for a detailed examination of the influence of the electrical variables in order to gain some understanding of the mechanism and experimental design criteria of the method. The grounds of selection were (*a*) high analytical accuracy and precision to minimise chemical aberrations, (*b*) one inactive electrode to simplify the effect of the electrical variables and (*c*) advantageous changes in potential to allow latitude for the detection and delineation of adverse effects, yet changes so restricted in range as to eliminate alterations in the nature of electrode surfaces, such as formation of platinum oxides. Preference was

* For details of Parts I and II of this series, see reference list, p. 431.

accordingly given to a reaction of type 2 (*b*)[1] with as nearly irreversible a primary anodic process as possible; to the reaction producing the greatest peak height on the differential potentiometric curve among the reactions examined; and to a reaction involving reactants that can readily be purified, dried and weighed, so being as nearly as possible ultimate titrimetric standards. The hydrazine - bromate reaction was chosen. Hydrazine appears to be completely non-oxidisable at the anode; the inflexions appearing on the anode curve being due rather to bromide ion, which, although not appreciably oxidisable at the experimental potentials, appears to control the anode potential with the help of free bromine produced in the reaction. The cathodic reaction is not the reduction of bromate ion, but, as the potentials indicate, reduction of free bromine produced chemically in the system.

The solutions are stirred, and the currents are normally well below diffusion level, so that concentration polarisation is negligible. An investigational approach from the viewpoint of straight-forward electrolysis was therefore adopted. On a basis of simple ohmic circuitry, it therefore appears that the variables are (*i*) the magnitude of the electrolysis current, (*ii*) the sizes of the electrodes, (*iii*) the source voltage, (*iv*) the ballasting resistance and (*v*) the distance apart of the electrodes. Although variables (*i*) and (*ii*) may be combined as current density, the current densities on both electrodes must be separately considered. It can be predicted from Ohm's law that the height of the differential peak will be increased (*a*) the greater the current density (the larger the current and the smaller the electrodes), (*b*) the greater the source voltage and the smaller the ballast resistance (greater current density and smaller ballast load) and (*c*) the greater the distance between the electrodes. From the concept of the apparent surface impedance of indicating electrodes in "dead" or irreversible systems, developed some years ago for irreversible reductants,[3] it may further be predicted that factor (*c*) should have little if any effect. This is because the ohmic resistance of the ionic solution is smaller by a factor of $10^{-6}$ than the apparent surface impedance of the electrodes. Consequently, effects predicted on a basis of the apparent resistance over the whole electrode-solution dimension are much greater than those predicted from the simple ohmic resistance of the ionic solution. Since the effect of this phenomenon is concentrated in the electrode - solution boundary layers, the physical distance between the electrodes has little influence. This hypothesis also explains why calculations based on the total-surface area of circular wire electrodes give results similar to those based on the exposed-surface area of flat electrodes having insulated backs and edges. Although, since it is the sigmoid of many imperfectly understood factors, the apparent electrode - surface impedance cannot be pressed quantitatively, the concept is qualitatively useful, and some idea of the magnitude may be formed by Ohm's law calculations from the changes in differential potential and electrolysis current through the end-point. For example, $\Delta E/\Delta I$ for the cerate - ferrocyanide reaction[1] is 24 megohms, and for the bromate - thallium[I] reaction[1] the resistance appears to be 23·4 megohms under the same electrical conditions. The apparent electrode - surface impedance bears some relationship to the source voltage and ballast resistance, as will be shown. For comparison purposes, the ballast resistance divided by the apparent electrode - surface impedance has been designated the "ballast ratio"; in the above-mentioned examples the ballast resistance was 300 megohms, giving ballast ratios of 12·5 and 12·8, respectively. It is not always possible, however, to predict the value of $\Delta I$ or to measure it with ordinary equipment, and it is convenient and often sufficiently informative in practice to work with the product of the source voltage and ballast resistance, designated the "ballast load" or volt-ohm load. A source of 600 volts in the above-mentioned examples gives a ballast load of $18 \times 10^{10}$ volt-ohms.

The purpose of this paper is to record an investigation with ordinary equipment of the various influential electrical factors in reactions of type 2 (*b*) and to discuss the effects of these factors on the experimental design criteria and analytical usefulness of differential electrolytic potentiometry.

## Apparatus

The apparatus and procedure used were essentially those previously described,[1] except that in the electrical circuit the ballast resistance was furnished by a decade resistance box made up from high-stability $\pm 1$ per cent. 2-watt resistors giving a range of 0 to 1000 megohms in 10-megohm steps and that facilities were included for using a Tinsley mirror galvanometer (type 4500) having a sensitivity of 1500 mm per $\mu$A for measurements in the lower current ranges. Facilities were also included for accurate positioning of the electrolysis electrodes.

### Bromate - hydrazine reaction

The chemical conditions and requirements of this reaction have been thoroughly investigated and will form the subject of a separate communication. The presence of bromide in addition to that produced by reduction of bromate is necessary for complete reaction, and it has been found convenient to work in solutions 1·0 *M* in hydrochloric acid and 0·1 *M* in potassium bromide at the end-point of the titration. Under these conditions, accuracy and precision (replicability) are satisfactory.

### Function of electrodes

If what has previously been said about the function of the electrodes[1] be allowed, then the electrolysis anode in the bromate - hydrazine reaction is electrolytically inactive and behaves principally as a damped indicator electrode. The chief influence on the form of the differential curve is exerted by the cathode. Consequently, if the anode be isolated from the titration solution its potential should remain steady, and the anode inflexion together with the upward peak of the differential curve should disappear. The differential curve should then become simply an inversion of the cathode curve, running negative when the cathode potential joins the ordinary indicator-electrode curve after the end-point. Curves having just this form, as shown at *a* in Fig. 1, were produced by placing the anode in a filter stick (porosity No. 4) filled with a supporting electrolyte of 1·0 *M* hydrochloric acid and 0·1 *M* potassium bromide to above the level of the titration solution to prevent diffusion of the titration solution into the anode compartment.

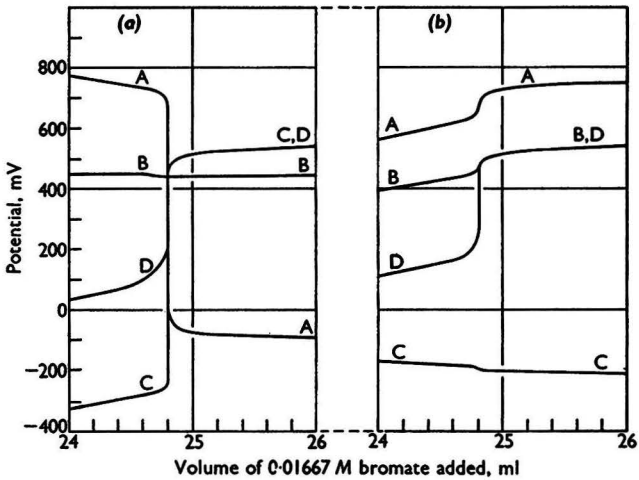


Fig. 1. (*a*) Isolation of the electrolysis anode; (*b*) isolation of the electrolysis cathode: curve A, differential potential; curve B, anode potential against calomel cell; curve C, cathode potential against calomel cell; curve D, indicator-electrode potential against calomel cell
Cathode area 0·105 sq. cm; anode area 0·107 sq. cm; current 0·100 μA, from 48 volts and 450 megohms

| | End-points | | | |
|---|---|---|---|---|
| | Potentiometric, ml | Anode, ml | Cathode, ml | Differential, ml |
| (*a*) | 24·805 | — | 24·805 | 24·805 |
| (*b*) | 24·805 | 24·80 | 24·79 | 24·805 |

Similar isolation of the cathode should give a differential potential that follows the anode potential. Although, bearing in mind the reflected magnification of the active-electrode curve, the cathode might be expected to show some disturbance at the end-point, the differential potential should be more or less steady. This was in fact so, as shown at *b* in Fig. 1. The supporting electrolyte in the filter stick showed no detectable generation of bromine when containing the isolated anode in a titration lasting 103 minutes.

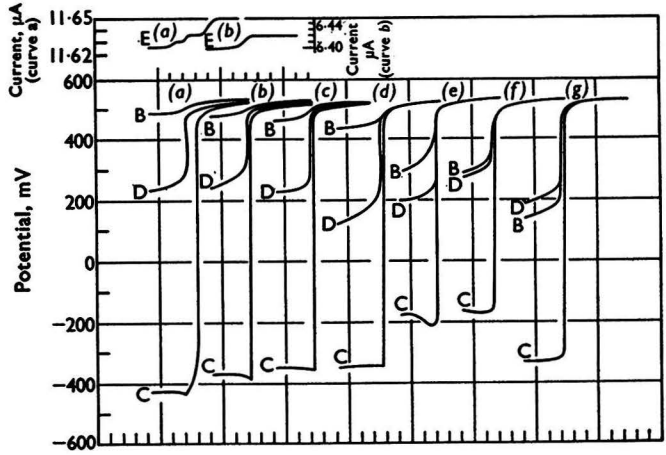


Fig. 2

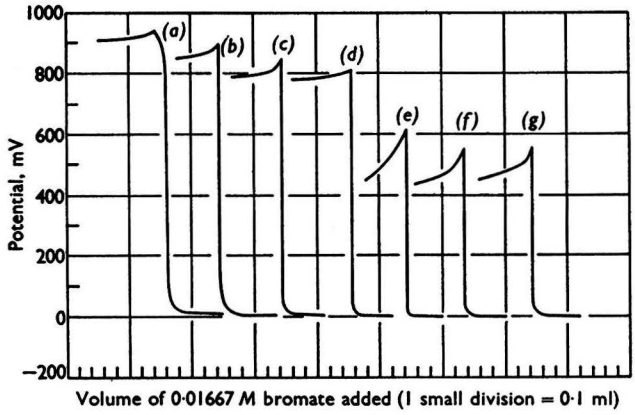


Fig. 3

Fig. 2. Response of individual electrodes at various currents: curves lettered as for Fig. 1, except that curve E is the electrolysis current

Anode and cathode 1 inch of 22 s.w.g. platinum wire, set 1 cm apart; electrode area 0·568 sq. cm

| | | (a) | (b) | (c) | (d) | (e) | (f) | (g) |
|---|---|---|---|---|---|---|---|---|
| Current, μA | | 11·64 | 6·41 | 1·59 | 0·615 | 0·07 | 0·014 | 0·001025 |
| Source potential, volts | | 1200 | 1200 | 300 | 120 | 12 | 12 | 1·018 |
| Ballast resistance, megohms | | 110 | 200 | 200 | 200 | 200 | 990 | 990 |
| Current density, μA per sq. cm | | 20·5 | 11·3 | 2·80 | 1·08 | 0·123 | 0·025 | 0·0018 |
| $\Delta E_e$, mV | | 14 | 15 | 30 | 40 | 140 | 160 | 322 |
| $\Delta E_e$, mV | | 880 | 858 | 852 | 832 | 716 | 652 | 815 |
| Differential peak potential, mV | | 945 | 895 | 848 | 812 | 610 | 552 | 558 |
| ΔV anode —cathode, ml | | 0·08 | 0·015 | 0·01 | 0·005 | 0 | 0·008 | 0·008 |
| Anode error, ml | | 0 | −0·005 | 0 | +0·005 | 0 | 0 | 0 |
| Cathode error, ml | | +0·08 | +0·01 | +0·01 | +0·01 | 0 | +0·008 | +0·008 |
| Differential error | inflexion, ml | +0·08 | +0·005 | — | — | — | — | — |
| | peak, ml | −0·005 | −0·002 | 0 | 0 | 0 | 0 | 0 |
| End-points | potentiometric, ml | 24·72 | 24·715 | 24·72 | 24·77 | 24·715 | 24·667 | 24·717 |
| | anode, ml | 24·72 | 24·71 | 24·72 | 24·775 | 24·715 | 24·667 | 24·717 |
| | cathode, ml | 24·80 | 24·725 | 24·73 | 24·78 | 24·715 | 24·675 | 24·725 |
| | differential— | | | | | | | |
| | inflexion, ml | 24·80 | 24·72 | — | — | — | — | — |
| | peak, ml | 24·715 | 24·713 | 24·72 | 24·77 | 24·715 | 24·667 | 24·717 |
| | current, ml | 24·72? | 24·69 | — | — | — | — | — |

Fig. 3. Differential curves corresponding to Fig. 2

### Magnitude of electrolysis current

A study of the effect of different current magnitudes was made, the electrodes used being set 1 cm apart and consisting of 1-inch lengths of 22 s.w.g. platinum wire. Families of curves for the individual electrode potentials are shown in Fig. 2, and the differential curves, separated for clarity, are shown in Fig. 3.

#### Differential curves—

Other things being equal (the ballast load was not maintained constant in these experiments), the height of the differential peak and the change in potential at the end-point increase with increasing current, as expected, but two additional important factors emerged. First, there is a limiting current above which the curve form changes and increasing errors may arise. Instead of rising to a peak and falling abruptly and vertically to near zero, the differential curve after the peak falls gradually, the spread increasing with increasing current, before making the abrupt descent, and the vertical part of the curve lies after the equivalence point. This is discussed later. The inflexion in the electrolysis current under these conditions is marked and often after the equivalence point. Under the conditions described, the maximum tolerable current is rather less than 6 $\mu$A, and the altered shape of the curve is clearly apparent at 11·64 $\mu$A.

Secondly, it was expected that, for very small currents, the differentiating effect of the electrolysis would decrease and become vanishingly small, but, although there is a decrease in the effect, it is by no means proportional, and perfect differential curves were formed at the lowest current detectable with the equipment described above ($1{\cdot}025 \times 10^{-9}$ amp). The significance of this phenomenon will be discussed in a later paper dealing with measurements made with d.c. amplifiers.

#### Anode curves—

The magnitude of the change in anode potential increases with decreasing current, slowly at first, and then more rapidly as the current becomes very small, which is contrary to the trend in cathode- and differential-potential changes. The anode reaction is therefore highly irreversible at large currents, although the curves still show slight inflexions, even with excessive currents. With large currents, the anode lead (error) is appreciable and the inflexion precedes the equivalence point. Below the maximum tolerable current, inflexion and equivalence points are in close agreement. With further decrease in current, the anode becomes increasingly active, inflexion becomes more pronounced and the anode-potential curve approaches closer to the ordinary potentiometric (indicator-electrode) curve. A curious and unexpected feature is that this change does not cease when coincidence of anode and indicator curves is achieved, but eventually, at $10^{-9}$ amp, the anode curve falls below the indicator-electrode curve (see curves at *g* in Fig. 2). This is attributed to a type of electron polarisation that will be enlarged upon later.

#### Cathode curves—

The magnitude of the negative reach of the cathode diminishes with decreasing current, as expected, but is somewhat dependent on the time allowed for equilibration at lower currents. With large currents, the cathode lag (error) is considerable and the inflexion is late. Below the maximum tolerable current, the inflexion coincides with the equivalence point. The diminished anodic inflexion at higher currents, which would reduce the height of the upward rise to the differential peak, is in some degree compensated for by a downward peak in the cathode curve. This cathode dip diminishes as the current decreases, vanishes at about one-tenth of the maximum tolerable current and is replaced by either a rising curve or some irregularities in potential.

#### Reproducibility and rate of equilibration of potentials—

After the end-point the potentials of all electrodes quickly become identical, except at very high currents, and the differential potential rapidly vanishes. The reproducibility of all electrode potentials is excellent after the end-point, and before the end-point the same is true of anode and cathode potentials, but not of indicator-electrode potentials on the reduced side. This is unimportant, since these potentials have no real meaning until sufficient bromine has

been generated for the reversible oxidant system to assume control of the potential and stabilise the electrode.[3] Before this point the indicator electrode exhibits a fugitive potential anywhere within a range of some 300 mV, largely dependent on the time allowed for equilibration. All electrodes after the end-point and the active electrode at all times reached equilibrium within 3 minutes. Before the end-point, the indicator electrode is sluggish and erratic and will continue to drift at more than 1 mV per minute for 3 hours or more. The anode at very low currents approaches the behaviour of the indicator electrode and so becomes sluggish, but not erratic. This effect is noticeable for the curve at *e* in Fig. 2 and pronounced for those at *f* and *g*. At higher currents, the anode rapidly attains equilibrium. Under similar conditions, the electrolysis electrodes attain equilibrium some 10 to 15 times more rapidly than does the indicator electrode. The rate at which the differential potential attains equilibrium is, of course, dependent on the equilibration rate of the anode and is rapid at high and moderate currents, but sluggish at very low currents. This, however, is no deterrent to rapid titration at low levels of current, since the fall in potential at the end-point is so large and its location so precise that complete equilibrium need not be awaited; 70 per cent. of the maximum attainable potential is reached in 5 minutes even with currents below $10^{-8}$ amp.

It may be concluded that, for a given reaction and set of electrodes, there is a maximum tolerable current, above which the form of the curves becomes distorted and errors may arise, and that the optimum working conditions for ease of manipulation, accuracy and rate of potential equilibration lie within 1 to 20 per cent. of this maximum tolerable current.

## Size of electrodes

### Use of micro electrodes—

The *IR* drop, *i.e.*, the differential potential, should be increased by reducing the size of the electrodes. Micro electrodes at moderate currents gave potential differences far beyond the range of the meters and erratic and erroneous results. More reasonable results were achieved with J-shaped electrodes consisting of 2 mm of 45 s.w.g. platinum wire placed 2 mm apart at a current of 0·100 $\mu$A, but curve distortion indicated that this current was still well above the maximum tolerable value. Current magnitude is therefore not the controlling parameter, but rather a function of current, electrode-surface areas and, perhaps, distance of separation. Reduction of the current to $13 \times 10^{-9}$ amp gave smooth and satisfactory curves on the verge of excessive current.

Setting the J-shaped electrodes closer together with a smaller overlap (1·1 mm) and still further reducing the current gave most satisfactory results. Under these conditions, the cathode curve on its positive-going change crossed the anode curve, so giving a small negative differential potential. This is attributed to the adhesion of the anode to the indicator-electrode behaviour and to its slightly lower rate of equilibration in circuit with a high-capacity calomel cell. That the anode - calomel circuit gave slightly spurious potentials was confirmed by the fact that the measured differential potential was less than the calculated algebraic difference between the measured anode and cathode potentials under these conditions.

It thus became clear that, provided the current was kept sufficiently small, extremely small electrodes could be used. A pair of electrodes, each consisting of 1 mm of 45 s.w.g. platinum wire, set 0·5 mm apart was made up, and titrations at 2 to $5 \times 10^{-9}$ amp were made for all the reactions previously described[1]; smooth curves resulted, giving excellent end-points. Reduction of the scale of working from macro to semi-micro (2 ml) and micro (0·1 ml) caused no trouble, so this form of paired micro electrodes was evolved into an ultra-micro titration platform, and ultra-micro titrations were performed with unexpectedly good results.[2]

### Relative surface area of electrodes—

Although it seemed that the cathode exerted the major formative influence on the differential curves, it was not unlikely that the anode made some contribution. A set of pairs of bright platinum-foil electrodes having nominal areas of 0·01, 0·1 and 1·0 sq. cm was prepared, the backs and edges being covered with fused glass, and series of titrations were performed at various fixed currents with the several permutations of electrode combination. The titration curves for one such series are shown in Figs. 4 and 5, the differential curves being separated for clarity. The selected current for this series is excessive with the smallest electrodes, but is chosen as illustrating most of the points that have emerged and as giving

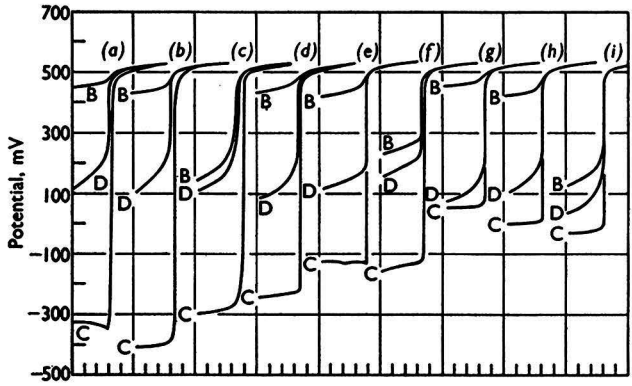


Fig. 4

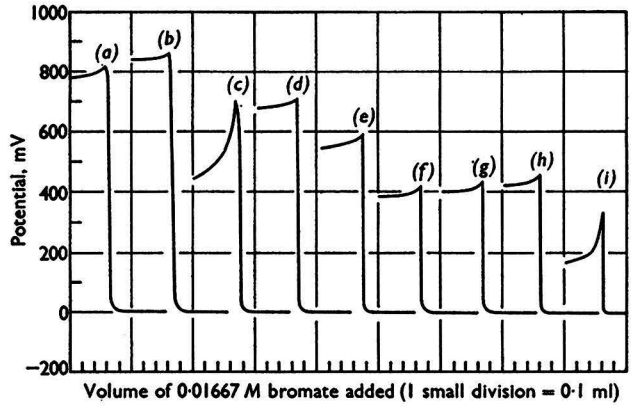


Fig. 5

Fig. 4. Behaviour of individual electrodes of various sizes at constant current: curves lettered as for Figs. 1 and 2

Current 0·100 $\mu$A, from 48 volts and 400 megohms; electrodes set 1 cm apart

| | (*a*) | (*b*) | (*c*) | (*d*) | (*e*) | (*f*) | (*g*) | (*h*) | (*i*) |
|---|---|---|---|---|---|---|---|---|---|
| Cathode area, sq. cm .. | 0·0195 | 0·0195 | 0·0195 | 0·105 | 0·105 | 0·105 | 1·00 | 1·00 | 1·00 |
| Anode area, sq. cm .. | 0·024 | 0·107 | 1·02 | 0·024 | 0·107 | 1·02 | 0·024 | 0·107 | 1·02 |
| Current density— | | | | | | | | | |
| cathode, $\mu$A per sq. cm | 5·12 | 5·12 | 5·12 | 0·952 | 0·952 | 0·952 | 0·100 | 0·100 | 0·100 |
| anode, $\mu$A per sq. cm .. | 4·16 | 0·935 | 0·098 | 4·16 | 0·935 | 0·098 | 4·16 | 0·935 | 0·098 |
| $\Delta E_a$, mV .. .. | 33 | 48 | 253 | 32 | 50 | 222 | 32 | 48 | 312 |
| $\Delta E_c$, mV .. .. | 822 | 879 | 759 | 720 | 634 | 625 | 441 | 491 | 528 |
| Differential peak potential, mV .. .. | 820 | 872 | 700 | 715 | 595 | 415 | 440 | 460 | 340 |
| $\Delta$V anode − cathode, ml | 0·01 | 0·03 | 0·065 | 0 | 0·005 | 0·01 | 0·017 | 0 | 0 |
| Anode error, ml .. | +0 | 0 | −0·02 | +0·01 | −0·005 | 0 | −0·012 | 0 | 0 |
| Cathode error, ml .. | +0·01 | +0·03 | +0·045 | +0·01 | 0 | +0·01 | +0·005 | 0 | 0 |
| Differential error— | | | | | | | | | |
| inflexion, ml .. .. | +0·015 | +0·035 | +0·04 | — | — | — | — | — | — |
| peak, ml .. .. | −0·01 | 0 | −0 | +0·005 | −0·002 | +0·005 | −0 | 0 | 0 |
| End-points— | | | | | | | | | |
| potentiometric, ml .. | 24·80 | 24·80 | 24·845 | 24·84 | 24·885 | 24·835 | 24·837 | 24·805 | 24·805 |
| anode, ml .. .. | 24·80+ | 24·80 | 24·825 | 24·85 | 24·88 | 24·835 | 24·825 | 24·805 | 24·805 |
| cathode, ml .. .. | 24·81 | 24·83 | 24·89 | 24·85 | 24·885 | 24·845+ | 24·842 | 24·805 | 24·805 |
| differential— | | | | | | | | | |
| inflexion, ml .. .. | 24·815 | 24·835 | 24·885 | — | — | — | — | — | — |
| peak, ml .. .. | 24·79 | 24·80 | 24·84+ | 24·845 | 24·88+ | 24·84 | 24·835 | 24·805 | 24·805 |

Fig. 5. Differential curves corresponding to Fig. 4

rapid equilibration with even the largest electrodes. (It should be noted that several hundred titrations have been carried out with many batches of standard solutions closely similar in concentration, so that variation in titre from experiment to experiment is without significance; the potentiometeric results were always within $\pm 0{\cdot}005$ ml of the theoretical values.)

ANODE CURVES—

The change in potential of the anode over the range $-0{\cdot}05$ to $+0{\cdot}05$ ml of titrant round the inflexion point, $\Delta E_a$, first increases slowly with decreasing anode current density and then rapidly as the anode current density becomes very low. As the anode current density decreases, there is a break-in, after which the anode rapidly becomes active, and the anode curve approaches the potentiometric curve, giving differential curves intermediate between those for reactions of types 1 and 2 (*b*). Indeed, below $2 \times 10^{-9}$ amp per sq. cm the anode curve crosses the potentiometric curve (see curves at *g* in Fig. 2).

Except at very low anode current densities, $\Delta E_a$ is notably constant for a given anode and is independent of cathode current density.

The anode lead (error) is generally small and may become a lag at high current densities, as in the curves at *d*. Although the anode inflexions are reasonably sharp and the precision of location is fair, the inflexions are small and the precision not as good as with the other curves; errors are therefore less easy to detect and assess accurately. There appears to be a tendency for the error to increase with increasing disparity in electrode areas.

CATHODE CURVES—

The negative reach of the cathode over the range $-0{\cdot}05$ to $+0{\cdot}05$ ml of titrant round the inflexion point, $\Delta E_c$, increases with increasing cathode current density. At moderate cathode current densities (about 1 $\mu$A per sq. cm) $\Delta E_c$ increases with increasing anode current density, but at low cathode current densities the relationship is reversed. At high cathode current densities $\Delta E_c$ at first increases and then decreases with increasing anode current density.

The cathode lag (error) increases with increasing cathode current density. At low cathode current densities it is negligible, but at high cathode current densities it is appreciable and increases rapidly with decreasing anode current density. At moderate cathode current densities, the lag is negligible at comparable anode current densities, but becomes detectable at high or low anode current densities.

DIFFERENTIAL CURVES—

The differential-potential peak increases sharply with increasing cathode current density and less sharply with increasing anode current density.

As the cathode current density increases, the form of the curve changes from a sharp peak and then a vertical drop to a peak and then a falling S-shaped inflexion. For curves of the latter type, if the peak be taken as the end-point there is little error, often negative, but if the falling inflexion is taken as the end-point, positive errors arise; these errors increase with increasing cathode current density and decreasing anode current density. There is also a loss of precision in locating the peak on such a curve, and any deviation from verticality is undesirable.

By using the pipette-dilution method,[4] the slope of the drop after the peak was checked under conditions corresponding to the curves at *d*, *e*, *f*, *g*, *h* and *i* in Fig. 4; it was found to be practically vertical, an increment of reagent considerably less than 0·01 ml being sufficient to bring the potential from the peak value to within a few millivolts of zero.

Curves having the shapes shown at *a*, *b* and *c* in Fig. 5 and at *a* and *b* in Fig. 3 were originally drawn with rounded tops, and the S-shaped falling inflexion was taken to be the end-point, which naturally gave positive errors. Experimentation with the shapes of the curves, however, showed that if the curve through the points plotted when the potential was rising was extended instead of being bent over and the curve through the points plotted when the potential was beginning to decrease just before its steep descent was likewise extrapolated backwards, the intersection of these two curves gave a peak, as shown in the examples quoted, in close agreement with the theoretical equivalence point. The validity and precision of this intrapolation were checked in three ways: (*i*) by repeated re-drawing of the curves by the same and different individuals, resulting in a replicability of about 0·005 ml, (*ii*) by careful large-scale plotting of anode and cathode curves, measuring the

difference in potential from these, and plotting the differential curve and (*iii*) by pipette-dilution titration, passing through this region in 0·005-ml increments, which again confirmed the existence and location of the peak. This peak is, of course, dependent on the anode and disappears when the anode is isolated (see curves at *a* in Fig. 1). Under these conditions no excess of current can be tolerated, as the point of inflexion has to be taken as the end-point. The maximum tolerable current (or current density) is defined as that at which deviation from verticality in the differential curve becomes just detectable.

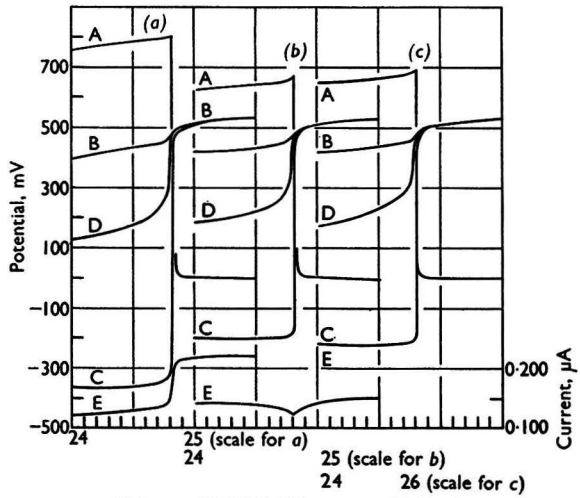


Fig. 6. Effect of ballast load: curves lettered as for Figs. 1 and 2
Cathode area 0·105 sq. cm; anode area 0·107 sq. cm; electrodes set 1 cm apart; current graphed

| | | (*a*) | (*b*) | (*c*) |
|---|---|---|---|---|
| Source potential, volts | | 2 | 12 | 168 |
| Ballast resistance, megohms | | 10 | 100 | 990 |
| Apparent electrode - surface impedance, megohms | | 11·68 | 68 | 690 |
| Ballast ratio | | 0·86 | 1·47 | 1·436 |
| Ballast load, volt-ohms | | $20 \times 10^6$ | $12 \times 10^8$ | $16{\cdot}6 \times 10^{10}$ |
| Mean current density, μA per sq. cm | | 1·65 | 1·23 | 1·885 |
| $\Delta E_e$, mV | | 825 | 681 | 710 |
| $\Delta E_a$, mV | | 46 | 40 | 45 |
| Differential peak potential, mV | | 810 | 678 | 694 |
| ΔV anode − cathode, ml | | 0·01 | 0·01− | 0 |
| Anode error, ml | | +0·005 | −0·003 | 0 |
| Cathode error, ml | | +0·015 | +0·007 | 0 |
| Differential error, ml | | 0 | 0 | 0 |
| End-points | potentiometric, ml | 24·805 | 24·803 | 24·805 |
| | anode, ml | 24·81 | 24·80+ | 24·805 |
| | cathode, ml | 24·82 | 24·81 | 24·805 |
| | differential, ml | 24·805 | 24·805 | 24·805 |
| | current, ml | 24·815 | 24·81 | — |

Series of experiments in which the cathode current density was held constant over various sizes of cathode and the area of the anode was varied led to similar conclusions to those outlined above. It is therefore evident that, alone, neither current nor cathode current density is the controlling factor, but rather a combination of current and area of both anode and cathode.

## Ballasting and source potential

Assuming that the distance apart of the electrodes is without marked influence, the supply voltage and the value of the ballasting resistance remain to be considered. Only supply voltages above the back e.m.f. of the cell will be considered here.

A sample set of curves, taken fairly close to the maximum tolerable current density with electrodes 0·1 sq. cm in area and different values of source voltage and ballast resistance are shown in Fig. 6. Strict comparison cannot be made with these curves, as the currents are rather different, but Fig. 6 shows the different behaviour of the current during titration. Broadly speaking, for a given reaction, current and set of electrodes, a decrease in ballast load produces the same effects as does an increase in current density.

Two further points emerge. First, the ballast ratio shows only small variation, but the apparent electrode - surface impedance is apparently proportional to the square root of the ballast load (this will be discussed in the further paper previously mentioned). Secondly, aside from the general effect noted above, there is a considerable qualitative effect. With the current used, equilibration is rapid with all electrodes, but, as the ballast load is decreased, the potentials become unsteady and erratic. The curves in Fig. 6 are smooth, but in sets *a* and *b*, particularly *a*, irregularities in potential are common before the end-points. These irregularities disappear after the end-points and are ironed out as the ballast load increases. It is therefore best to work with moderately high ballast loads, but a practical limitation renders extremely high ballast loads at high currents inconvenient, as leakage currents and hand-capacitance effects become more pronounced and less controllable.

Variations in current are evident at low ballast loads, becoming more pronounced as the ballast load decreases and giving inflexions often in agreement with the equivalence point. By using d.c. amplifiers, variation in current has been shown to persist with ballast resistances up to $10^{10}$ ohms.

## Conclusions

Experimental design criteria can now be formulated for the application of differential electrolytic potentiometry to reactions of type 2 (*b*), *i.e.*, those in which a reversibly electrolysable oxidant is used to titrate a reductant that is not reversibly electrolysed. The anode current density should be equal to or greater than the cathode current density, which should be within the range 0·1 to 1·5 $\mu$A per sq. cm, and the ballast load should be not less than $10^9$ and preferably $10^{10}$ volt-ohms. Higher current densities favour greater changes in potential and higher rates of potential equilibration, but the current should not be sufficiently large to cause deviation from verticality in the differential curve.

Increase in current density or decrease in ballast load gives rise to increases in the height of the differential peak and the value of $\Delta E_c$ and to a decrease in the value of $\Delta E_a$, so rendering the anodic process more irreversible. Cathode current density is not solely responsible for the form of the differential curve; the anode current density and ballast load also exert considerable influence.

I thank Imperial Chemical Industries Limited and the Baker Platinum Division of Engelhard Industries Limited for the loan of apparatus and instruments used in this work.

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Note—References 1 and 2 are to Parts II and I of this series, respectively.

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# The Gravimetric Determination of Mercury by Precipitation with Sodium Tetraphenylboron*

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Diphenylmercury is quantitatively precipitated when a solution containing mercuric ions is treated with an excess of sodium tetraphenylboron solution. A method in which mercury is determined as diphenylmercury is described.

Much research has been carried out on the use of sodium tetraphenylboron in chemical analysis since Wittig prepared this compound and recognised its properties in 1949. The reagent is an excellent precipitant for potassium, rubidium, caesium, thallium and ammonium ions and for numerous organic bases, including alkaloids and bases of pharmaceutical or biochemical interest. These compounds are usually determined by gravimetric analysis of the precipitates or by indirect methods, *e.g.*, argentimetric titration of the tetraphenylboron moiety of the precipitate.[1,2]

During an investigation into the possibility of a mercurimetric determination of tetraphenylborons we found that, when tetraphenylboron ion was titrated with a solution of mercuric nitrate or perchlorate, the reaction proceeded in two steps. Both steps can be followed when the titration is carried out potentiometrically or polarometrically.[3] In the first step, 3 equivalents of $Hg^{2+}$ are consumed per mole of tetraphenylboron, 1·5 moles of diphenylmercury and 1 mole of phenylboric acid being produced. As the titration proceeds, $Hg^{2+}$ ions react with diphenylmercury and phenylboric acid; as a result, a phenylmercuric salt is formed in the second step, with the consumption of a total of 8 equivalents of $Hg^{2+}$ per mole of tetraphenylboron.[3] There are also two steps in the reverse titration, *i.e.*, that of mercuric ions with a solution of sodium tetraphenylboron. A phenylmercuric salt is formed initially and reacts with further additions of tetraphenylboron to form diphenylmercury quantitatively in the second step. For reactions in this direction, precipitation of mercury as diphenylmercury requires the addition of 1 mole of tetraphenylboron for each 2 moles of mercuric salt; the equation for the over-all reaction is—

$$2Hg(NO_3)_2 + Na[B(C_6H_5)_4] + 3H_2O = 2C_6H_5{\cdot}Hg{\cdot}C_6H_5 + H_3BO_3 + NaNO_3 + 3HNO_3.$$

Since a gravimetric determination of $Hg^{2+}$, as diphenylmercury, after precipitation by phenylboric acid had been described,[4] we decided to test the possibility of a gravimetric determination with sodium tetraphenylboron as precipitant.

## Method

### Reagents—

Water distilled from an all-glass apparatus was used throughout.

*Sodium tetraphenylboron solution*—A freshly prepared 2 per cent. solution in twice-distilled water; if turbid, the solution was clarified by treatment with alumina.[5] The sodium tetraphenylboron used (Kalignost) was obtained from Heyl and Co., Hildesheim, Germany.

*Standard mercury solutions*—Weighed amounts of thrice-distilled mercury were dissolved in nitric acid, and the solutions were heated to expel oxides of nitrogen and then diluted with distilled water to the desired volumes.

### Procedure—

To the sample solution, which should contain between 5 and 100 mg of $Hg^{2+}$ at a concentration of about 1 mg or less per ml and should be nearly neutral or faintly acid, add a few millilitres of a saturated solution of analytical-reagent grade sodium acetate and then an excess

* Part of a communication presented at the Third Congress of Analytical Chemistry, Prague, September 1st to 8th, 1959.

of sodium tetraphenylboron solution, drop by drop, with stirring. Separate the precipitate on a sintered-glass crucible (medium porosity), wash with a saturated aqueous solution of analytical-reagent grade diphenylmercury, allow to dry in a desiccator, and then weigh.

1 mg of diphenylmercury ≡ 0·5654 mg of mercury.

## Discussion of the method

### Conditions of precipitation—

Results were good when sample solutions were buffered with sodium acetate. In acid solutions results were high, probably owing to the formation of the free acid, $H[B(C_6H_5)_4]$, which is insoluble and may be carried down with the precipitate of diphenylmercury; precipitates from acid solutions did, in fact, contain different amounts of boron. Further, in unbuffered solutions there may be local formations of acidity,[6] in accordance with the equation—

$$Na[B(C_6H_5)_4] + 4Hg(NO_3)_2 + 3H_2O = 4C_6H_5{\cdot}HgNO_3 + NaNO_3 + 3HNO_3 + H_3BO_3.$$

In moderately acid solutions (pH 1 to 5) there is a positive error, which increases with the time of contact between the precipitate and the solution. However, when the precipitate is separated immediately, reproducible results can be obtained. In presence of sodium acetate solution, results were correct even when some hours elapsed between precipitation and filtration. Precipitation is satisfactory in the pH range 6·5 to 9·0.

The excess of precipitant should not be too great, as some of the reagent may be adsorbed on the precipitate; further, the reagent is relatively expensive. The necessary excess can be obtained by adding the precipitant from a graduated pipette. During the addition, the precipitate first formed re-dissolves, owing to the formation of a phenylmercuric salt. When the turbidity is persistent, the volume of precipitant added should be noted, and a further addition equal to 1·5 times the volume already added should be made.

If the precipitate is dried at temperatures between 60° and 110° C, results are somewhat low; correct results are obtained after drying at room temperature in a desiccator. If dried under reduced pressure with silica gel as desiccant, precipitates usually attain constant weight after 4 to 6 hours; drying overnight may be convenient.

### Interference—

The determination cannot be carried out in presence of cations forming insoluble precipitates with sodium tetraphenylboron, *e.g.*, silver, thallium, potassium, caesium, rubidium and ammonium, or of anions forming insoluble phenylmercuric salts, *e.g.*, chloride, bromide, iodide and thiocyanate. Mercurous ions should not be present, as they react with the reagent to form a grey precipitate consisting of diphenylmercury and metallic mercury. Interference from metal ions precipitated as hydroxide or basic salt at the pH used can be overcome by using an appropriate chelating agent; in preliminary experiments, results were satisfactory in presence of an excess of various ions when ethylenediaminetetra-acetic acid was used.

## Results

Results found by the proposed method for standard solutions of mercuric nitrate or perchlorate are shown in Table I. The error does not usually exceed 1 per cent.

### Table I

Recovery of mercury by the proposed method

| Mercury present, mg | Weight of precipitate, mg | Mercury found, mg | Error, % |
|---|---|---|---|
| 5·0 | 8·8 | 4·97 | −0·6 |
| 5·0 | 8·9 | 5·03 | +0·6 |
| 10·0 | 17·9 | 10·10 | +1·0 |
| 10·0 | 17·7 | 10·00 | 0·0 |
| 15·0 | 26·4 | 14·95 | −0·3 |
| 15·0 | 26·6 | 15·05 | +0·3 |
| 20·0 | 35·5 | 20·00 | 0·0 |
| 20·0 | 35·7 | 20·10 | +0·5 |
| 30·0 | 53·0 | 29·95 | −0·2 |
| 30·0 | 53·2 | 30·05 | +0·2 |

When mercury was determined in various mercury compounds, organic mercurials and pharmaceutical preparations containing mercury, after suitable mineralisation, there was good agreement with results found by standard methods.

I thank Heyl and Co., Hildesheim, Germany, for a gift of Kalignost.

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# An Extension of the Gradient Elution Principle

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Elution of components on chromatographic and ion-exchange columns with an eluting solution of gradually varying composition is a well established technique that can greatly assist the separation of components. Hitherto, two homogeneous eluting agents have been mixed progressively during the elution process and, in this way, an appropriate concentration gradient has been achieved. Sometimes the progressive mixture of three eluting agents can be useful, the third being added at a much slower initial rate. To provide an advance on existing apparatus, even when employed in mixing two multi-component eluting-agent systems, a series type of mixing apparatus is necessary, in which the first eluting agent is mixed with the second and simultaneously the mixture is fed to the third. The various concentration patterns arising from simple methods of mixing are calculated and discussed.

In the chromatographic separation of a mixture, a component that is strongly "adsorbed" may require a considerable volume of a single eluting agent to carry it through the column. Consequently, undesirable dilution of this component occurs. If more than one component is strongly held, separation of these components may be poor, although complete separation has been effected for the compounds that have travelled more rapidly through the column. Asymmetric adsorption isotherms, which exaggerate the "tailing" of a component, may prevent separation of similar substances, even if these travel down the column at a reasonable rate. Such undesirable factors can sometimes be overcome by use of the gradient elution principle, in which the column is eluted by a solution of varying composition. In this way the eluting power of the solution for the slower-moving components is gradually increased as the chromatographic process proceeds, so that undesirable dilution of later components is avoided. Also, by reducing the tailing effect, separations may be produced that would not be possible in the absence of the concentration gradient in the eluting solution. Wide applications of this method include separations of alkali metals,[1] rare earths,[2,3] organic acids,[4,5] silicones,[6] oxyhaemoglobin[7] and deoxyribonucleic acid.[8] Gradient elution sometimes provides satisfactory resolution when numerous other variations of the chromatographic method fail,[9] and, in the opinion of some workers, superior results are always obtainable by variation in composition of the eluting solution.[10] Other types of gradient, *e.g.*, a temperature gradient along the column,[11] have occasionally been employed.

The way in which the proportion of the two eluting agents varies with time may be restricted by the type of mixing apparatus. Two methods of mixing are commonly employed. In the simplest method, a separating funnel equipped with a stirrer and originally containing $V$ ml of component A, acts as a mixing chamber. As each drop leaves the funnel, an equal-sized drop of second component B enters from a second funnel. The volume of liquid in the mixing funnel is kept constant by preventing the ingress of air. If it is assumed that the

two solvents mix without over-all change in volume, then the fraction by volume of component A in the liquid leaving the mixing chamber and entering the column after time $t$ is given by $V_A/V = \exp(-kt/V)$, when the eluting solution leaves the mixing chamber at a constant rate $k$ ml per unit time.

This "constant-volume" method of mixing, although effective for many purposes, restricts the shape of the component-composition graph to an exponential type in which the concentration of the second component rises rapidly in the early stages and much more gradually later. Increase in concentration of component B is approximately linear only in the early stages. Usually either a linear or a "concave" composition graph is preferable to the "convex" graph always obtained with this type of apparatus. However, there is little doubt that this method has been used more frequently than any other.

Much greater flexibility in the type of gradient can be achieved by introducing component B at a rate different from that at which the mixture leaves the mixing chamber. Equations for the mixing of two eluting agents under such conditions have been given by Lakshmanan and Lieberman[12] and also by Colás.[13] In practice, simple automatically acting apparatus is used, which may impose greater restrictions. Syphoning under atmospheric pressure is the basis of a commonly used mixing method of considerable value. Here, a boiling tube (reservoir) of constant cross-sectional area containing component B and a cylindrical separating funnel, equipped with a stirrer and containing component A, are juxtaposed so that the liquid surfaces are at the same level. The liquids are then connected by a narrow-bore U-shaped syphon tube. This should preferably be filled with component B, as, in this way, sharp density gradients, which would result in liquid transfer, never exist in the syphon tube. As liquid is allowed to drop at a constant rate from the funnel, so syphoning transfers component B into the funnel, and the levels drop at identical rates in the two vessels. Naturally neither level must drop below the bottom of the syphon tube, and "topping up" is not permissible during the process. If it is assumed that no over-all change in volume occurs on mixing, the proportion by volume of component A in each drop entering the column is given by—

$$F_A \equiv \frac{V_A}{V_1} = \left[1 - \frac{ka_1t}{V(a_1 + a_2)}\right]^{\frac{a_2}{a_1}}$$

where $V_A$ = volume in millilitres of component A in funnel at time $t$ (from beginning of the process),

$V_1$ = total volume in millilitres of liquid in funnel at this time

$$\left(V_1 = V - \frac{ka_1t}{a_1 + a_2}\right),$$

$V$ = volume in millilitres of liquid originally in funnel,

$a_1$ = cross-sectional area in square centimetres of the funnel,

$a_2$ = cross-sectional area in square centimetres of the reservoir and

$k$ = rate in millilitres per unit time at which the mixture leaves the funnel.

The fraction $F_B$ of component B in each drop is equal to $1 - F_A$.

The shape of the component-composition graph with this method of mixing depends on the ratio of cross-sectional areas of the two vessels, being linear if these areas are equal, concave (*i.e.*, $d^2F_B/dt^2$ is positive throughout the process) if $a_1 > a_2$ and convex ($d^2F_B/dt^2 < 0$) if $a_1 < a_2$. This method is as simple as the constant-volume method and is more flexible in the type of gradient that it can produce.

Occasionally it is advantageous to employ a wider range of variations in composition of eluting solution than is provided by the mixture of two eluting agents. In this way it may be possible to effect separations on one column that might otherwise require some preliminary treatment or more than one chromatographic procedure. Stepwise elution with various eluting agents is occasionally used and would certainly be improved by a gradient process. Also, if more than two eluting agents are used, the first and last need not be completely miscible, provided an adequate excess of an intermediate eluting agent is interposed. It is considered that all contingencies could be covered by the use of three eluting agents, and that the introduction of further agents would merely add complication. The mixing methods to be described have been applied in these laboratories for separating mixtures of products produced in organic reactions when investigation was also required of the more

complex impurities formed in trace amounts. They could also be applied to preliminary separations of small amounts of natural material.

Simultaneous mixing of three eluting agents at different rates can be effected by extensions of the two sets of apparatus already described. Parallel mixing in which components B and C are simultaneously, but separately, added to component A can easily be carried out, but usually provides no advance over the gradual mixing of two multi-component eluting solutions. Only series mixing in which component C is added to component B and, simultaneously, this mixture is added to component A readily provides a gradient ranging through three components, the concentration of component B at first rising and then falling.

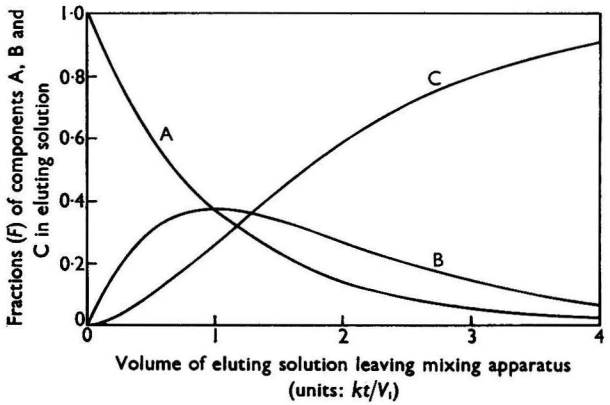


Fig. 1. Composition of eluting solution produced by constant-volume mixing method when identical volumes are used in the first two funnels ($V_2 = V_1$)

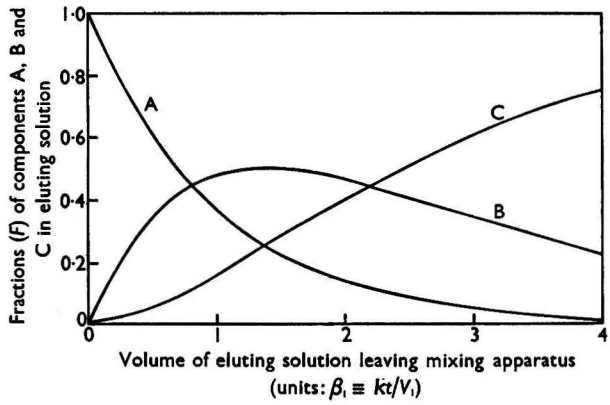


Fig. 2. Composition of eluting solution produced by constant-volume mixing method when $V_2 = 2V_1$

## CONSTANT-VOLUME METHOD

Three separating funnels (exact size and shape are not important) are, for the purpose of clarity, labelled 1, 2 and 3 and contain $V_1$ ml of component A, $V_2$ ml of component B and $V_3$ ml of component C, respectively. Funnels 1 and 2 are equipped with magnetic stirrers or stirrers possessing air-tight glands. Funnel 1 is fitted to the top of the column, funnel 2 to funnel 1 and funnel 3 to funnel 2, so that, with the taps open, for every drop that enters the column from funnel 1, equal amounts are transferred from funnel 3 to funnel 2 and from funnel 2 to funnel 1. Then, if transference to the column is slow, stirring efficient,

temperature constant and no over-all changes in volume occur on mixing, the composition by volume of the liquid in funnel 1 after time $t$ is given by—

$$F_A \equiv \frac{V_A}{V_1} = \exp\left(-\frac{kt}{V_1}\right) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$F_B \equiv \frac{V_B}{V_1} = \frac{V_2}{V_2 - V_1}\left[\exp\left(-\frac{kt}{V_2}\right) - \exp\left(-\frac{kt}{V_1}\right)\right] \quad .. \quad .. \quad .. \quad .. \quad (2)$$

when $V_1 \neq V_2$, and, when $V_1 = V_2$

$$F_B \equiv \frac{V_B}{V_1} = \frac{kt}{V_1}\exp\left(-\frac{kt}{V_1}\right) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

The fraction by volume $F_C$ of component C is given by $1—F_A—F_B$. In equations (1), (2) and (3), $V_A$, $V_B$ and $V_C$ are the volumes of liquids A, B and C, respectively, that are mixed in vessel 1 after time $t$. The mixture enters the column at the constant rate $k$ ml per unit time.

Relative concentrations when $V_1 = V_2$ are shown in Fig. 1. Although of occasional use, the concentration of component B remains too low (maximum value for $F_B$ being 0·37) and the concentration of component C rises too rapidly for general application. In the more useful instance when $V_2 = 2V_1$, component B attains a maximum concentration ($F_B = 0{\cdot}5$) when $kt/V_1$ has the value 1·39. This maximum is, moreover, very flat and $F_B$ has a value greater than 0·45 when $0{\cdot}9 < kt/V_1 < 2{\cdot}1$ (see Fig. 2).

## Syphon method

Two reservoirs (labelled 2 and 3) are juxtaposed and attached to a separating funnel (vessel 1). Each vessel must be of constant cross-sectional area throughout the regions to be occupied by the columns of liquid undergoing the syphoning process. Vessels 1 and 2 are equipped with stirrers, and small narrow-bore syphon tubes connect vessels 1 and 2 and vessels 2 and 3. Components A, B and C are placed in vessels 1, 2 and 3, respectively, the positions of the vessels being altered until the levels are equal. Just before the chromatographic run is begun, the syphon from tube 3 to tube 2 is primed with component C and that from tube 2 to funnel 1 is primed with component B. In this way only minute density gradients can occur in the syphon tubes and they have a negligible effect on the over-all process. Liquid is allowed to drip at constant rate from the funnel 1 into the column, the liquids in vessels 1 and 2 being stirred. As the three liquid surfaces are open to atmospheric pressure, levels slowly drop in all three vessels. The fractions of the three components in the separating funnel and, therefore, in an issuing drop after time interval $t$ are given by—

$$F_A \equiv \frac{V_A}{V_1} = \left(1 - \beta_1\right)^{\eta - 1} \quad .. \quad .. \quad .. \quad .. \quad (4)$$

$$F_B \equiv \frac{V_B}{V_1} = \frac{k(\eta - 1)}{U_1 \eta}\left(1 - \beta_1\right)^{\eta - 1}\int_0^t \left(1 - \beta_1\right)^{-\eta}\left(1 - \beta_2\right)^{\frac{a_2}{a_1}} dt \quad .. \quad .. \quad (5)$$

$$F_C = 1 - F_A - F_B$$

where $V_A$, $V_B$ and $V_C$ = volumes of components A, B and C, respectively, in funnel 1 after time $t$,

$a_1$, $a_2$ and $a_3$ = cross-sectional areas of vessels 1, 2 and 3, respectively,

$V_1$ = total volume in vessel 1 after time $t$,

$$V_1 = V_A + V_B + V_C = U_1(1 - \beta_1),$$

$U_1$ and $U_2$ = original volumes of liquids in vessels 1 and 2, respectively,

$k$ = rate in volume per unit time at which the mixture leaves vessel 1 and enters the column,

$\eta = (a_1 + a_2 + a_3)/a_1$,

$\beta_1 = kt/U_1\eta = ka_1t/U_1(a_1 + a_2 + a_3)$ and

$\beta_2 = ka_2t/U_2(a_1 + a_2 + a_3)$.

Although in the general case, when $a_3/a_2$ and $\eta$ have values that need not be whole numbers, the integral in equation (5) cannot be expressed in terms of elementary functions, it can readily be evaluated in important special cases. Three such cases are shown below—

(*i*) When $a_1 = a_2 = a_3$,

then $F_A = (1 - \beta_1)^2$ and $F_B = \beta_1 (2 - \beta_1 - \beta_2)$

$[\beta_1 = kt/3U_1$ and $\beta_2 = kt/3U_2]$.

(*ii*) When $a_2 = a_3$ ($= a$ say) and $\eta \neq 2$,

then $F_A = (1 - \beta_1)^{\eta-1}$

and $F_B = (1 - \beta_2) + \gamma^{-1} (1 - \beta_1) [1 - (1 + \gamma) (1 - \beta_1)^{\eta-2}]$

$[\beta_1 = ka_1t/U_1 (2a + a_1)$, $\beta_2 = kat/U_2 (2a + a_1)$

and $\gamma = U_2 (2a - a_1)/U_1a]$.

(*iii*) When $a_2 = a_3 = a_1/2$ (here $\eta = 2$),

then $F_A = 1 - \beta_1$

and $F_B = \beta_1 - \beta_2 - 2{\cdot}303\ \beta_1^{-1}\beta_2 (1 - \beta_1) \log_{10} (1 - \beta_1)$

$[\beta_1 = kt/2U_1$ and $\beta_2 = kt/4U_2]$.

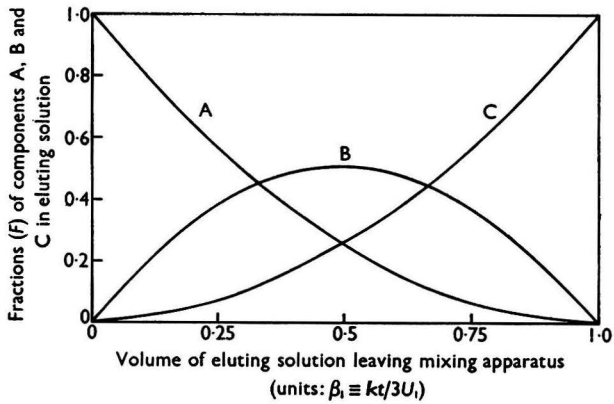


Fig. 3. Composition of eluting solution produced by syphon method with vessels of equal cross-sectional area ($a_1 = a_2 = a_3$) and with equal initial volumes in the first two vessels ($U_2 = U_1$)

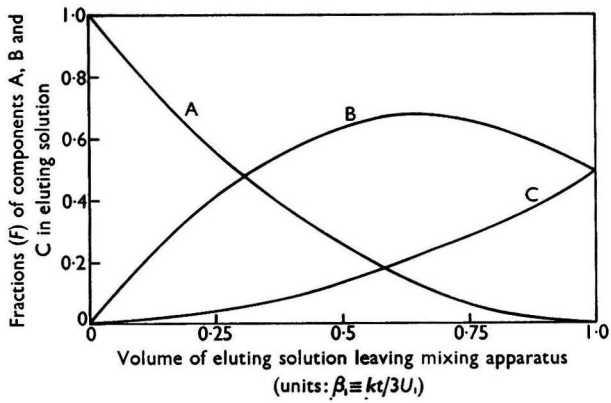


Fig. 4. Composition of eluting solution produced by syphon method with vessels of equal cross-sectional area ($a_1 = a_2 = a_3$) and when $U_2 = 2U_1$

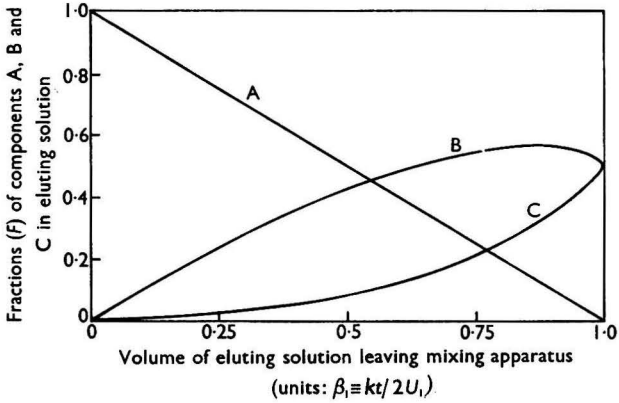


Fig. 5. Composition of eluting solution produced by syphon method when $a_1 = 2a_2 = 2a_3$ and $U_2 = U_1$

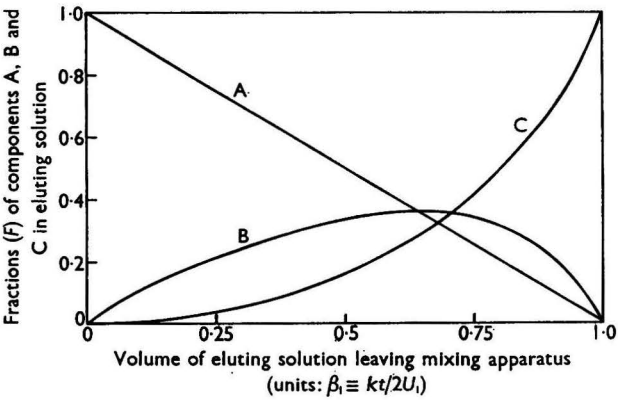


Fig. 6. Composition of eluting solution produced by syphon method when $a_1 = 2a_2 = 2a_3$ and $U_2 = 2U_1$

Examples of composition curves are shown in Figs. 3 to 6. If the three vessels have identical cross-sectional areas, then the three component-composition graphs are quadratic curves (see Figs. 3 and 4). If, in addition, equal volumes are present in vessels 1 and 2, component B reaches its maximum concentration (50 per cent.) half-way through the process (see Fig. 3). By increasing the initial volume of solvent B, its maximum concentration is increased. If the cross-sections are such that $\eta = 2$, the concentration of solvent A decreases linearly. This is shown in Figs. 5 and 6. When the constant-volume method (*e.g.*, see Figs. 1 and 2) is used, the mixing and elution process may be continued indefinitely, so that, if desired, the gradient could be applied near the beginning of a chromatographic run. In contrast, the syphon method (see Figs. 3 to 6) necessarily terminates when $\beta_1$ becomes unity. In practice, alteration in the cross-section of the funnel near the tap will produce marked changes in the composition of the eluting solution just before this stage is reached. The process should, of course, be stopped before the liquid levels have fallen to places where the cross-sectional areas of any of the vessels undergo change. Figs. 1 to 6 may be used to assess approximate shapes of the component-composition graphs produced in other circumstances.

Advantages that accrue from using superimposed gradients have been suggested previously by Bock and Nan-Sing Ling[14] and Piez.[15] Theories on the mechanism of transport of solutes through columns under the action of a solvent of varying composition have been

outlined by Drake[16] and by Freiling,[17] following the qualitative treatment by Alm, Williams and Tiselius.[18]

I thank the Department of Scientific and Industrial Research for a special research allocation.

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# Notes

## THE DETERMINATION OF CAPTAN IN PLANT EXTRACTS

SPRAY residues of the fungicide captan, N-trichloromethylthio-4-*cyclo*hexene-1 : 2-dicarboxyimide, are generally determined by methods based on the formation of a colour with resorcinol.[1,2] Burchfield and Schechtman[3] have described a simpler method, based on the reaction of captan with pyridine and tetraethylammonium hydroxide to form a coloured complex, and have applied this technique to the determination of captan in soil. We have found their method to be suitable for determining captan in spray residues, as it is not susceptible to interference from plant extracts or other spray chemicals likely to be present on the surface of the plant. Certain refinements in technique are also suggested.

### Method

REAGENTS—

*Benzene*—Fractionate before use.

*Pyridine*—Fractionate in presence of potassium hydroxide before use.

*Tetraethylammonium hydroxide solution*—Dilute the commercially available 25 per cent. aqueous solution to 0·073 *M* (check by titration against standard acid), add charcoal, set aside for 15 minutes, and filter through a Whatman No. 50 filter-paper. Repeat this procedure until the solution has a negligible optical-density blank value at 430 m$\mu$.

*Pyridine - tetraethylammonium hydroxide solution*—Add 3 volumes of the 0·073 *M* tetraethylammonium hydroxide to 7 volumes of the pyridine. Prepare this solution freshly before use.

*Captan*—Recrystallise from benzene until the product has a constant melting-point (173° to 174° C).

PROCEDURE—

Transfer a suitable aliquot of a solution of captan in benzene to a round-bottomed flask. Place the flask on a water bath, and evaporate the benzene in a stream of clean dry air. Just before evaporation is complete, remove the flask from the water bath, and allow the final droplet

to evaporate slowly at room temperature; over-heating of the dried deposit causes an incomplete colour reaction. Add 10 ml of pyridine - tetraethylammonium hydroxide solution, swirl to dissolve the residue, set aside for about 3 minutes, and measure the optical density of the solution in a 2-cm cell at 430 m$\mu$ with a spectrophotometer. (The pyridine - tetraethylammonium hydroxide solution alone has a negligible blank value at this wavelength.) A calibration graph obeyed Beer's law over the range 0 to 75 $\mu$g of captan.

## Discussion of the method

We have found that the colour produced by the reaction begins to fade after 15 minutes and not 30 minutes, as reported by Burchfield and Schechtman.[3] These workers found the absorption maximum to be at 415 m$\mu$, a value later amended[4] to 427 m$\mu$. From the absorption spectrum shown in Fig. 1, we have taken the maximum to be 430 m$\mu$.

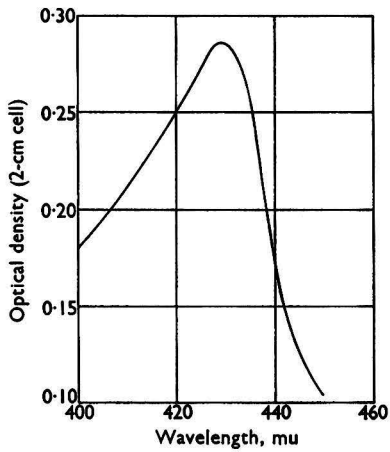


Fig. 1. Absorption spectrum of the product of the reaction between pyridine - tetraethylammonium hydroxide solution and 29 $\mu$g of captan

### Effects of other pesticides—

Burchfield and Schuldt[5] have shown that several spray chemicals containing active halogen react with pyridine and alkali to form coloured complexes. A 500-$\mu$g portion of each of six compounds, all of which contained active chlorine groups and could be used in conjunction with captan sprays, was added to a solution of 50 $\mu$g of captan in benzene. The materials examined were gamma-BHC, chlorobenzilate, chlorbenside, DDT, dieldrin and endrin, none of which developed an appreciable blank value or interfered in the determination of captan.

### Effects of fruit and leaf extracts—

Extracts from fruit were prepared by dipping strawberries (50 g) and apples (20 g) into two successive portions of benzene at 50° to 60° C. Each solution was filtered through benzene-extracted cotton-wool, and an aliquot of a solution of captan in benzene was added. Each mixture was gently shaken with 0·5 g of charcoal (Darco G60, obtained from the Darco Carbon Corporation, New York) for 20 minutes and then filtered through a Whatman No. 50 filter-paper; the charcoal was washed with successive portions of warm benzene. The filtrates were evaporated and treated by the proposed procedure, the final solutions being filtered through a loosely packed plug of cotton-wool to remove waxy particles before optical-density measurements were made. The recoveries of captan are shown in Table I. With the extract of strawberries, a large residue of wax was obtained, which prevented complete reaction between the captan and the pyridine - tetraethylammonium hydroxide solution. Some of this wax was precipitated by adding 15 to

20 ml of redistilled analytical-reagent grade acetone to between 2 and 3 ml of the concentrated benzene extract and then setting aside at 5° C overnight. The wax was separated by filtration, and the solution was evaporated as before. However, the results in Table I show that slight losses occurred when this procedure was used.

TABLE I

RECOVERY OF CAPTAN FROM PLANT EXTRACTS

| Sample | Amount of captan added, μg | Amount of captan found, μg | Recovery, % |
|---|---|---|---|
| Apple fruits .. .. | 25 | 25, 24·5 | 100, 98 |
| | 50 | 49, 51 | 98, 102 |
| Apple leaves .. .. | 25 | 25, 24 | 100, 96 |
| | 50 | 48·5, 49 | 97, 98 |
| Strawberry fruits .. | 50 | 47, 46 | 94, 92 |
| Broad-bean leaves .. | 25 | 25, 25·5 | 100, 102 |
| | 50 | 48, 49 | 96, 98 |
| Potato leaves .. .. | 50 | 50,48 | 100, 96 |

For the analyses of the leaf extracts reported in Table I, 5-g portions of fresh leaves were dried in air and then extracted with benzene in a continuous percolator. Aliquots of captan solution were added, and the solutions were then decolorised and analysed as described above. The benzene extracts of the leaves of some varieties of apple, *e.g.*, Bramley, had to be left in contact with the charcoal overnight for complete decolorisation.

The results in Table I show that the recovery of captan from plant extracts is satisfactory, except perhaps in the presence of high concentrations of plant wax.

We thank Mr. D. Odhiambo for experimental assistance.

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## THE RAPID DETERMINATION OF TOLUENE AND STYRENE VAPOURS IN THE ATMOSPHERE

THE method[1] for determining the concentration of benzene vapour in an atmosphere is rapid; it involves the use of easily portable apparatus and is sufficiently accurate for estimating the safety of an atmosphere likely to contain benzene.

This Note describes modifications made to the procedure that permit the same apparatus to be used for the rapid determination of toluene or styrene vapour.

### PREPARATION OF STANDARD ATMOSPHERES

Each of two streams of air from separate blowers was passed at a controlled rate of flow, via a constant-head device, through tubes containing calcium chloride and charcoal and through a flowmeter; one stream then passed through a weighed U-tube containing a pool of toluene or styrene and became almost saturated with vapour. The two streams were mixed at a T-junction, and the mixture was passed into a 1500-ml flask fitted with two exit tubes. One exit tube was connected to a meter measuring the total volume of air passing through the flask, and the other was used to bleed off small volumes of air for the determination. After each run, the concentration of vapour in the air passing through the apparatus was calculated from the weight of hydrocarbon lost from the U-tube and the total throughput of air.

By controlling the rates of flow of the streams of air, concentrations of toluene vapour ranging from 80 to 200 p.p.m. v/v and of styrene vapour ranging from 30 to 200 p.p.m. v/v were prepared.

### Determination of toluene vapour

The formolite reagent used for determining benzene vapour (a 5 per cent. solution of formalin in concentrated sulphuric acid) cannot be used for toluene because (*a*) it is more sensitive to toluene than to benzene and (*b*) the safety threshold limit for toluene vapour (200 p.p.m.) is higher than that for benzene (25 p.p.m.). With the apparatus used for determining benzene vapour,[1] the colour produced by a reasonable number (about five) of inflations of the aspirator bulb with an atmosphere containing the maximum permissible concentration of toluene is therefore much too dark for comparison with a standard colour.

The sensitivity of the formolite reagent was found to depend on its content of sulphuric acid rather than of formaldehyde. It was also found that replacement of part of the sulphuric acid in the reagent by syrupy phosphoric acid reduced the sensitivity without introducing undesirable complications, *e.g.*, when water was used as diluent, precipitation occurred during absorption of toluene and colour comparison became impossible.

The sensitivity of the reagent was adjusted by using various mixtures of sulphuric and phosphoric acids so that, at the threshold concentration of toluene vapour, a reasonable tint for comparison purposes was produced in the reagent by five inflations of the aspirator bulb. The tint was analysed in terms of absorption coefficients, and an inorganic solution was then prepared to match it; this solution was used as the standard colour.

#### Reagents—

*Orthophosphoric - sulphuric acid mixture*—Measure 100 ml of orthophosphoric acid, sp.gr. 1·75, and 100 ml of sulphuric acid, sp.gr. 1·84, into separate measuring cylinders. Transfer the orthophosphoric acid to a 250-ml conical flask immersed in ice - water mixture, and carefully add the sulphuric acid, with constant shaking. Rinse both measuring cylinders with the acid mixture, and return the rinsings to the flask. This mixture can be prepared in bulk and stored.

*Standard colour solution*—Mix 64·3 ml of 0·1 *M* cobalt sulphate, 1·7 ml of *M*/60 potassium dichromate and 34·0 ml of 0·125 *M* ammonium nickel sulphate, all solutions being prepared from analytical-reagent grade salts. This solution can be stored indefinitely.

*Formaldehyde solution, 40 per cent. w/v.*

#### Procedure—

Use the apparatus previously described for determining benzene vapour.[1] Place 0·5 ml of formaldehyde solution in the bubbler, and dilute to 10 ml with orthophosphoric - sulphuric acid mixture. Note the number of inflations of the bulb required to produce a colour in the reagent mixture equivalent to that of the standard solution, and read the concentration of toluene in the atmosphere from the figures below—

| Number of inflations .. .. | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|
| Toluene concentration, p.p.m. .. | 250 | 200 | 160 | 140 | 125 | 110 | 100 |

Occasionally check the bulb of the aspirator to ensure that the time taken for one inflation is 2 minutes and that 120 ml of air are aspirated per inflation.

### Determination of styrene vapour

The formolite reagent was unsatisfactory for determining styrene because the colour produced by an atmosphere containing 100 p.p.m. of styrene (the safety threshold limit) was too faint to be of any practical use unless fifteen to twenty inflations were made, thereby prolonging the test unduly. Concentrated sulphuric acid was found to be a more sensitive reagent; a yellow colour was formed, which could be easily reproduced as a standard from stable inorganic compounds. The colour produced by five or six inflations with an atmosphere containing 100 p.p.m. of styrene was, in fact, too intense for satisfactory comparison. However, when the acid was slightly diluted with water, the solution so obtained gave, after five inflations at 100 p.p.m. of styrene, a colour suitable for comparison, which became substantially deeper during a further inflation. This colour, as in the test for toluene, was analysed with a photo-electric absorptiometer, and an inorganic standard solution was prepared.

#### Reagents—

*Sulphuric acid, 89·5 per cent. w/v*—To 15 ml of distilled water in a 250-ml conical flask slowly add, with shaking and cooling, 80 ml of cooled sulphuric acid, sp.gr. 1·84. Check the concentration of the solution (33·1 *N*) by titration or by measuring its specific gravity (1·8172 at 15° C),

and adjust if necessary. (An increase of 1 per cent. in the sulphuric acid content of the solution causes an increase of almost 10 per cent. in the intensity of the colour produced by a given concentration of styrene.)

*Standard colour solution*—To 6·9 ml of $M/60$ potassium dichromate add 4·3 ml of 0·1 $M$ cobalt sulphate, and dilute to 250 ml in a calibrated flask.

PROCEDURE—

Use the apparatus described for determining benzene vapour.[1] Place 10 ml of 89·5 per cent. sulphuric acid in the bubbler, and note the number of inflations of the bulb required to produce a colour equivalent to that of the standard solution. Read the concentration of styrene in the atmosphere from the figures below—

| Number of inflations .. .. | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|
| Styrene concentration, p.p.m. .. | 165 | 125 | 100 | 85 | 70 | 60 | 44 | 50 |

Occasionally check the bulb of the aspirator, as described for determining toluene.

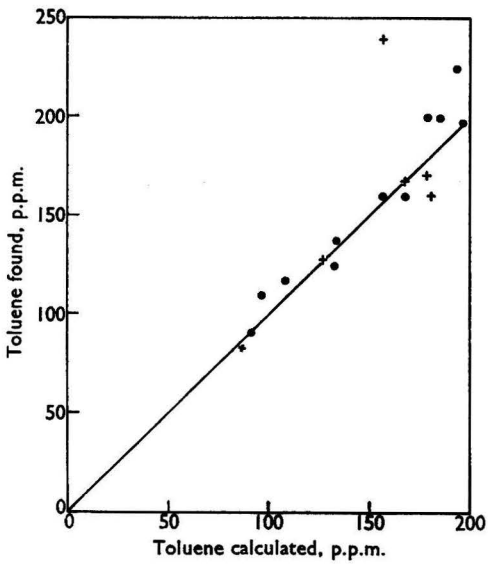


Fig. 1. Graph of toluene calculated against toluene found by: +, butanone method; ●, proposed method

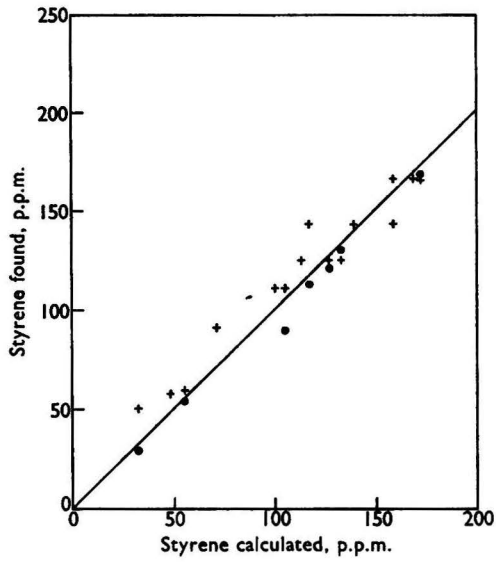


Fig. 2. Graph of styrene calculated against styrene found by: +, proposed method; ●, nitration method

## DISCUSSION OF THE METHODS

The procedure for determining toluene was checked by comparison with a method in which the toluene is nitrated and the dinitrotoluene formed is determined colorimetrically with butanone[2]; a graph of the results found by both methods plotted against the calculated concentrations of toluene is shown in Fig. 1. The procedure for determining styrene was similarly checked by comparison with a nitration method,[3] and the results are shown in Fig. 2.

The reagent mixture used for determining toluene is comparatively insensitive to benzene vapour, the presence of which in the test atmosphere causes only a small error in the concentration of toluene found. Xylene vapour, however, produces a colour similar to that produced by a comparable concentration of toluene. A concentration of 100 p.p.m. of styrene produced no colour in the reagent mixture after six inflations of the bulb.

Concentrations of 100 p.p.m. each of benzene, toluene and α-methylstyrene produced no colour in the solution used for determining styrene after six inflations of the bulb, but vinyltoluene produced a colour closely similar in shade and intensity to that produced by the same concentration of styrene with the same number of inflations.

The apparatus used can be obtained from Messrs. Siebe Gorman and Co. Ltd., Chessington, Surrey; glass colour standards that can be used in place of the inorganic solutions are available from Tintometer Ltd., Salisbury, Wilts.

This work was carried out on behalf of the Toxic Gases Committee of the Ministry of Labour and National Service, and we thank the Department of Scientific and Industrial Research for permission to publish this Note.

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## THE ABSORPTIOMETRIC DETERMINATION OF PALLADIUM AS ITS 2-MERCAPTO-4:5-DIMETHYLTHIAZOLE COMPLEX

Reagents suggested for the identification or determination of palladium[1,2,3] include compounds containing the *p*-nitrosophenylamino group, *p*-NO—$C_6H_4$—N=, *p*-dimethylaminorhodanine[4,5] and its diethyl analogue,[6] *p*-dimethylaminobenzylidinethiobarbituric acid[7] and phenothiazine.[8,9] However, gold and sometimes copper and iron interfere, and a quantitative determination is susceptible to variations in the concentrations of acid and salt present.

Ryan[10,11,12] stated that the determination of palladium with 2-mercapto-4:5-dimethylthiazole was not affected by changes in acidity and concentration of salt, and it was decided to study this method in detail, checking the effects of the concentrations of acid, salt and ethanol and extending the absorption measurements to the ultra-violet region.

### Experimental

Reagents—

*2-Mercapto-4:5-dimethylthiazole solution*—Purify the reagent by twice recrystallising it from hot water. Dissolve 0·5 g of pure crystals in 50 ml of 95 per cent. ethanol, and dilute to 100 ml with distilled water.

*Standard palladium solution, 10 μg per ml, in 0·05 N hydrochloric acid*—Dissolve 0·1665 g of palladium chloride in 250 ml of hot 2 *N* hydrochloric acid, cool, and dilute to 1 litre. Dilute 50 ml of this solution to 500 ml with distilled water.

Choice of light source and filters—

Solutions containing 200 μg of palladium were treated by Ryan's procedure.[10] The results obtained by using a Spekker absorptiometer fitted with a mercury-vapour and a tungsten-filament lamp and various filters are shown in Table I; these results confirmed that absorption occurred mainly in the ultra-violet region.

Colour development and stability—

The optical densities of a series of solutions containing 100 to 1000 μg of palladium per 100 ml were measured over a period of 1 hour. With the mercury-vapour lamp and Wood's glass filters, there was a slight increase in intensity over the first 5 minutes, but with the tungsten-filament lamp and Ilford No. 601 filters, maximum intensity was reached immediately.

Concentration of acid—

To confirm that the absorption was independent of the concentration of hydrochloric acid present, the optical densities of a series of solutions containing 100 to 500 μg of palladium in different concentrations of hydrochloric acid and in mixtures of hydrochloric and nitric acids were measured; the results are summarised in Table II.

TABLE I

EFFECT OF LIGHT SOURCE AND FILTER ON OPTICAL DENSITY

Each solution contained 200 μg of palladium. Optical-density measurements were made with a Spekker absorptiometer

| Lamp | Filter | Wavelength of maximum transmittance, mμ | Absorptiometer-drum reading (4-cm cell) |
|---|---|---|---|
| Mercury vapour | Wood's glass | 365·0 | >1·3 |
| | Chance OV1 + Wratten No. 2 | 407·8 | 0·784 |
| Tungsten filament | Kodak No. 1 | ~420 | 0·602 |
| Mercury vapour | Chance OB2 + Wratten No. 50 | 435·8 | 0·490 |
| Tungsten filament | Ilford No. 601 | ~430 | 0·475 |
| | Kodak No. 2 | ~430 | 0·360 |
| | Ilford No. 602 | ~470 | 0·250 |

TABLE II

EFFECT OF VARIATION IN CONCENTRATION OF ACID

| Concentrated hydrochloric acid present, ml per 100 ml | Concentrated nitric acid present, ml per 100 ml | Absorptiometer-drum reading (tungsten-filament lamp) in presence of— | | Absorptiometer-drum reading (mercury-vapour lamp) in presence of— | |
|---|---|---|---|---|---|
| | | 200 μg of palladium (4-cm cell*) | 500 μg of palladium (2-cm cell†) | 100 μg of palladium (4-cm cell‡) | 500 μg of palladium (1-cm cell‡) |
| 1·0 | — | — | — | 0·722 | — |
| 2·0 | — | 0·500 | 0·762 | 0·729 | 0·921 |
| 5·0 | — | — | 0·773 | 0·748 | 0·943 |
| 10·0 | — | 0·500 | 0·776 | 0·765 | 0·962 |
| 20·0 | — | — | 0·773 | 0·841 | 0·991 |
| 2·0 | 2·0 | 0·518 | — | — | — |
| | 5·0 | 0·548 | 0·788 | — | 1·026 |
| | 10·0 | 0·405 | — | — | — |

* Ilford No. 601 filter. † Kodak No. 1 filter. ‡ Wood's glass filter.

Over the range 1 to 20 ml of concentrated hydrochloric acid per 100 ml of solution, there was no change in the absorption measured in the visible region, but a marked increase occurred when measurements were made in the ultra-violet region. The presence of up to 5 ml of concentrated nitric acid gave increased optical-density values, but higher concentrations gave lower values; this effect was considered to be caused by oxidation of the reagent. All nitric acid must therefore be removed before colour development.

CONCENTRATION OF ETHANOL—

Ryan[10] stated that the presence of ethanol was necessary to prevent precipitation of the complex, but that its concentration affected the absorption of the solution. Two series of solutions containing 200 and 1000 μg of palladium, respectively, and having ethanol contents from 0 to 30 ml per 100 ml were prepared, and measurements on these confirmed that the optical densities were affected; however, no precipitation of the complex was noticed in absence of ethanol, even after the solutions had been set aside for 1 hour. No precipitation was observed when the concentration of palladium was increased to 1600 μg per 100 ml or when the concentrated hydrochloric acid content was increased from 1 to 20 ml. However, when 5- and 10-ml portions of concentrated nitric acid were added, turbidity was immediately observed.

CONCENTRATION OF SODIUM CHLORIDE—

Experiments in which sodium chloride was added to the solution confirmed Ryan's findings that the optical density was unaffected by the presence of this compound.

CALIBRATION AND ADHERENCE TO BEER'S LAW—

The Spekker absorptiometer was calibrated by using solutions containing 2 ml of concentrated hydrochloric acid and 1 ml of 2-mercapto-4 : 5-dimethylthiazole solution for each 500 μg of palladium present and measuring the optical densities against a reagent blank solution after 5 minutes. The results (see Fig. 1) showed that, when the tungsten-filament lamp was used with Ilford

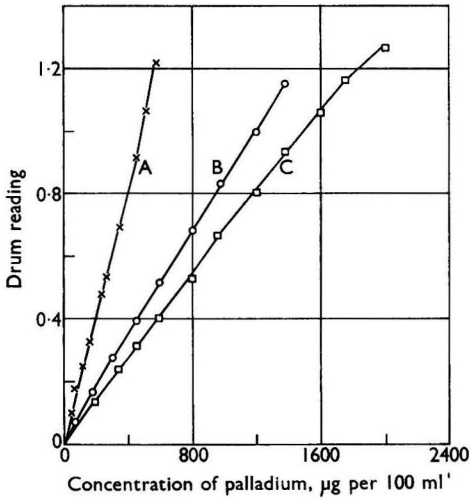


Fig. 1. Calibration graph for determining palladium as its complex with 2-mercapto-4 : 5-dimethylthiazole: curve A, mercury-vapour lamp and Wood's glass filters; curve B, tungsten-filament lamp and Kodak No. 1 filters; curve C, tungsten-filament lamp and Ilford No. 601 filters

No. 601 or Kodak No. 1 filters, the absorption deviated slightly from Beer's law. However, Beer's law was obeyed when measurements were made in the ultra-violet region with the mercury-vapour lamp and Wood's glass filters.

A comparison of sensitivities produced the results shown in Table III.

TABLE III

AMOUNTS OF PALLADIUM CORRESPONDING TO ABSORPTIOMETER-DRUM READING OF 0·10

| Lamp | Filter | Length of cell, cm | Amount of palladium corresponding to absorptiometer-drum reading of 0·10, μg |
|---|---|---|---|
| Mercury vapour.. | .. Wood's glass | 4 | 13 |
| | | 1 | 50 |
| Tungsten filament | .. Kodak No. 1 | 4 | 30 |
| | | 1 | 120 |
| | .. Ilford No. 601 | 4 | 40 |
| | | 1 | 155 |

CONCLUSIONS

When the absorption of the palladium - 2-mercapto-4 : 5-dimethylthiazole complex is measured in the ultra-violet region by using a mercury-vapour lamp and Wood's glass filters, there is an increase of 160 per cent. in sensitivity and the solutions obey Beer's law. Increases in the concentration of hydrochloric acid cause slightly higher optical-density values, and the acidity must therefore be controlled; this constitutes a disadvantage not arising when a tungsten-filament lamp is used.

Addition of ethanol to prevent turbidity is unnecessary, but nitric acid must be removed.

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## THE DETERMINATION OF NITROGEN IN BORON NITRIDE

BORON nitride prepared at a low temperature reacts readily with hydrochloric acid in a sealed tube at 200° C.[1,2,3] If prepared at a high temperature, the product is less reactive, but attempts have been made to decompose it by Kjeldahl digestion[4] and by fusion with sodium or potassium hydroxide.[5,6] Neither method is completely satisfactory for the determination of nitrogen, since a residue remains even after prolonged Kjeldahl digestion, and sodium and potassium hydroxide melts have a marked tendency to froth. The drawbacks to both these procedures have been overcome, and the revised methods are described below.

### FUSION WITH ALKALI

In contrast to sodium and potassium hydroxides, lithium hydroxide monohydrate is granular and has a relatively high melting-point. Consequently, the free escape of ammonia during fusion is permitted. When lithium hydroxide was used for the fusion, the nitrogen content found in four determinations on a sample of commercial boron nitride stated by the manufacturers (the Carborundum Co., Niagara Falls, New York State) to have a purity of 99 per cent. was 55·1 ± 0·2 per cent.

APPARATUS—

A silver hygrometer thimble, 20 mm in diameter and 40 mm high, contained in a glass test-tube, 55 mm × 150 mm, fitted with a ground-glass joint carrying two tubes is used for the fusion. The nitrogen-inlet tube extends to within 70 mm of the bottom of the test-tube, and the outlet tube is bent downwards to dip into a 200-ml beaker. A silica crucible half filled with sand is used as a heating bath.

REAGENTS—

*Hydrochloric acid*, 0·25 N.
*Boric acid solution, 2 per cent. w/v*—Neutralised to the change-point of methyl orange.
*Lithium hydroxide monohydrate.*
*Methyl orange solution, 0·1 per cent. w/v.*

PROCEDURE—

Place a 0·2-g sample of boron nitride on about 1 g of lithium hydroxide in the bottom of the thimble, and cover with a further 2 g of lithium hydroxide. Place the thimble in the test-tube, and stand the latter in the sand-bath. Fit the ground-glass joint, and arrange the outlet tube to dip just below the surface of 50 ml of 2 per cent. boric acid solution and 2 drops of methyl orange solution contained in the 200-ml beaker. Adjust the nitrogen supply to about 2 bubbles per second, and purge the apparatus for at least 5 minutes. Heat the sample to between 500° and 550° C. After 30 minutes, titrate with 0·25 *N* hydrochloric acid, and continue the titration at 10-minute intervals until there is no further increase in titre over 30 minutes.

### Decomposition by acid

The reaction appears to be a hydrolysis and to proceed in accordance with the equation—

$$BN + 3H_2O = H_3BO_3 + NH_3.$$

The use of Kjeldahl digestion with an oxidant as catalyst would therefore hardly be expected to effect complete decomposition of boron nitride.[4] In confirmation of this view, residues remained when the usual catalysts, copper sulphate, mercuric oxide and selenium, were used and also when 0·2 g of boron nitride was heated with 6 ml of 98 per cent. w/v sulphuric acid in a sealed tube for 8 hours at 400° C. A clear solution was obtained, however, with 80 per cent. w/w sulphuric acid after heating for 8 hours at 320° C. In four determinations on the commercial material previously mentioned the nitrogen content found by this procedure was 55·4 ± 0·1 per cent.

### Reagents—

*Sulphuric acid, approximately* 80 *per cent. w/w*—Pour 100 ml of concentrated sulphuric acid into 50 ml of water.

*Boric acid solution,* 4 *per cent. w/v.*

*Sodium hydroxide solution,* 45 *per cent. w/v.*

*Hydrochloric acid,* 0·25 N.

*Methyl orange solution,* 0·1 *per cent. w/v.*

### Procedure—

Deposit a 0·2-g sample of boron nitride in the bottom of a Carius tube by means of a weighing stick held on an extension rod. Add 6 ml of 80 per cent. sulphuric acid, taking care not to wet the upper part of the tube. Seal the tube, and place in a Carius furnace set to reach 320° C in about 2 hours, maintain at this temperature for 8 hours, and then allow the furnace to cool. Carefully open the tube, and wash the contents into the flask of a distillation unit. Make up the volume to approximately 250 ml, and add 30 ml of 45 per cent. sodium hydroxide solution. Distil the ammonia into 80 ml of 4 per cent. boric acid solution, and collect 120 ml of distillate. Titrate against 0·25 *N* hydrochloric acid with methyl orange solution as indicator. A blank determination must be made, and its value subtracted from the titre.

### Conclusions

Hydrolysis by 80 per cent. w/w sulphuric acid is preferable, because visibly complete solution is obtained and there is no chance of catalytic cracking of the ammonia. Fusion with lithium hydroxide has, however, the advantage that nitrogen and boron may be determined on the same 0·2-g sample.

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## THE APPLICATION OF CELLULOSE PHOSPHATE TO THE TESTING OF HIGH-PURITY WATER AND THE DEVELOPMENT OF A SPOT TEST OF HIGH SENSITIVITY

In the determination of minute traces of metals in water a concentration step is usually introduced. Standard techniques include solvent extraction of coloured complexes and ion-exchange separations. The sensitivity of spot tests on ordinary filter-paper is frequently too low to be of any value, but the use of cellulose phosphate greatly increases the sensitivity of the test, largely because of the ion-exchange properties of this material.[1] The fact that modified cellulose paper with a relatively high ion-exchange capacity (0·02 milli-equivalent per sq. cm) can be prepared is a further advantage

and the hydrophilic nature of the absorbent results in an extremely high rate of exchange. Absorption of metals occurs during contact of their solution within the thickness of the disc of modified cellulose. The design of the apparatus used restricts the area of that part of the paper occupied by absorbate to a circle 1 cm in diameter, thereby increasing the concentration of metal and, in turn, the sensitivity of the spot test. It has been found possible to detect $0{\cdot}4 \times 10^{-9}$ parts of copper or $2 \times 10^{-9}$ parts of iron in a 50-ml sample when the area occupied by absorbate is sprayed with a colour-forming reagent. A set of similarly prepared standards is necessary.

## Method

### Apparatus—

The polythene block used to hold the disc of cellulose phosphate paper is shown in Fig. 1.

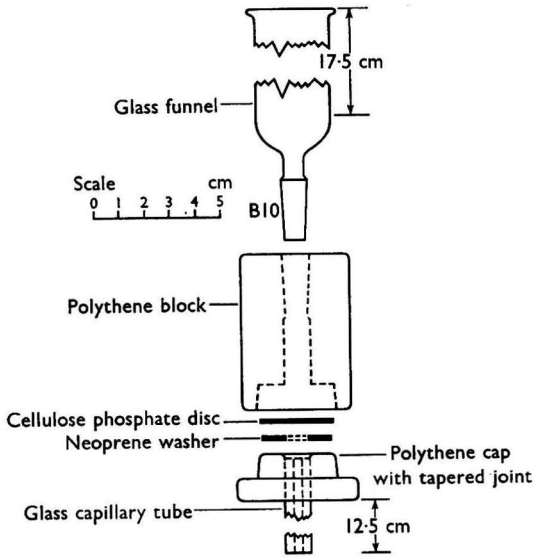


Fig. 1. Apparatus used for spot tests

The depth and taper of the cone joint on the lower part of the block were such that hand-pressure was sufficient to ensure adequately tight contact on the edges of the paper disc. A V-shaped cut-out in the outer edge of the neoprene washer allowed the wet paper disc to be easily removed when absorption was complete, and the capillary tube increased the rate of flow of water through the disc.

### Reagents—

*Cellulose phosphate*—Cellulose phosphate paper, as discs 3 cm in diameter and containing 10 per cent. (dry basis) of phosphate, was supplied by W. & R. Balston Ltd.

*Hydrochloric acid, sp.gr.* 1·18—Analytical-reagent grade.

*Rubeanic acid solution,* 0·05 *per cent. w/v, in* 80 *per cent. v/v aqueous ethanol.*

*Potassium ferrocyanide solution,* 2 *per cent. w/v, in* 0·2 N *hydrochloric acid.*

*Standard copper solution,* 0·10 *mg per ml*—Prepare by dissolving analytical-reagent grade cupric sulphate in water.

*Standard iron solution,* 0·10 *mg per ml*—Prepare by dissolving analytical-reagent grade ammonium ferric sulphate in 0·1 *N* hydrochloric acid.

### Procedure for determining copper—

Place a disc of cellulose phosphate paper on the neoprene washer, and insert firmly into the polythene block. Adjust the pH of the sample, if necessary, to greater than 4 by adding dilute ammonia solution, and pour into the funnel (avoid air locks by pouring the first portion slowly down the side of the funnel). After passage of the solution, carefully remove the disc, and place

on a clean sheet of filter-paper. Expose the disc to ammonia vapour, spray with rubeanic acid solution, and determine copper by comparing the colour produced with that of a previously prepared standard.

PROCEDURE FOR DETERMINING IRON—

Assemble the apparatus as described above, add 1 per cent. v/v of hydrochloric acid to the sample, and pour into the funnel as before. After absorption, remove the disc, dip into potassium ferrocyanide solution, and set aside for 2 minutes on filter-paper. Wash with distilled water to remove the excess of reagent solution, and determine iron by comparison against a previously prepared standard.

PREPARATION OF STANDARDS—

Prepare standards as described above, but use de-ionised water. Solutions containing copper must be used immediately after preparation to avoid loss of metal by adsorption on the walls of glass vessels. Standards may cover the ranges 0·02 to 5·0 $\mu$g of copper and 0·1 to 10·0 $\mu$g of iron; standards for iron must be thoroughly washed. Standards may be permanently mounted on filter-paper between glass. Always compare standards and samples in diffuse transmitted light from the upper side of the disc; most of the metal is absorbed near one surface.

## DISCUSSION OF THE METHOD

A series of tests was performed by one operator on samples prepared by an independent observer. Groups of four tests, each on a 50-ml sample of water, were completed in 30 minutes. The results for copper were—

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Copper present, parts per thousand million .. | 0·8 | 2·6 | 12 | 22 | 36 | 50 | 60 |
| Copper found, parts per thousand million .. | 1·0 | 3·0 | 10 | 26 | 32 | 50 | 65 |

and those for iron were—

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Iron present, parts per thousand million .. .. | 2 | 6 | 14 | 22 | 50 | 80 |
| Iron found, parts per thousand million .. .. | 3 | 6 | 14 | 26 | 42 | 65 |

The maximum error, $\pm 20$ per cent., is reasonable for a visual-comparison method at these extremely low levels. The lower limits of detection were $0{\cdot}4 \times 10^{-9}$ parts of copper and $2 \times 10^{-9}$ parts of iron in a 50-ml sample. This was found to be the practical maximum volume for a test completed in a reasonable time. The volume of water that would spread to a circular area 1 cm in diameter on ordinary filter-paper is about 0·01 ml; by passing 50 ml of water through the same area of cellulose phosphate, the sensitivity of the spot test is thereby multiplied by 5000.

The tests mentioned above were performed on distilled and de-ionised water. Determinations of copper in hard tap-water were unsuccessful because of competition by calcium for the ion-exchange capacity, but the same water, when softened, gave good results. Determinations of iron in hard or natural water were successful because iron is absorbed from acid solution by cellulose phosphate[1] under these conditions, but calcium and magnesium are not.

This simple technique requires only a minimum of apparatus of small surface area, a small volume of sample and a short time of operation; the risk of contamination is therefore reduced to a minimum. Since the test is performed directly on the absorbent, possible contamination from impurities in the eluting agents used in orthodox ion-exchange techniques is avoided.

This Note is published by permission of the Director of the National Chemical Laboratory and of the United Kingdom Atomic Energy Authority.

The experimental assistance of Miss J. M. Hawker and Miss C. Atkinson and the kindness of W. & R. Balston Ltd. in providing the samples of cellulose phosphate are gratefully acknowledged.

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## THE DETECTION OF PHOSPHATE ESTERS ON PAPER CHROMATOGRAMS

PHOSPHATE esters, *e.g.*, fructose-1:6-diphosphate, glucose-1-phosphate, glucose-6-phosphate and phytic acid, are usually detected on filter-paper by hydrolysis to orthophosphoric acid, which forms a complex with the molybdate of an acid molybdate spray or dip.[1] The molybdophosphate complex is then reduced to an intensely blue compound by treating the paper with hydrogen sulphide or by exposure to ultra-violet light. After either treatment the background colour of the paper gradually becomes blue, the process being accelerated when the paper is left exposed to light, and later identification of any faintly coloured spots is made difficult. A further disadvantage is that many naturally occurring acids, *e.g.*, citric, ascorbic and tartaric, also form a blue colour, although this does not usually happen until a short time after the paper has been treated.

To overcome these disadvantages the method described below has been developed.

### METHOD

PROCEDURE—

Dry the filter-paper in air, and dip in a mixture of 5 ml each of 60 per cent. perchloric acid and 20 per cent. w/v ammonium molybdate solution, 10 ml of *N* hydrochloric acid and 80 ml of acetone. Allow the paper to dry in air for about 5 minutes, expose to ultra-violet radiation,[2] immediately dip in a 2·5 per cent. w/v solution of α-benzoinoxime in methanol, and again allow to dry in air.

α-Benzoinoxime reacts with molybdenum under acid conditions to form a white precipitate,[3] so providing a white background that persists for some weeks, especially if the paper is left exposed to light. Spots formed by 1 per cent. solutions of citric, ascorbic and tartaric acids do not show up after the paper has been dipped in a solution of α-benzoinoxime.

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## THE PHOTOMETRIC DETERMINATION OF TITANIUM WITH TIRON

YOE and Armstrong[1] developed a method in which disodium 1:2-dihydroxybenzene-3:5-disulphonate (tiron) in a solution buffered to pH 4·7 was used for the photometric determination of titanium. Although this procedure was accepted by some later workers,[2,3] it has the disadvantages stated below.

(*i*) It has been shown that, if the solution attains a pH of 4 or more before the reagent solution is added, not all of the titanium reacts, presumably owing to the formation of metatitanic acid. The reagent solution is therefore added to a strongly acid solution, and adjustment to the chosen pH is subsequently made by adding ammonium hydroxide. However, this procedure can lead to an increase in temperature accompanied by partial decomposition of the reagent and hence to high readings.

(*ii*) Sodium dithionite, used to reduce iron to the ferrous state and so to prevent interference, slowly produces a precipitate of sulphur at pH 4·7, a fact noted by Yoe and Armstrong and confirmed in this laboratory.

If the procedure could be carried out at a pH below 4, precipitation of metatitanic acid would be avoided, the solution could be buffered and cooled before the reagent solution was added, and a more stable reducing agent could be used. Yoe and Armstrong[1] stated that a pH of 4·3 was necessary for full colour development, but gave no figures for colour intensity at pH values below this. The variation in colour intensity with acidity at pH values less than 4·3 was therefore studied.

In preliminary experiments, separate solutions containing 30 μg of titanium in dilute sulphuric acid were adjusted to the required pH values, treated with 5-ml portions of a 4 per cent. solution of tiron and diluted to 100 ml. The optical densities of the solutions were measured in 4-cm cells

at 405 m$\mu$ with a Unicam SP500 spectrophotometer, and the pH values of the final solutions were checked by means of a glass electrode. (The wavelength chosen for optical-density measurements was not that of maximum absorption for the complex, but permits ready conversion to a filter instrument having a mercury lamp.)

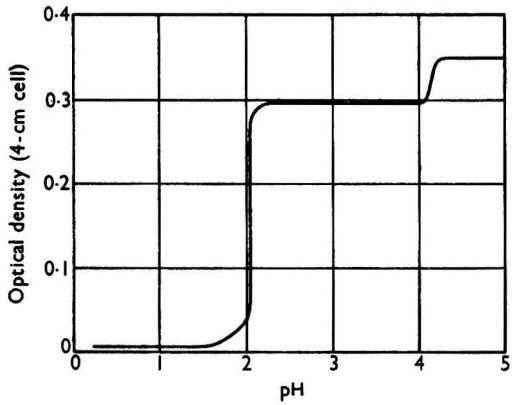


Fig. 1

Fig. 1. Variation in optical density at 405 m$\mu$ with pH of a solution containing 30 $\mu$g of titanium per 100 ml

The results (see Fig. 1) confirmed that a pH of 4·3 was necessary for maximum colour development, but also showed a "plateau" between pH 2·3 and 4·0, which had not previously been reported. The decrease in sensitivity (about 15 per cent.) is more than offset by the advantages of working at a lower pH. In this range of pH, 5 mg of iron per 100 ml can be readily reduced to give a clear and stable solution by treatment in the cold with 20 mg of ascorbic acid or by warming with 5 ml of hydroxylamine hydrochloride solution.

With the wide tolerance for pH, the use of a buffer solution is not necessary, and the pH can be adjusted to 3 $\pm$ 0·2 with use of an external indicator paper. Colour develops rapidly at this lower pH and is stable for at least 24 hours; Beer's law is obeyed up to at least 80 $\mu$g of titanium per 100 ml, the maximum concentration tested. The intensity of the colour is dependent on temperature, *e.g.*, the optical density (4-cm cell) of a solution containing 18 $\mu$g of titanium per 100 ml was 0·165 at 17° C and increased by approximately 0·005 for each 2° C rise in temperature up to 25° C.

The proposed method has been applied to the analysis of nickel, with good results.[4]

I thank Dr. J. A. M. van Moll, Head of the Material Research Laboratory, and the Directors of The Mullard Radio Valve Company Ltd. for permission to publish this Note.

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# Book Reviews

ADVANCES IN MASS SPECTROMETRY. Edited by J. D. WALDRON. Pp. xvi + 704. London, New York, Paris and Los Angeles: Pergamon Press Ltd. 1959. Price 120s.

In 1958, the Mass Spectrometry Panel of the Hydrocarbon Research Group of the Institute of Petroleum organised its third conference, this time in collaboration with Committee E-14 on Mass Spectrometry of the American Society for Testing Materials. The conference was held from the 24th to the 26th of September, inclusive, at Senate House, University of London, and opened with an address by Professor Sir Cyril Hinshelwood, P.R.S. Forty-one papers, including eleven from workers in Great Britain and fourteen from workers in the U.S.A., were read and discussed. The other participating countries were Canada, Germany, Holland, France and Belgium. All these papers are published in full (in English) in this book, together with edited reports of the discussions on the various papers. In addition, an extensive bibliography containing more than 2000 references to published papers and books on mass spectroscopy has been prepared by the Intelligence and Interchange Section of the Research Department of Metropolitan-Vickers Electrical Co. Ltd. A bibliography of this kind has, in fact, been a valuable feature of previous conference reports. The earlier work in this respect has been incorporated into the present bibliography, which has been extended to provide a fairly complete record of published work during the years 1938 to 1957, inclusive.

It can be seen from a perusal of this bibliography that the output of published work on the various aspects of mass spectroscopy is continuing at a very high level. For example, in each of the last two years referred to in the bibliography, 1956 and 1957, more than 150 papers were published in a wide variety of journals. From this, one could well argue a strong case for an international journal solely devoted to mass spectroscopy and cognate subjects. The desire for further conferences, at which all workers have the opportunity to pursue by formal discourse and informal argument the more abstruse phenomena of the subject and to report on technical progress in extending instrumental facilities and applications, remains as strong as ever. It seems likely that, from the collaboration of the Mass Spectrometry Panel and Committee E-14, a conference of this kind can be expected as a triennial event.

It is to be hoped, however, that this does not mean more and more voluminous, and hence more and more costly, conference reports. At the first conference in 1950, fourteen papers were presented; at the second conference in 1953, twenty-six papers; and at this, the third conference, forty-one papers—no sign of saturation yet. The present volume is, indeed, excellently produced; but, at the price of £6, many junior research workers may find it difficult to acquire a personal copy. This would be a pity, for this report will repay extensive and careful study.

Of course, in a record of this kind, written by experts for experts, a good working knowledge of the existing state of the art and science of mass spectrometry is assumed. The expert mass spectrometrist in a particular aspect of his subject is concerned in demonstrating to what extent he has been able to extend the boundaries of knowledge and techniques and then to indicate the new uncertainties thereby revealed as a temporary obstacle to further progress.

In instrument design, there is an unremitting search for greater speed in scanning the mass spectrum of a sample in order to follow the kinetics of fast chemical reactions; for higher mass resolution in both single-focusing and double-focusing instruments, not only for the quantitative analysis of compounds of high molecular weight, but also for the identification of unknown organic chemicals from sufficiently accurate mass measurements of the "parent" or molecular ion and a knowledge of the different nuclear-packing fractions of the isotopes; and naturally for greater sensitivity and analytical accuracy. Some of these demands are to some extent mutually exclusive, and frequently one has to compromise according to the nature of the work. Several papers deal with experimental and theoretical studies of image defects in various designs for both first- and second-order focusing. It appears to be quite practical to-day to use a double-focusing instrument with a mass resolution of 10,000 for analytical work on organic compounds. However, there are groupings, such as $N_2$—$C_2H_2O$, that would require a resolution of 100,000 or more. Some atomic masses have been determined with a precision of about one part in two million.

The analysis of solids is dealt with in several papers on the use of techniques such as the triple-filament surface-ionisation source, isotope dilution and spark-source spectrometry with both conducting and non-conducting solids. The analysis of heavy oils and corrosive gases receives attention. There is a also a paper on the use of rhenium (in place of tungsten) filaments and low ionising voltages for analysing liquid products from the hydrogenation of coal.

It is interesting to note that the time-of-flight mass spectrometer appears now to have achieved a degree of maturity in development sufficient to invite favourable comparison with magnetic spectrometers.

Although it is clear that there can be no quantitative predictions as to absolute mass spectra for a particular mass species, and that observed mass spectra are bound to depend to a considerable extent on the conditions of operation in the mass spectrometer, much interesting research is directed towards elucidating the major processes of ionisation and dissociation of molecules by electron impact. There are several papers devoted to studies on the interpretation of mass spectra. Mass discrimination in the ion source has, it appears, no appeal to-day as a subject for research. A whole session was devoted to ionisation and electron-impact studies.

The conference concluded with a session on the use of the mass spectrometer in fundamental research, dealing with such subjects as the isotopic analysis of trace amounts of rare gases, applications to geology and surface chemistry and studies of the reactions of atoms and free radicals and of the thermodynamic properties of various elements.

It is apparent that there is a wealth of material here for prolonged study by all workers on mass spectrometry, and that such conferences provide valuable opportunities for the interchange of information between all users of mass spectrometers in the many different fields of application.

It is, perhaps, a pity that there is no subject index, but this would not have been easy to compile. Only a few minor misprints have been noted.

G. P. BARNARD

BRITISH PHARMACEUTICAL CODEX, 1959. Published by direction of the Council of the Pharmaceutical Society of Great Britain. Pp. xxx + 1301. London: The Pharmaceutical Press. 1959. Price 70s.

This is the seventh edition of the British Pharmaceutical Codex, the sixth having been published in 1954. It is interesting to compare this edition with the first publication in 1907. It seems that the character of the book has changed over the years, and, whereas the first edition contained much casual information of interest and value to the pharmacist or physician, the monographs in recent editions have become standarised in form, and more attention has been devoted to standards and methods of analysis. Though often irrelevant and sometimes inaccurate, the deleted information certainly added to the interest (and sometimes to the entertainment) of the book.

In the new edition are described forty-eight new synthetic drugs, of which nine are included in the British Pharmacopoeia, 1958; the descriptions of twenty-two synthetic drugs and twenty-five of natural origin have been deleted. Now that the fashion in medicine has changed from bloodletting to blood transfusion, *Hirudo medicinalis*, the common leech, has finally received its quietus.

There is little new in the analytical methods. Many more drugs are assayed by non-aqueous titration; eleven of the new drugs are standardised by the determination of nitrogen, which hardly justifies the name of an assay. Fluorocortisone acetate is assayed by means of its fluorine content, which is determined by combustion in oxygen and then titration with thorium nitrate solution.

The high standard of production and accuracy has been maintained. NORMAN EVERS

XV CONGRESSO INTERNACIONAL DE QUIMICA PURA E APLICADA (*Quimica Analytica*): Actas do Congresso (*8 a 16 de Setembro de* 1956). Volume II. Pp. 1048. Lisbon: General Secretary of the XVth International Congress of Pure and Applied Chemistry, Instituto Superior Technico. 1957. Price, together with volumes I and III, 320 Escudos.

This volume contains Section IV, Optical methods (64 pages), Section V, Radiochemical methods (34 papers) and Section VI, Organic complexes (37 papers). Twenty-two of these papers have been published elsewhere and twenty-two appear only as short summaries. A complete list of papers and authors appears in the June issue of *Analytical Abstracts*.

NORMAN EVERS

# Publications Received

A Statistical Manual for Chemists. By Edward L. Bauer. Pp. x + 156. New York and London: Academic Press Inc. 1960. Price $4.75; 38s.

The Radiochemistry of Cadmium. By James R. DeVoe. Pp. vi + 57. Washington, D.C.: National Academy of Sciences—National Research Council. 1960. Price 75 cents. *Nuclear Science Series: NAS-NS-3001.*

The Radiochemistry of Arsenic. By Harold C. Beard. Pp. vi + 27. Washington, D.C.: National Academy of Sciences—National Research Council. 1960. Price 50 cents. *Nuclear Science Series: NAS-NS-3002.*

The Radiochemistry of Francium. By Earl K. Hyde. Pp. vi + 34. Washington, D.C.: National Academy of Sciences—National Resarch Council. 1960. Price 50 cents. *Nuclear Science Series: NAS-NS-3003.*

Henry Cavendish: His Life and Scientific Work. By A. J. Berry, M.A. Pp. 208. London: Hutchinson & Co. (Publishers) Ltd. 1960. Price 35s.

The Analysis of Fats and Oils. By V. C. Mehlenbacher. Pp. xvi + 616. Illinois, U.S.A.: The Garrard Press. 1960. Price $12.00.

Chromatographic Reviews: Progress in Chromatography, Electrophoresis and Related Methods. Volume II. Edited by Michael Lederer. Pp. viii + 195. Amsterdam, London, New York and Princeton: Elsevier Publishing Co.; London: D. Van Nostrand Co. Ltd. 1960. Price 46s.

Organosilicon Compounds. By C. Eaborn, Ph.D., D.Sc. Pp. x + 530. London: Butterworths Scientific Publications. 1960. Price 80s.

Thioacetamide as a Sulfide Precipitant: An Annotated Bibliography. Compiled by W. C. Broad and A. J. Barnard, Jr. Pp. ii + 10. New Jersey, U.S.A.: J. T. Baker Chemical Company. March, 1960. Gratis.

Chemical Analysis of Cast Iron and Foundry Materials. By W. Westwood, B.Sc., A.I.M., and A. Mayer, B.Sc., F.R.I.C. Second Edition. Revised by W. E. Clarke, F.R.I.C., and H. Green. Pp. 586. London: George Allen & Unwin Ltd. 1960. Price 63s.

Systematic Analysis of Surface-active Agents. By Milton J. Rosen and Henry A. Goldsmith. Pp. xviii + 422. New York and London: Interscience Publishers Inc. 1960. Price $13.50; 102s.

Biochemical Preparations. Volume 7. Editor-in-Chief: Henry A. Lardy. Pp. x + 102. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1960. Price $5.25; 42s.

The Pharmaceutical Pocket Book. Published by the direction of the Council of the Pharmaceutical Society of Great Britain. Seventeenth Edition. Pp. xii + 576. London: The Pharmaceutical Press. 1960. Price 30s.

Chromatographie en Chimie Organique et Biologique. Volume II. Applications en Chimie Biologique. Edited by E. Lederer. Pp. xvi + 876. Paris: Masson et Cie. 1960. Price (paper) 130 NF; (cloth boards) 140 NF.

Gas Chromatography Abstracts 1958. (References to the end of 1958.) Compiled and edited by C. E. H. Knapman; Assistant Editor: C. G. Scott. Pp. viii + 262. Sponsored by The Gas Chromatography Discussion Group (Associated with the Hydrocarbon Research Group of the Institute of Petroleum). London: Butterworths Scientific Publications. 1960. Price 42s.

Électrochimie Théorique. By Eugène Darmois and Genevieve Darmois. Pp. viii + 239. Paris: Masson et Cie. 1960. Price 39 NF.

## REPRINTS OF "THE DETERMINATION OF VITAMIN $B_{12}$"

**By W. H. C. Shaw and Christine J. Bessell**

Reprints of the Review Paper, "The Determination of Vitamin $B_{12}$" by W. H. C. Shaw and Christine J. Bessell, published in this issue of *The Analyst* (pp. 389–409), will be available shortly from the Assistant Secretary, The Society for Analytical Chemistry, 14 Belgrave Square, London, S.W.1, at 5s. per copy, post free. A remittance for the correct amount, made out to The Society for Analytical Chemistry, must accompany the order; these reprints are not obtainable through Trade Agents.

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