[and "The Analyst" is now Mansion House 6608.]

Volume 71 See Special Notice on p. 300 June, 1946

No. 842, Pages 251—300

THE ANALYST

The Journal of

The Society of Public Analysts

and other Analytical Chemists

Publication Committee

Chairman: J. R. NICHOLLS, D.Sc., F.R.I.C.

N. L. ALLPORT, F.R.I.C.
F. W. F. ARNAUD, F.R.I.C.
A. L. BACHARACH, M.A., F.R.I.C.
R. C. CHIRNSIDE, F.R.I.C.
B. S. COOPER, B.Sc., F.Inst.P.
H. E. COX, D.Sc., Ph.D., F.R.I.C.
BERNARD DYER, D.Sc., F.R.I.C.
F. W. EDWARDS, F.R.I.C.
B. S. EVANS, M.B.E., M.C., D.Sc., F.R.I.C.
E. B. HUGHES, D.Sc., F.R.I.C.
D. W. KENT-JONES, B.Sc., Ph.D., F.R.I.C.
S. ERNEST MELLING, F.R.I.C.
C. A. MITCHELL, M.A., D.Sc., F.R.I.C.
G. W. MONIER-WILLIAMS, O.B.E., M.C., Ph.D., F.R.I.C
A. MORE, I.S.O., A.R.C.S., A.R.T.C., F.R.I.C.
W. H. SIMMONS, B.Sc., F.R.I.C.
ERIC VOELCKER, A.R.C.S., F.R.I.C.
K. A. WILLIAMS, B.Sc., F.R.I.C.
G. H. WYATT, B.Sc., Ph.D., F.R.I.C.

Hon. Secretary:
L. EYNON, B.Sc., F.R.I.C.
7-8, Idol Lane, London, E.C.3

Hon. Treasurer:
G. TAYLOR, F.R.I.C.
Peek House, 20, Eastcheap, London E.C.3

Editor: J. H. LANE, B.Sc., F.R.I.C.
7-8, Idol Lane, London, E.C.3
Telephone: Mansion House 6608

Abstractors

J. ALLEN
L. A. DAUNCEY, B.Sc.
E. B. DAW, B.Sc., A.R.I.C.
J. GRANT, Ph.D., M.Sc., F.R.I.C.
A. O. JONES, M.A., F.R.I.C.
J. W. MATTHEWS, Ph.D., D.I.C., F.R.I.C.
E. M. POPE, B.A., B.Sc.
C. F. PRITCHARD, B.Sc., A.R.I.C.
F. A. ROBINSON, M.Sc.Tech., F.R.I.C.
J. T. STOCK, M.Sc., F.R.I.C.
D. R. WOOD, F.R.I.C.

Published for the Society by
W. HEFFER & SONS LTD., Cambridge, England

IMPORTANT NOTICE TO SUBSCRIBERS

(*Other than Members of the Society*).

All Subscriptions and renewals to the Journal should be sent through a Bookseller or direct to

W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.

Price 3/6; or to Subscribers in advance post free 35/- per annum.

N.B.—Members send their subscriptions to the Hon. Treasurer.

ENTERED AS SECOND CLASS AT NEW YORK, U.S.A., POST OFFICE
PRINTED IN ENGLAND

FOR ACCURACY AND RAPIDITY

WHATMAN FILTER PAPERS

High Grade

ARE ENTIRELY DEPENDABLE

HARDENED. PURIFIED. "ASHLESS". PAPER.
W. & R. BALSTON, Ltd.
GENUINE WHATMAN
FILTER PAPER
No 542

Each grade is distinctively labelled

STOCKED BY LABORATORY FURNISHERS THROUGHOUT THE WORLD

IN SEALED BOXES

THE WHATMAN range covers all requirements of the chemist in every branch of industry and research.

In the event of difficulty in obtaining supplies, free samples, or copy of our descriptive booklet and price list, please write the Sole Sales Representatives:

H. REEVE ANGEL & COMPANY, LTD.
9, BRIDEWELL PLACE, LONDON, E.C.4.

Made only by
W. & R. BALSTON, Ltd.
KENT

LITMUS

PURE GRAN

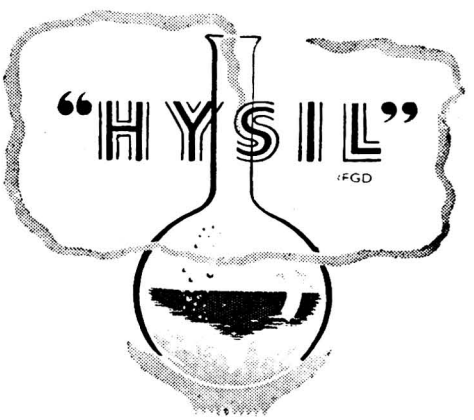
•

LITMUS PAPERS
AND OTHER
INDICATORS

•

JOHNSON & SONS
MANUFACTURING CHEMISTS LTD
HENDON, LONDON, N.W.4

Established 1743



LABORATORY GLASSWARE

USED WHEREVER

GLASS AND HEAT MEET

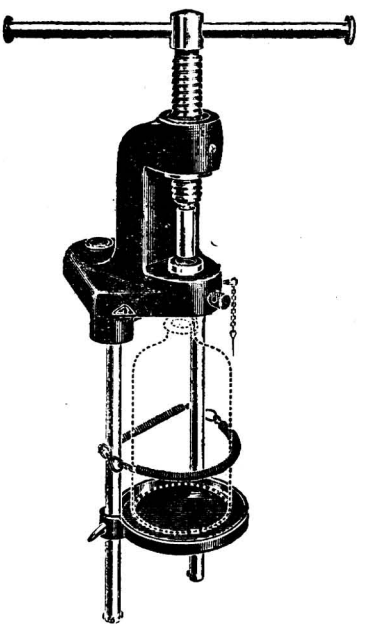
Made by Chance Brothers Ltd.
Head Office and Works: Smethwick, Birmingham
London Office: 28 St. James's Square, S.W.1

A Chance PRODUCT

Stocks held by all recognised Laboratory Furnishers.

THE MICROID NALIK PRESS

An improved British-made Press for use in extruding the alkali metals as wire



Descriptive leaflet GT. 1353/01 free on application

The Microid Nalik Press is suitable for use in extruding sodium and potassium as wire 0.5 mm. diam. and lithium as wire 1.0 mm. diam. The powerful dehydrating and reactive properties of these elements are much enhanced by the great increase in surface area.

The Nalik Press has a number of features not found in the sodium presses hitherto imported. It has: (a) an adjustable bottle stand, (b) a stainless steel one-piece mould, (c) a fully-floating stainless steel plunger, (d) it is simply secured to the bench by one bolt.

GRIFFIN and TATLOCK Ltd

Established as Scientific Instrument Makers in 1826

LONDON Kemble St., W.C.2 | **MANCHESTER** 19, Cheetham Hill Rd., 4 | **GLASGOW** 45, Renfrew St., C.2 | **EDINBURGH** 7, Teviot Place, 1

BIRMINGHAM: STANDLEY BELCHER & MASON LTD., Church Street, 3

CAMBRIDGE POLAROGRAPH

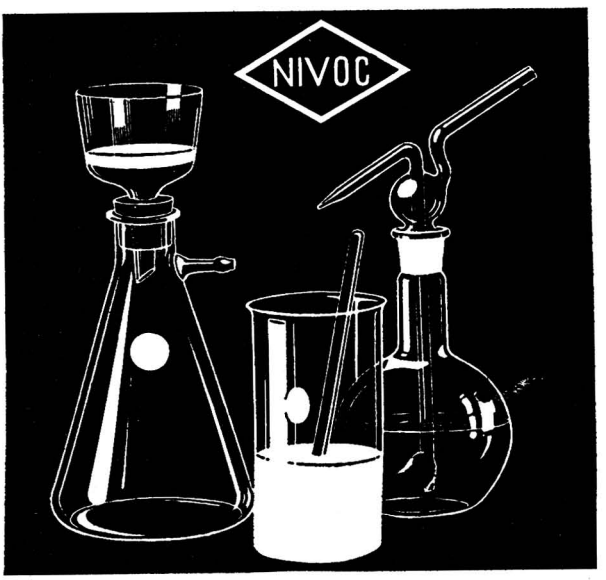
(HEYROVSKY DROPPING MERCURY ELECTRODE METHOD)

For the rapid electro-chemical analysis of solutions, enabling traces of metals and many acid radicals and organic substances to be determined. It has valuable applications in

PHYSICAL CHEMISTRY	METALLURGY
MEDICINE	MINERALOGY
OIL DISTILLING	SUGAR
DYEING & BLEACHING	EXPLOSIVES
CERAMICS	GASES
WATER PURIFICATION	AGRICULTURE
etc.	etc.

CAMBRIDGE
INSTRUMENT CO. LTD.
13 GROSVENOR PLACE, LONDON, S.W.1
WORKS: LONDON & CAMBRIDGE

THIS PUBLICATION 109-U gives full details, with valuable notes on technique.



Registered NIVOC *Trade Mark*

for
COMPLETE MAINTENANCE
of the
ANALYTICAL
and
CONTROL LABORATORY

W. & J.
GEORGE & BECKER
LTD
Established 1872
17-29 Hatton Wall, London E.C.1
Telephone: CHAncery 6011-4
157 Gt. Charles St., Birmingham 3
Telephone: CENtral 7641-3

LABORATORY FURNISHERS • MANUFACTURERS OF SCIENTIFIC APPARATUS • BALANCES AND WEIGHTS ; SUPPLIERS OF CHEMICALS AND ANALYTICAL REAGENTS

THE ANALYST

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

AN Ordinary Meeting of the Society was held at 6 p.m. on Wednesday, May 1st, at The Chemical Society's Rooms, Burlington House, London, W.1. The President, Dr. G. W. Monier-Williams, occupied the chair. The following papers were presented and discussed: "The Microbiological Assay of Amino Acids. I. The Assay of Tryptophan, Leucine, *Iso*leucine, Valine, Cystine, Methionine, Lysine, Phenylalanine, Histidine, Arginine and Threonine," by E. C. Barton-Wright, D.Sc., F.R.I.C.; "The Microbiological Assay of Amino Acids. II. The Distribution of Amino Acids in the Wheat Grain," by E. C. Barton-Wright, D.Sc., F.R.I.C., and T. Moran, D.Sc., Ph.D.

NEW MEMBERS

Douglas Geoffrey Glenn Allen, M.A. (Cantab.), A.R.I.C.; Stanley Thomas Patrick Brightwell, M.Sc. (Lond.), A.R.C.S., D.I.C., A.R.I.C.; William George David Carpenter, A.R.I.C.; Michael Robin Alexander Chance, B.Sc. (Lond.); Stanley Frederick Chancellor, A.R.I.C.; Henry Oswald Jackson Collier, B.A., Ph.D. (Cantab.); William Bryan Emery, B.Sc., A.R.I.C.

DEATHS

WE record with deep regret the deaths of

George Edward Boizot
Alexander Bruce
John Loudon Buchanan
John Alfred Foster
Alfred Douglas Heywood
Edward Hinks
Philip Henry Jones

Symposium on Chromatography

The following four Papers on Chromatography were read and discussed at the Meeting of the Physical Methods Group in London on Tuesday, February 26th, 1946. The Discussion follows the last of the four, on p. 265.

General Principles of Chromatographic Analysis

BY F. A. ROBINSON

CHROMATOGRAPHIC adsorption was first used in 1906 by the Russian botanist, Tswett. He poured a petroleum ether extract of green leaves on to a column of finely powdered calcium carbonate and observed the formation of a pale-yellow ring in the upper part of the column with two green zones immediately below it and three yellow bands below these. On washing the column with the pure solvent, a process now known as "development," all the bands became completely separated from one another, enabling the column to be cut up and the individual pigments eluted from each separate portion. This is an example of what might be termed "classical" chromatography applied to the separation of coloured pigments. Chromatography has been extensively employed for the characterisation of the individual components of naturally occurring pigments such as carotenoids and pterins; in fact it is difficult to see how progress in either of these fields would have been possible had the technique not been available. It has also been used for the isolation of compounds present in minute amounts in natural products or in synthetic mixtures, and for the purification of compounds that cannot readily be purified by crystallisation.

The application of chromatography is by no means confined to the investigation of coloured substances, however, in spite of what the name suggests, for it has also been proved of great value in the isolation and purification of polycyclic hydrocarbons, sterols, alkaloids, vitamins and hormones. In practice, the only difference between the chromatography of

coloured and colourless substances is that with the former the position of each component on the column is self-evident, whereas with the latter various devices have to be employed to indicate the position of the bands.

The earliest procedure for separating colourless components was to cut the column empirically, and test the eluate of each part by a suitable physical or chemical test. This method is not particularly satisfactory, however, for as often as not the component sought is contaminated by another constituent of the mixture and a second or even third chromatogram has to be prepared to effect complete separation.

Where the procedure is applicable, a particularly simple method[1] of detecting the position of the bands is examination of the column in light from a mercury vapour lamp; this reveals the presence of any substance that fluoresces in ultra-violet light, and often produces a series of coloured bands sufficiently distinctive to enable the column to be cut, as in "classical" chromatography.

Another method of detecting the position of a particular band is the so-called "brush method,"[2] in which the column is extruded from the tube and painted with a suitable reagent; antimony trichloride solution, for example, can be used to detect the position of a vitamin A band. This method has the disadvantage that the progress of development cannot be followed.

In some instances, instead of chromatographing the substances themselves it is preferable to chromatograph suitable coloured derivatives. For example, carbonyl compounds can be converted into dinitrophenylhydrazones,[3] and sugars or amino acids reacted with azobenzene-4-carboxylic acid chloride.[4] The resulting coloured derivatives are then chromatographed, the column is cut up according to the position of the coloured bands and the individual compounds are liberated from each fraction by hydrolysis.

Another satisfactory technique for indicating the position of a substance is to find a dyestuff with the same adsorption affinity and add this to the mixture being chromatographed. The dyestuff is adsorbed on the column in substantially the same position as the substance to be isolated. Brockmann,[5] for instance, used Sudan III to indicate the position of the vitamin D_3 band during the process of isolating it from fish liver oil.

A large variety of adsorbents has been used in chromatography, depending on the nature of the substances to be separated. The most strongly adsorbed of all organic compounds are acids and bases; then come hydroxyl-, amino- and thio-compounds, then aldehydes, ketones and esters, and finally halogen derivatives and hydrocarbons. In a homologous series adsorption affinity increases with the number of conjugated double bonds, and unsaturated compounds are more strongly adsorbed than the corresponding saturated compounds. Increasing the number of atomic rings increases adsorption affinity, and so does the introduction of polar groups. With positional and *cis-trans* isomers, the one with the greater dipole moment will be the more strongly adsorbed.

Strongly adsorbed substances such as carotenoids can readily be chromatographed on weak adsorbents such as calcium carbonate. Indeed the use of a strong adsorbent with these substances would result in the bands being bunched together at the top of the column instead of being distributed over its whole length. On the other hand, with a feeble adsorbent all the material may pass into the filtrate. Only experience will indicate the most suitable adsorbent for a particular purpose. The materials with the highest adsorption affinity are charcoal, activated alumina and some of the specially prepared adsorbent earths, whilst the weakest of all adsorbents are the carbohydrates such as sucrose, starch and inulin. Intermediate in activity are magnesia, lime, silicic acid and calcium phosphate at the upper end of the scale, and at the lower end talc and the alkali metal and alkaline earth carbonates.

It must be realised that the activity of an adsorbent varies with the mode of preparation, and aluminium oxides with varying degrees of activity are made[6] by heating aluminium hydroxide to strong-red heat and then partially deactivating it by shaking for shorter or longer periods with moist air. For this reason the use of standardised adsorbents is recommended. Brockmann[6] standardised alumina and other adsorbents by noting their behaviour towards a series of dyestuffs with increasing adsorption affinity, whilst Müller[7] advocated a calorimetric method based on the observation that the amount of heat evolved on treating different grades of alumina with light petroleum is proportional to their relative adsorption affinities.

Another method of comparing the properties of different batches of adsorbent was adopted by Le Rosen,[8] who made use of the following four terms to define density, porosity and adsorptive power:

$$S = \frac{\text{length of adsorbent column containing unit volume of solvent}}{\text{length of unfilled tube required to contain same volume of solvent}}$$

V_c = rate of flow (mm./min.) of developing solvent through column when a state of constant flow has been reached

T_{50} = time (sec.) required for solvent to penetrate 50 mm. into an initially dry column (9×75 mm.) under the vacuum of a water pump

$$R = \frac{\text{rate of movement (mm./min.) of front edge of adsorbate zone}}{V_c}$$

With different batches of silicic acid S had much the same value, but the values of V_c and T_{50} varied considerably. Adsorbents for which V_c was between 10 and 50 mm./min. and T_{50} between 20 and 100 sec. gave the best results. The value of R varied with the treatment to which the adsorbent had been subjected; values of 0·1 to 0·3 gave satisfactory chromatograms, that is, the rate at which the zone passes down the column should be one-tenth to one-third the rate of flow of the developing solvent.

The choice of solvents for use in the development of chromatograms, that is, in separating the adsorbed material into distinct bands or zones, is also wide. In general, the less polar the solvent, the more readily will the solute be retained on the column, and the more polar the solvent, the more readily will the solute be eluted. The weakest eluants are the four grades of light petroleum, but these are used for the initial adsorption rather than for development. Benzene, carbon tetrachloride, cyclohexane, ether and acetone are more commonly used for development, and a mixture of light petroleum and benzene is particularly popular. Stronger eluants are ethylene dichloride, chloroform, the alcohols and water, whilst the most active of all are pyridine, acids, and mixtures of acids with bases.

One of the most useful methods of separating the components of a colourless mixture is based on such differences in eluant power and is known as "fluid chromatography." In this method a series of solvents of gradually increasing eluant power is poured through the column in succession, each eluate is collected separately and its constituents are examined.

Charcoal has not been used to any great extent in chromatography because of the difficulty of detecting the position of the bands, although its high adsorption affinity would be an obvious advantage in many instances. During the last two or three years, however, Tiselius[9] has described a method of overcoming this difficulty by forcing the solution to be examined under pressure upwards through a column of charcoal, and examining in an optical system the liquid which emerges at the top. A number of *Schlieren* boundaries are formed, their relative positions depending on the degree of adsorption of the individual components, which are more or less retarded as compared with the movement of the meniscus of the supernatant liquid. The volume of liquid between each boundary and the meniscus is known as the "retardation volume," and is greatest with the substances most tenaciously held by the charcoal. The retardation volumes of a series of amino acids were measured and found to vary from 0 for glycine to 122 ml. per g. for hippuric acid. A method of separating lysine, valine and alanine was described.

Tiselius[10] has also introduced another technique termed "displacement chromatography." With strong adsorbents it is often difficult to find solvents of sufficient eluant power to overcome the adsorption affinity of the adsorbate, and Tiselius adopted the procedure of developing charcoal columns with a solution of a strongly adsorbed substance, such as ephedrine; this was found to elute the less strongly adsorbed fractions from a mixture of mono- and polysaccharides.

Of theoretical, though probably not of practical, importance is the reversed chromatogram[11] in which an adsorbent is poured into a column of the solution to be analysed; the component with the highest adsorption affinity is carried to the bottom, whilst that with the lowest is either adsorbed in the top layers or remains behind in solution.

Chromatography has also been applied to the separation of substances in the vapour phase. If a mixture of benzene and cyclohexane,[12] together with a carrier gas such as hydrogen or nitrogen, is passed through a tube containing silica gel, the emergent gases contain cyclohexane almost free from benzene. Benzene and carbon tetrachloride can be similarly separated. Again, when alcohol - water vapour is passed through a column of charcoal,[13] the ratio of alcohol is higher in the adsorbate than in the vapour phase, whereas in static adsorption the adsorbate is richer in water. This is due to the fact that although charcoal adsorbs water preferentially from the vapour, the rate of adsorption of alcohol is considerably

greater than that of water, so that in the continuous flow method the former displaces the latter; the ratio of alcohol in the adsorbate is, in fact, increased by increasing the rate of passage through the adsorbent. Similar results are obtained with propanol - water vapour, whilst with mixtures of water, methanol and ethanol, or of water, ethanol and butanol, almost complete adsorption of the higher alcohol occurs in each instance.

The processes so far described result in the separation of substances according to their relative adsorption affinities, but the chromatographic principle, that is, the continuous flow of a solution through a stationary column of solid, has also been used to separate substances by taking advantage of other properties.

The use of columns of zeolite for the recovery from solution of basic substances such as aneurine is now a fairly familiar procedure in analytical chemistry; it is an example of ion exchange chromatography in which sodium ions are displaced by the basic substance. Ion exchange resins[14] are now being used for the same purpose, *e.g.*, Amberlite I.R. 100 for adsorbing basic substances, and Amberlite I.R. 4 for acidic substances. They have been used for the separation of amino acids and vitamins.

Alumina has also been used for the separation of amino acids,[15] the process depending on ion exchange rather than adsorption. Ordinary alumina contains sodium ions which can be displaced by basic amino acids, whilst acid-washed alumina contains hydrogen ions which can be displaced by acidic amino acids. Neutral amino acids can be separated from one another by chromatographing on acid-washed alumina in presence of 10% formaldehyde,[16] which shifts the pK into the acid region. Under these conditions glycine and serine are strongly adsorbed, whilst alanine, valine, leucine and proline are not.

A third type of chromatography, discussed in more detail by Synge,[29] is partition chromatography, in which a column of solid such as silica gel,[17] paper[18] or starch[19] is used to support an aqueous phase, enabling substances to be separated by distribution between water and an organic solvent on the chromatographic principle. The result is the same as that obtained by repeatedly distributing the solutes between the two liquid phases, and such a chromatogram, as pointed out by Martin and Synge, is analogous to a fractionating column. Recently, this type of chromatogram has been further developed by adsorbing strong buffer solutions on the silica gel in place of water,[20] or by mixing the carbonate of an alkali or alkaline earth with the silica.[21] The mechanism is obviously complex, the result probably being due to a combination of partition and neutralisation by the buffer or metal carbonate.

An interesting development in chromatography is the use of columns of specific organic reagents for the separation of inorganic ions. Thus columns of 8-hydroxyquinoline[22] have been used for the separation of VO_3', WO_4', $Cu^{\cdot\cdot}$, $Bi^{\cdot\cdot\cdot}$, $Ni^{\cdot\cdot}$, $Co^{\cdot\cdot}$, $Zn^{\cdot\cdot}$, $Fe^{\cdot\cdot\cdot}$ and $UO_2^{\cdot\cdot}$, which are adsorbed in that order; this corresponds mainly to the relative solubilities of the hydroxyquinolates in water, but also appears to depend on pH. Columns of violuric acid and of 5-oxo-4-oximino-3-phenyl-isoxazoline[23] have been used for the separation of $Na^{\cdot}$, $K^{\cdot}$, $Mg^{\cdot\cdot}$ and $Ca^{\cdot\cdot}$ from one another.

The first attempt to explain the phenomena observed during the process of chromatography was made by Wilson,[24] who developed equations to express the distribution of one solute and of two solutes on a column. He assumed that equilibrium between the adsorbed material and the solution was reached instantaneously, that the volume of the interstices between the particles of adsorbent was negligible and that the effects of diffusion could be neglected. From these equations, Wilson predicted that the chromatogram formed from a solution containing only one solute should consist of a single band, which would maintain a constant width and sharply defined edges as long as the solvent used for development was the same as that used in forming the band. This prediction was tested experimentally by Cassidy and Wood,[24] who found that, although the behaviour of the front edge of the zone was in accordance with Wilson's prediction, the rear edge became diffuse on development; this discrepancy was undoubtedly due to the fact that some of the assumptions made were unjustifiable. Wilson also predicted that the first colour zone of a mixed chromatogram formed before development occurs should contain some of each solute initially present in proportions determined by the initial concentrations and the adsorption isotherms, whereas it had previously been supposed that this zone contained only the most strongly adsorbed solute. This prediction was verified by experimental observation.

A different set of assumptions was made by Martin and Synge,[17] who took into account the effects of diffusion and the possibility that equilibrium was not attained instantaneously,

but assumed that the solute had a linear adsorption isotherm. The column was regarded as consisting of a number of theoretical plates and the problem was treated as analogous to that of a fractionating column. Although adsorption isotherms are seldom linear, and the results from this mode of treatment were therefore only an approximation, they did emphasise the extraordinarily high efficiency of chromatographic separations; whereas the height equivalent to a theoretical plate (H.E.T.P.) of the best distillation columns is of the order of 1 cm., that of a chromatogram was found to be 0·002 cm.

De Vault[25] and Weiss[26] criticised Wilson's theory and showed that his assumptions were not justified. The former developed equations for both the front and rear boundaries, and showed that development of an initially sharp band would be expected to give a diffuse rear boundary, although the front boundary would continue to be sharp. Weiss reached similar conclusions and emphasised the fundamental character of the threshold volume (V_t, that is, the volume of solvent required to develop the column until the front edge of the zone reaches the bottom). Weil-Malherbe[27] measured the threshold volumes for benzpyrene under different conditions and concluded that, where the only variable in a chromatographic system is the nature of the solvent, the threshold volume is a measure of eluant power. Similarly, where the solvent and solute are the same it is possible to calculate from the threshold volume the amount of adsorbent and the minimum size of column necessary to ensure the full development of a solution.

It is clear, therefore, that considerable progress has been made towards an understanding of the theory of chromatography and that it is now possible to express the behaviour of a simple chromatogram more or less exactly in mathematical terms. With complicated chromatograms, however, the mathematical treatment would be extremely involved; the only really satisfactory method of determining the optimal conditions to be employed in such instances remains—and for some time is likely to remain—the method of trial and error.

References

1. Winterstein, A., and Schön, K., *Z. physiol. Chem.*, 1934, **230**, 139; Karrer, P., and Schöpp, K., *Helv. chim. Acta*, 1934, **17**, 693.
2. Zechmeister, L., Cholnoky, L., and Ujhelyi, E., *Bull. Soc. Chim. biol.*, 1936, **18**, 1885.
3. Strain, H. H., *Science*, 1936, **83**, 241.
4. Coleman, G. H., and Farnham, A. G., *Proc. Iowa Acad. Sci.*, 1941, **48**, 246; Karrer, P., Keller, R., and Szönyi, G., *Helv. chim. Acta*, 1943, **26**, 38.
5. Brockmann, H., *Z. physiol. Chem.*, 1936, **241**, 104; Brockmann, H., and Busse, A., *Id.*, 1937, **249**, 176.
6. Brockmann, H., and Schodder, H., *Ber.*, 1941, **74**, 73.
7. Müller, P. B., *Verhandl. Ver. schweiz. Physiol.*, 1942, **21**, 29; *Helv. chim. Acta*, 1943, **26**, 1945.
8. Le Rosen, A. L., *J. Amer. Chem. Soc.*, 1942, **64**, 1905; 1945, **67**, 1683.
9. Tiselius, A., *Arkiv. Kemi, Mineral. Geol.*, 1940, **14B**, No. 22; 1941, **14B**, No. 32; 1941, **15B**, No. 6; *Science*, 1941, **94**, 145.
10. Tiselius, A., and Hahn, L., *Kolloid-Z.*, 1943, **105**, 177; Tiselius, A., *Arkiv. Kemi, Mineral. Geol.*, 1943, **16A**, No. 18.
11. Lowman, A., *Science*, 1942, **96**, 211.
12. Hesse, G., and Tschacholin, B., *Naturwiss.*, 1942, **30**, 587.
13. Amphoux, B., *Ann. chim.*, 1944, **19**, 1.
14. Myers, R. J., Eastes, J. W., and Myers, F. J., *Ind. Eng. Chem.*, 1941, **33**, 697; Cleaver, C. S., Hardy, R. A., and Cassidy, H. G., *J. Amer. Chem. Soc.*, 1945, **67**, 1343.
15. Turba, F., and Richter, M., *Ber.*, 1942, **75**, 340; Wieland, T., *Naturwiss.*, 1942, **30**, 374; *Z. physiol. Chem.*, 1942, **273**, 24.
16. Schramm, G., and Primosigh, J., *Ber.*, 1943, **76**, 373.
17. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, 1941, **35**, 1358; Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Id.*, 1943, **37**, 79.
18. Consden, R., Gordon, A. H., and Martin, A. J. P., *Id.*, 1944, **38**, 224.
19. Elsden, S. R., and Synge, R. L. M., *Id.*, 1944, **38**, ix; Synge, R. L. M., *Id.*, 1944, **38**, 285.
20. Levi, A. A., Terjesen, S. G., and I.C.I. Ltd., Brit. Pat. 569,844.
21. Catch, J. R., Cook, A. H., and Heilbron, I. M., *Nature*, 1942, **150**, 633; Therapeutic Research Corporation Ltd., Heilbron, I. M., Cook, A. H., and Catch, J. R., Brit. Pat. 558,320.
22. Erlenmeyer, H., and Dahn, H., *Helv. chim. Acta*, 1939, **22**, 1369.
23. Erlenmeyer, H., and Schoenauer, W., *Id.*, 1941, **24**, 878; Erlenmeyer, H., and Schmidlin, J., *Id.*, 1213.
24. Wilson, J. N., *J. Amer. Chem. Soc.*, 1940, **62**, 1583.
25. Cassidy, H. G., and Wood, S. E., *Id.*, 1941, **63**, 2628.
26. De Vault, D., *Id.*, 1943, **65**, 532.
27. Weiss, J., *J. Chem. Soc.*, 1943, 297.
28. Weil-Malherbe, H., *Id.*, 1943, 303.
29. Synge, R. L. M., ANALYST, 1946, **71**, 256.

GLAXO LABORATORIES, LTD.
GREENFORD, MIDDLESEX

Partition Chromatography

By R. L. M. SYNGE

Robinson[34] has described how, on developing a chromatogram in which the distribution isotherms between the moving and stationary phases for the substances undergoing analysis are linear, relatively sharp bands will result. Deviations of the isotherms from linearity, as with adsorption of the usual Freundlich type, lead to much spread bands with sharp front edges and elongated "tails," while deviations in the opposite sense, when they occur, give conversely spread bands with sharp rear edges and diffuse "fronts."

A. J. P. Martin and I were led by considerations of this kind, stimulated by practical work with counter-current liquid - liquid extraction trains,[1] to the idea that delicate separations based on the distribution of solutes between two liquid phases should be capable of realisation in a chromatographic set-up, since Henry's law, which is approximately obeyed in a large number of systems, provides linear distribution isotherms. Our expectations were fulfilled in 1941, when we tried chromatograms in which powdered silica gel was saturated with the aqueous phase of a chloroform - water mixture, the chloroform phase being used for development of the chromatogram.[2] Since then a considerable number of applications of partition chromatography have been described, and I will enumerate these briefly, before passing on to a discussion of the physico-chemical processes occurring in partition chromatograms. To understand these processes is important, since on this basis the analyst can decide whether a problem may best be tackled by partition chromatography or some other technique and can further be guided as to choice of materials and mode of procedure.

First, however, it should be said that the application of partition chromatography seems to be limited in its present form to substances that can diffuse freely through the gel-like materials used in the chromatograms. Since the pores of these are of molecular dimensions, the materials so far employed are inherently unsuited for the fractionation of colloid substances. Further, the very large surfaces exhibited by the gel substances will provide adsorption on the gel structures, and in certain instances this adsorption, more than the partition between the two liquid phases, may dominate the effective distribution isotherm in the chromatogram. Such adsorption effects have often been noticed in partition chromatography, and have been partially mitigated by incorporating eluants in the developing liquid and, in the case of silica gel, by special precautions in its preparation. But it will probably turn out that there are groups of substances whose molecular size or extreme adsorption renders them unsuited for partition chromatography, while nevertheless being capable of undergoing useful separations through distribution between bulk liquid phases. For such substances, counter-current liquid - liquid extraction arrangements are likely to find extensive use on the laboratory scale.[1,3,4,5]

So far, only silica gel, cellulose and starch grains have been described for use as supporting substances in partition chromatography. All these tend to hold the more polar (aqueous) member of liquid phase pairs. There is a very real need for study of the application of hydrophobic gel structures. With the more polar as the flowing phase, many new separations, for example of higher fatty acids and sterols, will become amenable to partition chromatography.

There seems no reason why partition chromatography should not be effected with a gaseous moving phase.

Partition chromatography permits the location of colourless acid or basic substances by incorporation of an indicator in the aqueous phase of the chromatogram. In addition to this, the usual methods for locating colourless substances are applicable.

Silica Gel—Using silica gel, the original[2] separation of acetylamino acids has been extended to the quantitative analysis of some amino acids[6,7,8,9,10,11,12,13,14,15,15a] and to separations of peptides,[16] but *cf.* [11]. For these purposes various special indicators have been developed.[10,17]

Sanger[18] has worked out how to separate the yellow N-2:4-dinitrophenyl derivatives of nearly all the naturally occurring amino acids and has thus, by substitution and subsequent hydrolysis, been able to identify the free amino groups of insulin,[18] "gramicidin S"[19] and a number of other proteins.

Wieland and Fremerey[20] have separated amino acids as their blue copper complexes.

Elsden,[21] following a suggestion of Lester Smith,[22] has worked out an analytical procedure

for micro-determination of the normal fatty acids from acetic to valeric. These acids are weaker than the acetylamino acids; the indicators used with the latter are therefore insensitive and bromocresol green has been employed, *cf.*[22a].

Bell[23] has applied partition chromatography to the separation of methylated sugars and has thus been able to improve the accuracy of end-group assay of methylated polysaccharides while employing smaller quantities than previously.

CELLULOSE—Using cellulose, in the form of filter paper, Consden, Gordon and Martin,[24] *cf.* [25], have worked out a very valuable qualitative ultra-micro testing procedure for the identification of amino acids and peptides. A great feature of their arrangement is that each fraction obtained chromatographically can be re-analysed with a second solvent simply by turning the sheet of paper which constitutes the chromatogram through a right angle before the second run. In this way a "two-dimensional" chromatogram, giving a characteristic arrangement of spots on the sheet of paper, results. The spots are located by spraying the dried sheet with a solution of ninhydrin or other colour test reagent. The method has found valuable applications in the protein field and in pathological work, many of which are not yet published.[11,12,26,27] Special mention should be made of Edman's use of the method in the purification and amino acid analysis of hypertensin[28] (angiotonin). It is reasonable to expect applications in fields other than protein chemistry.

STARCH GRAINS—Using starch grains, Elsden and I[29,11] have realised on a preparative scale some of the separations worked out, as mentioned, with paper on the ultra-micro scale.

An important application, using a buffered aqueous phase in the chromatogram, has been evolved by workers at the Blackley I.C.I. Laboratories—unpublished[30]; *cf.* [22,31]. I understand that this has proved of value in the purification and separation of the penicillins. I will explain the underlying principle shortly.

Turning to a theoretical consideration of partition chromatography, it is evident from what has been said by Robinson,[34] that the band-rate of each substance is determined by its effective distribution isotherm between stationary particles and moving liquid. Equilibrium must be attained within a fraction of a second. Given this condition, if the effective distribution isotherm is approximately linear fairly sharp bands will result. Deviations from linearity will have the effects already mentioned.

We can now examine the various physico-chemical equilibrium factors which jointly determine the isotherm.

(1) *Distribution of a single molecular species between the two liquid phases*—This equilibrium usually obeys Henry's law and, ideally, should determine the distribution isotherm in ordinary partition chromatography. The partition of organic molecules between liquid phases has not been studied in the detail which it merits. The simple theory, as developed by Brønsted,[32,33] suggests that for particular solvent systems the coefficient for a given molecule can be calculated from constants for each of its component groups (much like the parachor). This theoretical basis is very advantageous for planning analytical operations with new substances. It also leads to the generalisation that the partition coefficient depends on the nature of the various groups in the molecule more than on their geometrical relationship. In adsorption work geometrical relations are of greater importance. One can picture crudely that, in adsorption, groups are presented to a flat surface, so that only a part of the molecule determines the effect, while in partition, the whole of the molecule is in contact with solvent. Thus for separating the fatty acid series one would choose partition chromatography, whereas for separating structural *ortho*, *meta* and *para* isomers one would choose adsorption chromatography.

Much remains to be done in assessing the affinity of different solvents for different radicles and towards improving the theory of solubility in mixed solvents.

(2) *Adsorption on the gel structure*—This factor, when of the Freundlich type, spreads the bands, and is thus deleterious. Linear adsorption slows the bands, without spreading them, but may be deleterious by having a greater effect on a faster than on a slower moving component, thus impairing separation.

(3) *Equilibria between different molecular species*—These effects are well exemplified in the partition chromatography of organic acids, where the occurrence of double, associated molecules in the organic solvent phase and ionic dissociation in the aqueous phase both lead to deviations of the isotherm from linearity in the same sense and thus, in ordinary partition chromatograms, to "tailing" of the bands.

The ionic dissociation effect may be controlled by loading the aqueous phase with a suitable unextractable buffer mixture. Then, in the aqueous phase, the *p*H will be constant, therefore the ratio of un-ionised to ionised acid will be maintained constant, since

$$\frac{[H^{\cdot}] \times [A']}{[HA]} \text{ is constant.}$$

Thus, by using buffered columns, three desirable effects may be realised when the ionised molecular species does not appreciably enter the organic solvent phase:

(*a*) The bands of ionising substances may be rendered sharp by promoting linearity in the effective distribution isotherm.

(*b*) By controlling the *p*H of the buffer, the distribution coefficient of ionising substances may be varied over a wide range. Thus substances which in the un-ionised form would travel too fast for separation in the chromatogram may be slowed to any desired rate.

(*c*) Ionising substances differing in *p*K but having similar partition coefficients in the un-ionised state may be separated on the basis of their different ionising strengths by partition chromatography at a suitable *p*H. Craig and colleagues[5] have presented a detailed treatment of the theory of such separations and have described applications using bulk liquid extraction.

In general, other chemical equilibria than ionisation are amenable to the same sort of treatment, provided that equilibrium is established sufficiently rapidly. One can envisage use being made of complex formation and other chemical reactions for determining distribution isotherms in partition chromatograms; the range of molecular or atomic (isotopic) properties on whose basis separations may be effected chromatographically is thus indefinitely extended.

References

1. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, 1941, **35**, 91.
2. —— —— *Id.*, 1358.
3. Cornish, R. E., Archibald, R. C., Murphy, E. A., and Evans, H. M., *Ind. Eng. Chem.*, 1934, **26**, 397.
4. Craig, L. C., *J. Biol. Chem.*, 1944, **155**, 519.
5. Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., *Id.*, 1945, **161**, 321.
6. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, 1943, **37**, 79.
7. —— —— —— *Id.*, 86.
8. —— —— —— *Id.*, 313.
9. —— —— —— *Id.*, 538.
10. —— —— —— *Id.*, 1944, **38**, 65.
11. Synge, R. L. M., *Id.*, 285.
12. —— *Id.*, 1945, **39**, 363.
13. Bailey, K., *Adv. Protein Chem.*, 1944, **1**, 289.
14. Chibnall, A. C., *Procter Memorial Lecture*, 1945, *J. Int. Soc. Leather Trades Chem.*, 1946, **30**, 1.
15. Hanby, W. E., and Rydon, H. N., *Biochem. J.*, 1946, (in press).

15*a*. Blackburn, S., Consden, R. and Phillips, H., *Id.*, 1944, **38**, 25.

16. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Id.*, 1943, **37**, 92.
17. Liddell, H. F., and Rydon, H. N., *Id.*, 1944, **38**, 68.
18. Sanger, F., *Id.*, 1945, **39**, 507.
19. —— *Id.* (in press).
20. Wieland, T., and Fremerey, H., *Ber.*, 1944, **77**, 234.
21. Elsden, S. R., *Biochem. J.*, 1946 (in press).
22. Smith, E. L., *Id.*, 1942, **36**, Proc. xxii.

22*a*. Ramsey, L. L., and Patterson, W. I., *J. Assoc. Off. Agric. Chem.*, 1945, **28**, 644.

23. Bell, D. J., *J. Chem. Soc.*, 1944, 473.
24. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.
25. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Id.*, 1943, **37**, Proc. xiii.
26. Consden, R., Gordon, A. H., Martin, A. J. P., Rosenheim, O., and Synge, R. L. M., *Id.*, 1945, **39**, 251.
27. Dent, C. E., 1946. (Applications in clinical pathology—to be published.)
28. Edman, P., *Arkiv. Kemi, Mineral. Geol.*, 1945, **22A**, No. 3.
29. Elsden, S. R., and Synge, R. L. M., *Biochem. J.*, 1944, **38**, Proc. ix.
30. Levi, A. A., Terjesen, S. G., and I.C.I. Ltd., Brit. Pat. 569,844.
31. Catch, J. R., Cook, A. H., and Heilbron, I. M., *Nature*, 1942, **150**, 633 (*cf.* Brit. Pat. 558,320).
32. Brønsted, J. N., *Z. phys. Chem., Bodenstein Festband*, 1931, p. 257.
33. —— *Comptes rend. Trav. Lab. Carlsberg*, 1938, **22**, 99.
34. Robinson, F. A. R., Analyst, 1946, **71**, 251.

Lister Institute for Preventive Medicine
London, S.W.1

Chromatography in the Analysis of Fatty Oils

By K. A. WILLIAMS

In the early days of the use of chromatography, adsorbents and solvents were chosen largely by a process of trial and error. With its more extended use certain regularities have become apparent in the behaviour of particular adsorbents towards compounds related to one another, but there is as yet no complete system of classification which makes it possible to predict the most suitable conditions for any desired new separation.

It is clear that the selective adsorption of one or more of the constituents of a solution depends on the relative affinities of the adsorbent and the solvent for the solutes. Thus solvents are chosen which have little or no affinity for the dissolved substances; they are usually non-polar in nature and may be typified by light petroleum and *n*-hexane. The adsorbents are more or less polar and include aluminium oxide, magnesium oxide, silica, sodium carbonate and a great variety of other substances. Adsorption occurs at the more reactive groups in the molecules adsorbed, for example at double bonds, carboxy groups and hydroxy groups. Different crystalline modifications of a particular adsorbent may act in different ways, but there is little precise information available to guide one in these variations.

We may regard chromatographic adsorption as the result of a competition in attraction between the adsorbent and the solvent for the solute, with accompanying dissolved substances influencing the degree of attraction. There seems to be little doubt that the contribution of each substance to the resultant affinity could be worked out on a quantitative basis, but this is not yet possible, and in many fields the basis remains qualitative or only very roughly quantitative at best.

The earlier applications of chromatography in the analysis of fatty oils were made on the unsaponifiable constituents. Heilbron,[1] reviewing the adsorption of the carotenes and related substances on magnesium oxide, notes that of the carotenes the γ-isomer is most strongly adsorbed, the β-isomer rather less strongly and the α-isomer least strongly. The varying affinity of magnesium oxide for these bodies is ascribed to their varying degrees of unsaturation and conjugation. Thus α-carotene, with the least affinity for the adsorbent, has 10 double bonds out of 11 conjugated; β-carotene contains 11 double bonds, all conjugated; and γ-carotene, the most strongly adsorbed, contains 12 double bonds, 11 of which are conjugated.

The introduction of hydroxy groups into the carotenes does not alter the order in which they are adsorbed, so that amongst the monohydroxy derivatives we find rubixanthin, corresponding to γ-carotene, more strongly adsorbed than kryptoxanthin, corresponding to β-carotene; amongst the dihydroxy derivatives zeaxanthin, corresponding to β-carotene, is more strongly adsorbed than lutein, corresponding to α-carotene.

The hydroxy groups do, however, raise the general level of adsorption affinity, monohydroxy-carotenes being more strongly adsorbed than carotenes, and dihydroxy-carotenes more strongly than monohydroxy-carotenes. The affinity of magnesium oxide for xanthophylls is so great that they can be adsorbed from ether solution, whereas the carotenes cannot be adsorbed from so polar a solvent and the non-polar light petroleum has to be employed if they are to be picked up by the column. Increase in oxygen content, in the absence of ester groupings, progressively augments the adsorption affinity. Thus astacene, a prominent colouring matter of herring oil,[2] is adsorbed as a red-violet layer on aluminium oxide and cannot be eluted by means of methyl alcohol containing a trace of ether, but requires the highly polar chloroform.

Esterification of the xanthophylls reduces the strength of their adsorption nearly to that of the carotenes.

Dealing more generally with the unsaponifiable matter of olive oil, wheat germ oil and certain marine animal oils, Drummond and his co-workers[2,3,4] dissolved the unsaponifiable matter in light petroleum and passed the solutions through aluminium oxide. They found that saturated hydrocarbons passed most readily through and that unsaturated hydrocarbons such as squalene and carotene passed through almost as easily. Unsaturated alcohols tended to be washed to the lower parts of the column, the extent of their movement being influenced largely by the amount of squalene in the solution. A broad band of sterols appeared about one-third of the way down the column, and lipochromes of the xanthophyll type were found mixed with the sterols or in neighbouring zones. Saturated alcohols were usually at the top

of the column. On re-fractionation of products removed from specific zones it was found that the relative order of adsorption might be changed, almost certainly owing to removal of the influence of co-solutes on the first adsorption.

Reference must next be made to the work of Walker and Mills,[5] who dissolved linseed oil in *n*-hexane and passed the solution through aluminium oxide. The column was divided into equal portions and oil adsorbed on each was recovered and re-fractionated. It proved possible to separate the original oil quantitatively into fractions containing 7, 6, 5 and 4 double bonds per molecule, and evidence was obtained of the presence of more highly unsaturated molecules. Thus one fraction would contain mono-oleo di-linolenin and di-linoleo mono-linolenin, both with 7 double bonds; a second would include mono-myristo-, palmito- and stearo- di-linolenin, tri-linolein and oleo-linoleo-linolenin, all containing 6 double bonds; and so on. The constituents of the oil were found to range themselves on the columns in descending order of unsaturation. This has a parallel to which attention may well be drawn in the catalytic hydrogenation of oils, where there is a marked selective hydrogenation of the more unsaturated fatty acids combined as esters before the less unsaturated ones react. Thus[6] if linseed oil is hydrogenated with nickel catalyst at about 180° C. its linolenic acid is reduced almost completely to acids of lower iodine value by the time that the iodine value drops to 120–130, and linolic acid disappears when the iodine value reaches about 50. The proportion of saturated acids does not rise to any marked extent until the linolenic acid has disappeared and the linolic acid has mostly been reduced. This parallel gives strong additional confirmation of adsorption on the catalytic metal as an essential stage in the hydrogenation reaction.

With fatty acids adsorption affinities are the reverse of those for fatty esters. Both on aluminium oxide and on magnesium oxide[7,8] the more saturated acids are most strongly adsorbed from light petroleum; fair, but not quantitative, separation is achieved in one passage over the adsorbent. Among the saturated higher fatty acids, those of higher molecular weight are more strongly adsorbed. Mono-glycerides are more strongly adsorbed than di-glycerides, and the latter more strongly than tri-glycerides, as is to be expected from the increased polarity conferred by additional alcoholic groups.

It will thus be seen that, in the absence of other polar groups, increasing adsorption affinity arises from increasing unsaturation, notably for hydrocarbons and esters. On the other hand the order is reversed for fatty acids and alcohols, the more unsaturated compounds being less easily adsorbed. This reversal has been ascribed to the effect of a chemical affinity of the adsorbent for acids, but it is doubtful whether such an explanation could hold with alcohols, and it is not supported by the failure of calcium carbonate to adsorb normal fatty acids from light petroleum.

The reversals become understandable if we postulate the occurrence of polar charges of differing sign and we have an adsorbent capable of attracting polar material of either positive or negative charge; thus double bonds of negative charge would be attracted in order of increasing unsaturation if they occur in molecules otherwise non-polar; but if the molecules also include a terminal group of strong positive polarity such as a carboxy or hydroxy group and the operative affinity is a function of the algebraic sum of the various polarities, increasing unsaturation could well lead to decreasing affinity. Absence of reversal of affinity gradation in passing from the carotenes to the xanthophylls, differing by a hydroxy group, would be due to this group having a positive polarity numerically less than the negative polarity arising from the double bonds.

It is interesting to note that reversal of the order in which related compounds are adsorbed may be brought about by changing the solvent. Thus dyes of the fluorescein series are adsorbed on aluminium oxide in order of increasing halogen content, but the order is reversed if the solvent is 60% aqueous pyridine. We may assume that the oxide will attract most strongly the dye held least strongly by the solvent. Then, if water has a small attraction for the dye, the order of adsorption would be expected to follow the halogen content. If, on the other hand, the solvent attracts the dyes strongly in order of increasing halogen content, as might be expected with pyridine, the adsorbent would remove from solution most easily the dye least strongly held by the solvent, and the order of adsorption is reversed.

It is known that electrical charges affect adsorption. Thus certain colouring matters found in fatty oils are positively charged and migrate to the negative pole on electrolysis. These colours are strongly adsorbed by fullers' earth, which bears a negative charge. On the other hand, negatively charged colouring matter found in sugars is not removed by the

earth, but is more readily attracted by carbon, an adsorbent without natural charge. If a positive charge is given to the carbon, its power of decolorising sugar is enhanced. The decolorising power of carbon towards oils is said to be reduced by addition of soluble acid to the oil, as the positive ions of the acid then compete with the colouring matter for a place on the adsorbent.

Results have been obtained in most of the analyses so far referred to by fractionation and re-fractionation on chromatographic columns; such examinations are lengthy and can be attempted only in instances where lengthy treatment is justified. Elaborate procedure is not required for a number of separations, and simple chromatographic methods are available for use in commercial analysis, leading to the removal and determination of a single constituent of a fat. Amongst these are the following.

(1) *Free Fatty Acids*—Sylvester, Ainsworth and Hughes[9] pass an ether solution of a fat through a column of aluminium oxide; the free fatty acids are quantitatively removed by the adsorbent, and the solvent is sufficiently polar to retain all other ether-soluble matter, so that this may be recovered quantitatively by washing the column with ether and evaporating off the solvent. The method gives an extremely satisfactory separation and is likely to be used extensively. It has obvious advantages over the usual method of titration with alkali, no assumption of molecular weight being involved; it is also preferable to extraction of the acids with aqueous-alcoholic alkali from light petroleum solution as this renders possible the inclusion of some glycerides of lower fatty acids with the free acids. The adsorption method also proves very satisfactory in the purification of the preliminary extract of unsaponifiable matter yielded by the S.P.A. method for the determination, replacing the lengthy technique of washing with water and alkali in that method.

The preferential adsorption of free fatty acids from mixtures with neutral fats has its parallel in the catalytic hydrogenation of such mixtures, for which Pelly[10] showed the free fatty acids to be preferentially hydrogenated by nickel catalyst. Here again there is confirmation that adsorption is of major importance in catalysis.

(2) *Hydrocarbons*—The passage of hydrocarbons through a column of aluminium oxide has been developed into a very useful method for their determination. Fitelson[11] concentrates squalene from olive and other oils by chromatographing a solution of the unsaponifiable matter in light petroleum through the oxide; the filtrate, containing the squalene and any other hydrocarbon present, is evaporated and recovered, operations being conducted in an inert atmosphere. Squalene may be identified by its crystalline hydrochloride, and its proportion determined from the iodine value obtained by the Rosenmund-Kuhnhenn method. The presence of this hydrocarbon has been conclusively demonstrated in notable quantities in olive oil, where it accounts for the high iodine value of the unsaponifiable matter, wheat germ oil, rice bran oil and many marine animal oils, and in much smaller quantities in many other oils.

Similar separations of hydrocarbons have been in use in my laboratory for some years for determining mineral oils in admixture with fatty oils and waxes. For example, adventitious hydrocarbons (mineral oil) can be thus determined in wool grease; the method may be used for determining hydrocarbons in distilled fatty acids so as to assess the degree of degradation that has taken place during manufacture, and in the determination of mineral oils in compounded oils and in certain mixtures, used in the leather industry, containing sulphonated oils.

(3) *Carotene, Xanthophylls and Vitamin A*—Several methods have been published dealing with the determination of these substances, and two are chosen for their illustration of principles. Barton Mann,[12] analysing the fat of egg yolk, prepares the unsaponifiable matter and adsorbs it from light petroleum on bone meal, previously defatted. Light petroleum elutes α- and β-carotenes quantitatively from the column. Carotene and vitamin A are eluted quantitatively by means of a solution of 2% of chloroform in light petroleum, this small proportion of chloroform being sufficient to overcome the affinity of the oxide for the vitamin. Sterols are also removed by the mixed solvent. Xanthophylls are removed from the column by acetone. This is a good illustration of the changes that can occur when eluting solvents are altered, the effect being controlled by the polarity of the solvents.

Cooley, Christiansen and Schroeder chromatograph a light petroleum extract from feeding stuffs on sodium carbonate, and thereby remove all chromogenic substances capable of interfering with the antimony trichloride test for blue value.[13]

(4) *Oxidised Acids*—The methods so far described have dealt with the adsorption of

substances completely in solution. There is a growing class in which adsorption is used to assist in the stripping of materials of low solubility from a solvent. The determination of oxidised fatty acids is an instance. These acids are characterised by their low solubility in light petrol and thus differ from the freely-soluble normal acids. The classic method for their determination consists in the preparation of the free fatty acids by saponification, and their extraction with the solvent. A precipitate forms, and after standing, is filtered off, washed, dried and weighed. Alternatively the washed precipitate may be extracted with ether, when a proportion will dissolve, this proportion depending on the degree of oxidation. Experience of the test makes it clear that the results are affected to a notable extent by the relative proportions of solvent and acids taken, as the normal fatty acids have a solubilising effect; if the oxidised acids are present in small proportion they may be incompletely precipitated.[14] Recent work in my laboratory has shown that a more satisfactory determination may be made if the light petroleum solution filtered from the oxidised acid precipitate is passed through a column of calcium carbonate, previously heated to about 200° C., for 2 hours. Washings from the precipitate are also passed through the filter, which is further washed with light petroleum and then eluted with ether. The ether eluate is evaporated and the residue dried and weighed, the weight being added to that of the precipitate. The material so separated appears to have a composition very similar to that of the main precipitate. All fatty acids passing the filter and caught on the column have so far been entirely ether-soluble. Calcium carbonate is used as adsorbent since, in contradistinction to alumina, it does not adsorb normal fatty acids from light petroleum.

Typical results obtained by the method are shown in Table I.

TABLE I

Sample	Total oxidised acids found, per cent.	
	Classic method	Adsorption method
Arachis oil	nil	0·40; 0·38
Sod oil	1·19	1·48; 1·47
Linseed oil (oxidised) ..	21·56	22·35; 22·38

The close relation between development of peroxide value and oxidised acids in fatty oils is similar to that noted in turbine lubricating oils.[15]

(5) *Impurities in Sulphur Olive Oil*—This oil contains impurities soluble in carbon disulphide and comparatively insoluble in light petroleum, and of a resinous nature. It is known that, as for oxidised acids, the extent of their precipitation from light petroleum is influenced by the relative amounts of oil and solvent. Agreement had been reached in 1939 for the use of 50 times as much light petroleum as oil in the determination of the impurities, there being evidence that further dilution caused no further precipitation. Attempts have now been made to remove higher amounts of impurity by chromatographic means. It was found that adsorption occurs when a mixture of the oil and light petroleum is passed through a column of calcium carbonate, and that the resinous impurities thus adsorbed can be eluted by carbon disulphide. The amount recovered is identical, within the limits of experimental error, with that found by the agreed filtration method; and it is concluded that the agreed method is satisfactory. In a typical experiment, 5 g. of a filtered sulphur olive oil were dissolved in 250 ml. of light petroleum and passed through calcium carbonate. A dark green band formed at the head of the column; the column was washed with 200 ml. of light petroleum and eluted with carbon disulphide. After evaporation of the solvent and drying, the residue amounted to 4·72%, and in a duplicate test to 4·74%. Determination by the agreed method (5 g. of oil, dissolved in 250 ml. of light petroleum, is allowed to stand for 30 min. and filtered through a paper previously dried and weighed in a bottle; the paper is washed well with cold light petroleum, dried to constant weight at 105°, and re-weighed in the bottle) gave 4·72%.

SUMMARY—The application of chromatography in the analysis of fatty oils is briefly reviewed, emphasis being laid on the principles involved. Methods are described for the determination of free fatty acids, carotene, xanthophylls and vitamin A, hydrocarbons, oxidised acids and certain impurities of sulphur olive oil. No exhaustive summary of the literature has been attempted, fuller accounts appearing in *Paint Technology*, **1945**, **10**, 85 and 107.

A quantitative basis needs to be established on which the relative affinities of solvents and adsorbents for solutes may rest. At present there is no clear indication where this is

to be sought; it may lie in parachors, di-pole data, surface potentials or elsewhere. It seems certain that its discovery will be assisted by what we know of forces existing near the surface of liquids and at the irregular surface boundaries of solids, the importance of whose fine cracks and projecting or partly isolated atoms is rapidly becoming recognised.

REFERENCES

1. Heilbron, I. M., *J. Soc. Chem. Ind.*, 1937, **56**, 160T.
2. Thorbjarnarson, T., Ruiz, A. Santos, and Drummond, J. C., ANALYST, 1935, **60**, 382.
3. Thorbjarnarson, T., and Drummond, J. C., *Id.*, 1935, **60**, 23.
4. Drummond, J. C., Singer, E., and Macwalter, R. J., *Biochem. J.*, 1935, **29**, 456.
5. Walker, F. T., and Mills, M. R., *J. Soc. Chem. Ind.*, 1942, **61**, 125; 1943, **62**, 106.
6. Williams, K. A., and Bolton, E. R., ANALYST, **49**, 460.
7. Kaufmann, H. P., *Fette und Seifen*, 1939, **46**, 268; 1940, **47**, 460.
8. Graff, M. M., and Skau, E. L., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 340.
9. Sylvester, N. D., Ainsworth, A. N., and Hughes, E. B., ANALYST, 1945, **70**, 295.
10. Pelly, R. G., *J. Soc. Chem. Ind.*, 1927, **46**, 449T.
11. Fitelson, J., *J. Assoc. Off. Agr. Chem.*, 1943, **26**, 499, 506.
12. Mann, A. Barton, ANALYST, 1943, **68**, 233.
13. Cooley, M. L., Christiansen, J. B., and Schroeder, C. H., *Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 669.
14. Dastur, N. N., and Lea, C. H., ANALYST, 1941, **66**, 90.
15. Williams, K. A., *Id.*, 1945, **70**, 409.

6, MILNER STREET
LONDON, S.W.3

February, 1946

Some Applications of Chromatographic Analysis in Industry

BY F. R. CROPPER

CHROMATOGRAPHIC analysis is a comparatively recent addition to the methods used by the industrial analyst for routine control testing and research purposes. The slowness with which chromatography was adopted was due partly to the fact that suitable adsorbents were expensive and had to be "recovered" for subsequent re-use, and were then found, as often as not, to be of different adsorptive power. Merck's Alumina "*nach* Brockmann" was the most popular adsorbent[1]; this was prepared by heating aluminium hydroxide to a strong red heat, and then partially deactivating by shaking with moist air for short or long periods, as necessary to give reproducible activity. Difficulties arose after 1939, when supplies of Merck's material ceased. In the Dyestuffs Division of Imperial Chemical Industries Ltd. this was overcome by preparing activated material by the process initiated by A. Stewart[2]; this process involves activation of alumina by heating to 360° C. to reduce the moisture content to 10%, and deactivation of the product to any desired degree by adding a little water, *e.g.*, 1–5%, and then mixing thoroughly. The deactivated grades are of reproducible quality, have a comparatively high adsorptive power and allow a fairly rapid rate of flow of solvent. A suitable activated alumina is obtainable from Peter Spence & Co. Ltd., Manchester, under the name Type "O" Activated Alumina, and the price is such that recovery by solvent extraction and reactivation is unnecessary. The activity of Merck's Alumina was controlled by chromatographic trials, using a solution of several Sudan colours.[1] The reproducibility of the Type "O" alumina, and of the partially deactivated products, may be controlled in an analogous way, using a solution of three azo compounds, namely, azobenzene, benzene-azo-β-naphthol and *p*-dimethylaminoazobenzene; a solution of these three colours in a mixture of chlorobenzene and light petroleum is added to the column, which is developed with the same solvent mixture; azobenzene quickly descends the column and is soon washed through completely, while benzene-azo-β-naphthol is held the most tightly. The position of the bands after washing with a known amount of solvent gives a semi-quantitative check on the adsorbent power. It does not follow, of course, that an alumina which is satisfactory for this separation will necessarily be the best for other types of separation.

Chromatographic analysis has been developed by A. Stewart* as a particularly valuable tool in the anthraquinone field. An example is provided by the analysis of technical 2-amino-anthraquinone, using Type "O" alumina plus 5% of added water as adsorbent and a mixture of toluene and pyridine as solvent. The column is developed until the main zone is completely

* Research Department, I.C.I. Ltd., Dyestuffs Division.

eluted, and the intensity of colour of the percolate is then measured on the Spekker absorptiometer. The column exhibited at the Meeting was prepared deliberately from a mixture of crude 1-amino- and 2-amino-anthraquinones, and was at a half-developed stage; the percolate contained the 1-amino-isomer, while the bands on the column corresponded to 2-aminoanthraquinone and 1 : 2-diamino- and 1 : 4-diamino-anthraquinones. In actual fact there is no 1-aminoanthraquinone in good quality technical 2-aminoanthraquinone. Quantitative analyses of many other coloured compounds are carried out in an analogous manner. In addition to control analyses of this kind, chromatography has been used for identification purposes. A competitive dyestuff, for example, may be separated into its several fractions, each of which may be identified separately; in one instance, hydrolysis of the dyestuff gave benzoic acid, a dibasic acid and a coloured mixture which was resolved from benzene - acetone solution into 1 : 4-diaminoanthraquinone (violet zone) and 1 : 5-diaminoanthraquinone (orange zone), indicating that the original dyestuff was an amide derived from 1 mol. of dibasic acid with 1 mol. each of 1-amino-4-benzoylaminoanthraquinone and 1-amino-5-benzoylaminoanthraquinone.

The chromatography of materials which are normally colourless in visible light usually necessitates the use of some alternative means of locating the zones; the easiest way, when dealing with fluorescent compounds, is to observe the column under an ultra-violet fluorescence lamp. This method was employed,[3] in conjunction with the ultra-violet absorption spectrograph, for the determination of anthracene in coal-tar and coal-tar distillates. The ultra-violet absorption spectrum of anthracene is highly characteristic, with intense maxima at 360 $m\mu$ and 380 $m\mu$ in chlorobenzene solution. The spectra of tar oils show these two maxima, but their relative intensities indicate that other components of the oil also absorb ultra-violet radiation at these wavelengths, thereby "distorting" the anthracene absorption curve. Separation of anthracene from these other components was effected by chromatography on alumina, using a mixture of chlorobenzene and light petroleum as solvent. The anthracene passes down the column fairly quickly, leaving all the more complex polynuclear hydrocarbons higher in the tube. In ordinary light, only narrow yellow or brown zones are visible at the top of the column, but in ultra-violet light several fluorescent zones appear*; the anthracene zone is located by its fluorescence, in the lower third of the column, the zone is removed mechanically from the tube and the anthracene is dissolved out with hot chlorobenzene. The amount of anthracene in the solution was determined by its ultra-violet absorption intensity. The absorption curves of the components in the other zones of the column showed that the compounds responsible for "distortion" of the original anthracene curve were adsorbed more strongly. It is believed that this is the first time that reliable figures have been obtained for the anthracene content of tar (1%) and of pitch (0.4%), in contradistinction to the amount which can be isolated in the form of crude pastes by a process of fractional distillation and chilling.

The activated alumina Type "O" is strongly alkaline, and is quite unsuitable for use for materials which are unstable in presence of alkali or which contain acidic groups. An acid-washed alumina can be prepared by boiling Type "O" alumina with dilute acetic acid, filtering, washing and again activating by heating to 360° C.; the product has a slightly acid reaction and its activity is rather less than that of the original Type "O" adsorbent. This material has been used[4] by J. Raventos† in biological studies on the breakdown products from thiobarbituric acids. The determination of barbituric acids and thiobarbituric acids in admixture was achieved by preparation of a chloroform extract of the biological fluid or tissue, and separation by chromatography on acid-washed alumina; the acids were held on the alumina while the pigment was washed out, and the thiobarbituric acid was then eluted by chloroform containing 2% of methanol. The barbituric acid was ultimately eluted by chloroform containing 10% of methanol. Each eluate was then tested by an appropriate colour reaction which, while satisfactory for a single component, would not have been applicable to the original mixed barbituric acid and thiobarbituric acid extract. It was shown that thiobarbituric acids are excreted partially as the non-thio analogue.

Adsorbents other than alumina have had a more limited application. Magnesium carbonate "Pond" is strongly adsorbent for some types of compound, and gives a satisfactory rate of flow of solvent. It has proved useful for chromatographic separations when alumina

---

* This column was demonstrated.

† Research Department, I.C.I. Ltd., Dyestuffs Division.

has not been permissible owing to formation of lakes, as is the case with hydroxyanthraquinone derivatives.

H. E. Stagg* has used filter paper extensively as adsorbent in tests for the quality of dyestuffs intermediates. These paper chromatograms, or capillary run-out tests, are carried out on filter paper circles placed between two flat glass plates, the upper of which has a central hole.[5] A concentrate of the alleged impurities in the intermediate is diazotised and coupled, or is coupled with a suitable diazo reagent, and a drop of the coloured solution is then placed in the hole; to this is added water or other suitable solvent, dropwise from a burette, to develop the chromatogram. As the water or other solvent diffuses from the central hole, the components of the mixture are carried in concentric rings towards the rim of the paper. The intensity of the impurity zones is then compared with a standard series of papers prepared in the same way from known synthetic mixtures. An alternative procedure involves only the dipping of a paper strip into the coupled mixture and observation of the rise of the various components by capillary attraction.

G-acid (β-naphthol-6 : 8-disulphonic acid) can be determined in R-acid (β-naphthol-3 : 6-disulphonic acid) by coupling with diazotised *m*-nitroaniline and running out on paper between glass plates; the range 0–4% of G-acid may be covered with an accuracy of about 0.5 in the percentage figure. The standard series of papers† shows a gradual increase in the intensity of the yellow zone derived from G-acid as the concentration of this acid rises.

Aniline, when present as impurity in aminoazobenzene, may be determined by diazotising and coupling with H-acid (1-amino-8-naphthol-3 : 6-disulphonic acid), and developing a paper chromatogram in the way described; the intensity of the red zone is a sensitive measure of the aniline content.† In the case of Tobias acid (β-naphthylamine-1-sulphonic acid) a concentrate is coupled, run out on paper and developed until the outer zone, due to the isomeric β-naphthylamine-8-sulphonic acid (not normally present), is well separated from the main zone; the outer zone is cut out and extracted and the colour of the extract is measured by the Spekker absorptiometer.

This account of chromatographic analysis in an industrial analytical laboratory is necessarily brief, but sufficient will have been said to show that chromatography is certainly earning its rightful place as one of the most valuable weapons in the hands of the industrial analyst.

References

1. Brockmann, H., and Schodder, H., *Ber.*, 1941, **74**, 73.
2. Stewart, A., and Imperial Chemical Industries Ltd., British Patent Specification No. 565,405.
3. Cropper, F. R., and Strafford, N., *J. Soc. Chem. Ind.*, 1944, **63**, 268.
4. Raventos, J., Personal communication and *Brit. J. Pharmacology and Chemotherapy* (1946—in press).
5. Brown, *Nature*, 1939, **143**, 377.

Imperial Chemical Industries Ltd., Dyestuffs Division
Analytical Laboratories, Blackley, Manchester, 9

Discussion on the Four Preceding Papers

The Chairman (Mr. R. C. Chirnside) said the papers read seemed to fit into each other very conveniently to provide a basis for discussing the subject.

Dr. F. R. Cropper said it appeared from Mr. Williams's remarks on the chromatographic separation of the glycerides of linseed oil that the bulk of the glycerides contain 7 double bonds per molecule. Examination of the chromatographic fractions by the ultra-violet absorption spectrograph, preferably after application of glycol saponification, would show how this and other fractions were constituted as regards linolic and linolenic acids, *e.g.*, oleo-dilinolenin or dilinoleo-linolenin. Did Mr. Williams know if any work had been done on these lines?

Mr. Williams said he knew of none. There were chemical methods of dealing with the problem, but to obtain sufficient of the chromatographic fractions for such work took a very long time. For example, with linseed oil, from 12 to 20 fractionations would be necessary in the first place to obtain sufficient material to fractionate in the second series, and six or twelve months' work might be involved.

Mr. M. R. Mills endorsed what Mr. Williams had said about the length of time required for this type of work. For the separation of the component glycerides of linseed oil a long series of experiments were necessary to obtain fractions representing only one level of unsaturation. In an instance with which he was concerned the final products were not large enough for exhaustive examination and had been used for thiocyanogen value determinations. The next step was obviously to explore the fatty acid composition at the different levels of unsaturation so that the glyceride species present in each could be identified. It seemed probable that more recent developments of chromatographic technique as applied to fatty oils would simplify the separation and give larger yields, thus opening up a wide field for future research.

---

* Analytical Department, I.C.I. Ltd., Dyestuffs Division.

† These papers were made available for detailed examination during the meeting.

Mr. G. H. OSBORN asked whether inorganic substances had been found amenable to chromatographic separation.

Mr. ROBINSON said he had mentioned as one such instance the separation of certain metals on a column of 8-hydroxyquinoline; banding occurred, with vanadium at the top and then tungsten, copper, nickel and cobalt, zinc, iron and uranium. With other adsorbents the method could be used to separate potassium, sodium, magnesium and calcium.

Dr. SYNGE said that, reading between the lines of the Smythe Report on atomic energy, he thought some revelations might be looked for in the inorganic field.

Mr. WILLIAMS mentioned that in a lecture before the Institute of Chemistry by A. H. Cook in 1941 reference was made to the separation of isotopes of lithium in columns about 30 feet in length.

Dr. CECIL L. WILSON said he had had some experience of inorganic chromatography. There was a fundamental difference between the underlying processes of chromatographic separations of organic and inorganic substances. Although banding occurs with both classes it is impossible with inorganic substances to get the clear separations, with blanks between, that are usually displayed by organic mixtures. Secondly, it is usually preferable to work with extraordinarily dilute solutions of inorganic ions. Inorganic separations had been found especially sensitive for the detection of very small amounts, sometimes as little as 1 p.p.m. of one ion in presence of another. Iron could thus be detected in trace amounts in copper. A very recent paper recommended the use of 8-hydroxyquinoline columns for routine qualitative separation of copper from cadmium, but this required further trial.

Dr. S. TORRANCE asked whether in inorganic ionic adsorption by special synthetic resins the adsorption occurred in a series of chromatic bands amenable to separation.

Mr. ROBINSON said the adsorption occurred in bands, and Amberlite I.R. 100 when used for adsorbing basic substances gave banding.

Dr. CROPPER asked if there was a real gap between the bands which made it easy to separate them.

Mr. ROBINSON said the bands were not separated by gaps as in the examples shown.

Dr. SYNGE thought that these ion exchange reactions were more amenable to treatment by the displacement chromatography of Tiselius, in which there was a procession of substances eluting one another as they passed down the column. Incidentally one often noticed that in ordinary organic chromatography, in the early stages where the mixture was very crude and one substance displaced another, sharp bands occurred near the top of the column due to substances that would never show up sharply if in the pure state. This displacement technique was particularly suitable for substances where the Freundlich index $1/n$ was very sharply different from unity and the adsorption isotherm was curved, as often with inorganic materials, and then the band developed a very sharp front; if its tail could be chased along with a more strongly adsorbed substance a very sharp and pure fraction might be obtained, although there was no space between the different substances.

Dr. J. G. A. GRIFFITHS said that, as a rapid sorting test for finding suitable adsorbents and eluants for chromatographic separations, the filter paper, in the technique in which a filter paper is placed between two glass plates one of which has a single perforation, may be replaced by a thin layer of finely powdered adsorbent such as alumina, bone-meal, etc. As with the filter paper, the chromatogram consists of a series of concentric circles round the hole in the glass plate.

Dr. S. JUDD LEWIS asked if chromatography could succeed in the detection of tea-seed oil in olive oil, and/or in the differentiation of the natural colouring constituents of jams, both of which were possible by absorption spectrography.

Mr. WILLIAMS said that he must pay a tribute to Dr. Judd Lewis's spectroscopic method for detecting tea seed oil, which was the first successful method to be used for that purpose. He had not had occasion to investigate the basis of the method fully. He suspected, however, that the difference between the spectrograms of tea-seed oil and olive oil was not basic, depending on the composition of the oils themselves, but rather due to the presence of colouring matters or the like usually associated with one or the other. If that were so the test would not stand up any better than some others that have been proposed, such as one based on fluorescence of tea-seed oil which it was later found possible to remove or to impart to olive oil also. On the subject of colours in jams, Mr. Williams said he had no knowledge of any chromatographic work, but it seemed a problem likely to respond to chromatography.

Mr. E. PAUL referred to the investigation of the colouring matter in cigarette tobacco. Part was easily dealt with in light petroleum solution, but a large proportion was soluble in water and very difficult to adsorb except on charcoal; alumina would not hold it.

Mr. WILLIAMS suggested that the water extract containing the colouring matter contained also large amounts of reducing sugars, and these, like other solutes, might interfere with adsorption of colouring matters.

Dr. T. C. J. OVENSTON said that explosives chemists like himself doubted whether alumina deserved the popularity it had gained as a chromatographic adsorbent. Some important advantages were offered by a less expensive commercial grade of silica gel. The instability of some substances when adsorbed on alumina had not been observed when this particular silica gel was used. He also asked if anyone else had ever obtained two bands from one homogeneous substance. This phenomenon had been observed after certain pre-washing treatments of the column, when the water content of the adsorbent exceeded a critical value. In such circumstances an uneven water distribution was set up, leading to irregular activity down the column, and this probably accounted for the abnormality.

Dr. CROPPER said he had tried silica on several occasions but had found it difficult to reproduce chromatograms owing to variation in particle size, and for his purposes alumina had been more satisfactory; he did not wish, however, to deprecate a plea for the more extended use of silica.

Mr. H. C. RAMPTON had had some experience on the analysis of hydrocarbon mixtures, using silica gel, and he agreed with what had been said about particle size; the smaller the particles the sharper the separation between the zones. As a petroleum technologist he was interested to know what was meant by "light petroleum" Petroleum products were now more variable in quality than before the war. That of b.pt. 60–80° C. might contain aromatics, and benzene would be liable to be adsorbed on the top part of a

chromatographic column. For chromatographic purposes he thought a pure hydrocarbon such as normal hexane would be preferable.

Mr. WILLIAMS said that by "light petroleum" was meant the "light petroleum" of the British Pharmacopoeia, which used to be called petroleum ether, and which is free from aromatics. The grade used boils between 40° and 60° C. He did not know its precise composition, but for his chromatographic work with fatty oils small differences in composition would not matter.

The Microbiological Assay of Amino Acids. I. The Assay of Tryptophan, Leucine, *Iso*leucine, Valine, Cystine, Methionine, Lysine, Phenylalanine, Histidine, Arginine and Threonine

BY E. C. BARTON-WRIGHT

(*Read at the Meeting of the Society on Wednesday, May* 1, 1946)

THE advances made in recent years in the successful assay of the components of the vitamin-B complex by means of the lactic acid-forming bacteria have now been extended to the quantitative estimation of a number of naturally occurring amino acids, including the eleven so-called "essential" amino acids. These methods have been chiefly developed in the U.S.A. The quantitative determination of any amino acid by chemical methods is difficult and tedious and requires considerable skill and practice. Moreover, the aliphatic amino acids, *e.g.*, *iso*leucine, leucine and valine, present special difficulties of their own. On the other hand, microbiological methods of assay for the determination of amino acids are rapid, specific and relatively simple to carry out and require no special or elaborate apparatus. Microbiological methods have the further advantage that they make it now possible to determine the amino acid content of foods directly; the presence of fats and carbohydrates often causes a good deal of interference with chemical methods.

A general survey has been made in these laboratories of microbiological methods of assay of amino acids, in an attempt to choose the most suitable procedures, more especially in connection with the estimation of the "essential" acids (*cf.* Rose[1]) arginine, cystine, histidine, *iso*leucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine and their application to the quantitative distribution of these acids in different parts of the wheat grain (see Part II, this vol., p. 278).

EXPERIMENTAL

Organisms—The organisms used in this work were the lactic acid-forming bacteria *Lactobacillus arabinosus* 17/5, *Leuconostoc mesenteroides* P-60 and *Streptococcus faecalis* (*lactis*) Rogers. Stock cultures of these organisms were carried on a yeast-water-glucose agar to which had been added 0·6% of sodium acetate. The cultures were preserved in a refrigerator at 4° C. approximately and renewed at weekly intervals. It was found necessary to grow *L. mesenteroides* P-60 on an agar slope instead of the more usual stab cultures used for lactic organisms.

ASSAY OF TRYPTOPHAN

The assay of tryptophan can be described apart from that of the remaining acids because it forms a special exception. Tryptophan is destroyed by prolonged hydrolysis with strong acids, *e.g.*, concentrated hydrochloric acid and 25% sulphuric acid, under the conditions employed for the hydrolysis of casein and other proteins. It therefore becomes possible to utilise *Lactobacillus arabinosus* 17/5 for the assay of this amino acid with only slight modification of the basal medium employed for the microbiological assay of nicotinic acid with this organism (Barton-Wright[2]). All that is necessary is to omit tryptophan from the medium and add excess of nicotinic acid. Such a medium has been described by Greene and Black,[3] but it will be found more advantageous to use a concentration of 2% of glucose and 2% of sodium acetate in place of the 1% of glucose and 0·6% of sodium acetate recommended by these workers. The standard curve obtained with the increased concentration

of glucose and sodium acetate is steeper and the titres higher. Moreover, the range of the assay is increased (Fig. 1).

TABLE I

COMPOSITION OF BASAL MEDIUM FOR ASSAY OF TRYPTOPHAN

The weights given are per ml. of basal medium

Acid-hydrolysed casein	6·0 mg.	*p*-Aminobenzoic acid	0·1 μg.
l-Cystine	0·2 ,,	Biotin	0·0005 μg.
Glucose	20·0 ,,	Adenine	0·01 mg.
Sodium acetate (anhydrous)	20·0 ,,	Guanine	0·01 ,,
Xylose	1·0 ,,	Uracil	0·01 ,,
Aneurine	0·1 μg.	Xanthine	0·01 ,,
Calcium *d*-pantothenate*	0·2 ,,	Sodium chloride	5·0 ,,
Pyridoxine	0·2 ,,	Ammonium sulphate	3·0 ,,
Riboflavine	0·2 ,,	Inorganic salt-solution A	See text
Nicotinic acid	0·4 ,,	,, ,, B	See text

* If calcium *dl*-pantothenate only is available, twice this amount must be used.

PREPARATION OF STOCK SOLUTIONS—The stock solutions described below are preserved in the presence of a thin layer of sulphur-free toluene and stored in a refrigerator. All stock solutions for this and the remaining assays are prepared with glass-distilled water.

Acid-hydrolysed casein—The method of preparation of a vitamin-free casein hydrolysate described by Barton-Wright[2] will be found suitable for the assay of tryptophan. A spray-dried vitamin-free casein hydrolysate, however, was mainly used in this work.

l-Cystine—*l*-Cystine (4·0 g.) is suspended in 500 ml. of hot water, 5 ml. of concentrated hydrochloric acid are added and the solution is made up to a final volume of 1000 ml. This solution keeps indefinitely in a refrigerator.

Glucose, sodium acetate, xylose, sodium chloride and ammonium sulphate are weighed out as required. Hydrated sodium acetate is used in preference to the anhydrous salt (1·66 g. of $CH_3COONa.3H_2O \equiv$ 1 g. of CH_3COONa).

Biotin—An ampoule containing 25 μg. of the free acid is made up to 250 ml. with water. One ml. of Salt Solution A is added prior to making up to volume. The solution contains 0·1 μg. of biotin per ml. It will maintain its activity for at least three months if preserved under toluene in a refrigerator.

p-*Aminobenzoic acid*—0·1 g. of *p*-aminobenzoic acid is dissolved in 100 ml. of water containing 1 ml. of glacial acetic acid. Before use this solution is diluted to one in ten, so that each ml. contains 100 μg.

Aneurine, nicotinic acid, calcium d-*pantothenate and pyridoxine*—A separate stock solution of each of these substances is prepared by dissolving 0·1 g. in 100 ml. of water. The stock solutions are diluted one in ten before use.

Riboflavine—One ml. of glacial acetic acid is added to 25 mg. of riboflavine and the solution is diluted to 1000 ml. with water. This solution contains 25 μg. per ml.

Adenine, guanine and uracil—A single stock solution of these three substances is prepared containing 1 mg. of each per ml. Solution is effected by heating with a few drops of concentrated hydrochloric acid.

Xanthine—The concentration of this solution is also 1 mg. per ml. Solution is effected by addition of a few drops of strong ammonia.

The various stock solutions of vitamins, with the exception of biotin, are renewed at weekly intervals and the adenine, guanine, uracil and xanthine solutions are renewed at fortnightly intervals. All stock solutions are preserved under toluene in a refrigerator.

Inorganic Salt Solution A—This is composed of 25 g. of K_2HPO_4 and 25 g. of KH_2PO_4 in 250 ml. of water.

Inorganic Salt Solution B—This solution has now been modified to contain 10 g. of $MgSO_4.7H_2O$, 0·5 g. of $MnSO_4.4H_2O$ and 0·1 g. of $FeCl_3$ in 250 ml. of water. Addition of a few drops of concentrated hydrochloric acid will prevent the formation of any precipitate in the mixture.

Standard tryptophan solution—0·2 g. of *dl*-tryptophan is dissolved in water with the aid of four or five drops of concentrated hydrochloric acid and the volume is made up to 100 ml. Only the *l*-enantiomorph is active for the growth of *L. arabinosus*. On this account 0·2 g. of *dl*-tryptophan is used for the preparation of the initial standard solution, which is equivalent to 0·1 g. of active *l*-tryptophan. Two dilutions are made from this stock solution to contain, respectively, 2 μg. of *l*-tryptophan per ml. (standard solution i) and 10 μg. of *l*-tryptophan per ml. (standard solution ii).

PREPARATION OF INOCULUM—The inoculum is best prepared by making a transfer from the agar stab culture to a tube of the Snell and Strong riboflavine medium (see Barton-Wright and Booth[4]). This is incubated for 18–20 hours at 37° C. It is then centrifuged aseptically and, after the supernatant liquor has been poured off and replaced by 10 ml. of sterile 0·9% saline solution, centrifuged again. This operation is repeated with a further 10 ml. of sterile saline solution. After the third centrifuging and replacement with 10 ml. of saline solution, 1 ml. of this suspension is diluted to 100 ml. with sterile saline solution and this dilute inoculum is used for subsequent inoculation.

The fermentations are carried out in chemical or bacteriological test tubes (18–20 mm. × 160–180 mm.) and the lactic acid produced is titrated against 0·1 *N* sodium hydroxide, with bromothymol blue as indicator. The titrations are carried out as described by Barton-Wright and Booth.[4]

PREPARATION OF HYDROLYSATES—Strong acids cannot be used for the hydrolysis of material in the assay of tryptophan because of partial or complete destruction of the tryptophan. Barium hydroxide was found to be the best hydrolysing agent.

Dry materials are finely ground and mixed with 4·2 g. of anhydrous or 7·7 g. of hydrated barium hydroxide. Ten ml. of water are added and the mixture is autoclaved at 15 pounds pressure for 6–10 hours. It must be remembered that racemisation takes place with this hydrolysing reagent and racemisation must be complete. Autoclaving for 4 hours was found to be too short a period, but 6 hours gave complete racemisation (see also Greene and Black[3]). 10 *N* Sulphuric acid is added to the hot hydrolysate until *p*H 4 is reached, to precipitate barium as sulphate. The precipitate is centrifuged off and well washed with water and the washings are added to the original hydrolysate. The hydrolysate is now shaken with two successive 100 ml. lots of ethyl ether in a separating funnel and then with 30 ml. of toluene. This ether and toluene treatment is necessary to remove any indole or anthranilic acid that may be present in the hydrolysate. Both indole and anthranilic acid can partially replace tryptophan for *L. arabinosus* (Snell[5]); indole shows some 75% and anthranilic acid some 50% of the activity of tryptophan. After the treatment with ether and toluene the solution is adjusted to *p*H 6·8 with sodium hydroxide and made up to volume. The concentration of *l*-tryptophan in the final volume of the hydrolysate should be as nearly as possible 1 μg. per ml. Since the *d*-enantiomorph is quite inactive the figure found by assay will have to be doubled to give the correct value.

ASSAY PROCEDURE—The following amounts of the stock solutions and other components will give sufficient medium for 100 tubes.

Acid-hydrolysed casein (or spray-dried hydrolysate)	60 ml. (6 g.)	*p*-Aminobenzoic acid solution	1·0 ml.
l-Cystine	50 ml.	Aneurine solution	1·0 ,,
Anhydrous glucose	20 g.	Calcium *d*-pantothenate solution	2·0 ,,
Anhydrous sodium acetate	20 ,,	Nicotinic acid solution	4·0 ,,
Xylose	1 ,,	Pyridoxine solution	2·0 ,,
Sodium chloride	5·0 g.	Riboflavine solution	8·0 ,,
Ammonium sulphate	3·0 ,,	Adenine + guanine + uracil solution	10·0 ,,
Biotin solution	5·0 ml.	Xanthine solution	10·0 ,,
		Inorganic salt solution A	5·0 ,,
		,, ,, ,, B	5·0 ,,

After mixing, the *p*H of the medium is adjusted to 6·8 with sodium hydroxide and the volume made up to 500 ml. with water. This gives a mixture having twice the concentration of the final assay medium. Five ml. of the mixture are transferred to each tube. Sixteen tubes are retained for the blanks and the determination of the standard tryptophan curve. The tubes are set up in duplicate and to them is added serially 5 ml. of water (= blank), 0·5, 1·0, 2·0, 3·0, 4·0, 5·0 of standard solution i and 1·2 ml. of standard solution ii of tryptophan (2 μg./ml. and 10 μg./ml.). To each tube sufficient distilled water is added to bring the final volume to 10 ml. The remaining tubes are used for the assay of the hydrolysates.

It is desirable to carry out each assay at four or five different concentration levels. It will be found convenient to take 1·0, 2·0, 3·0, 4·0 and 5·0 ml. of each hydrolysate for an assay, making up the final volume in each tube to 10 ml. with water. The assay tubes are also set up in duplicate. After the volumes in the tubes have been adjusted to 10 ml. with water the tubes are shaken so as to mix the contents well, plugged with cotton-wool and autoclaved for 10 minutes at 10 pounds pressure, cooled and inoculated with one drop of inoculum per tube from a sterile pipette. The tubes are incubated for 72 hours at 37° C. and the acid formed is titrated. Equally good results can be obtained by incubating at 30° C.

The standard curve (Fig. 1) is plotted in the usual way and the tryptophan content of each concentration level of hydrolysate directly read from it. The mean of at least three readings not differing among themselves by more than ±10% should be taken. Twice the mean value gives the true value of tryptophan. It is not possible with these amino acid assays to employ the slope-ratio method of computation (*cf.* Wood[6]), since the standard curves are slightly convex in shape and no part is strictly linear.

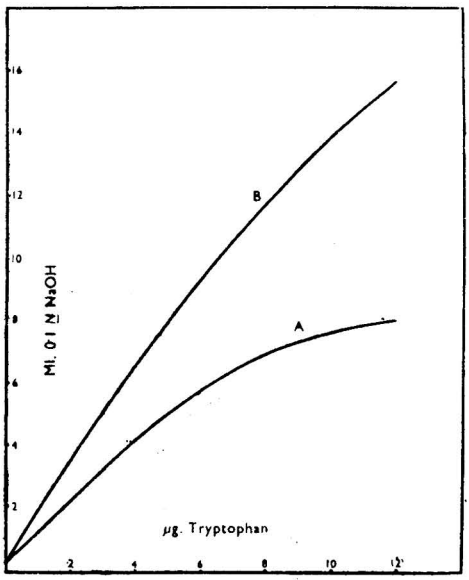


Fig. 1. Tryptophan.

A—Standard curve obtained on Greene and Black's medium.[3]

B—Standard curve obtained on medium with 2% of glucose and 2% of sodium acetate.

Assay of Leucine, Valine, *Iso*leucine and Cystine

Organism—*Lactobacillus arabinosus* 17–5 is also used for the assay of these amino acids.

Composition of Basal Medium—The basal medium is similar to that for tryptophan, except that the casein hydrolysate is replaced by a series of individual amino acids. A number of different media have been proposed for the assay of these amino acids, differing only in the concentration of the various amino acids. After a number of tests it was found that the medium recommended by Schweigert, McIntire, Elvehjem and Strong[7] for the assay of leucine and valine, with slight modifications, principally increasing the concentration of the vitamin supplements, the addition of 0·3% ammonium sulphate and the replacement of *l*-asparagine by *dl*-aspartic acid, gave excellent results for the four amino acids leucine, valine, *iso*leucine and cystine. It must be emphasised that only amino acids of the highest purity can be used in microbiological assays. Contamination of amino acids with others, which can be ignored in chemical determinations, willl lead to high "blanks" and invalid or vitiated assays.

Table II

Composition of Basal Medium for Assay of Leucine, Valine, *Iso*leucine and Cystine

The weights given are per ml. of basal medium

Amino acids		Other components	
l(+)-Glutamic acid	0·4 mg.	Glucose	0·02 mg.
dl-Aspartic acid	0·8 ,,	Sodium acetate (anhyd.)	0·02 ,,
dl-Lysine HCl	0·4 ,,	Aneurine	0·2 μg.
dl-Threonine	0·2 ,,	Calcium *d*-pantothenate	0·2 ,,
dl-Valine*	0·2 ,,	Pyridoxine	0·2 ,,
dl-*Iso*leucine*	0·2 ,,	Riboflavine	0·4 ,,
dl-α-Alanine	0·2 ,,	Nicotinic acid	0·4 ,,
l(−)-Cystine*	0·1 ,,	*p*-Aminobenzoic acid	0·1 ,,
l(−)-Leucine*	0·1 ,,	Biotin	0·0005 μg.
dl-Methionine	0·1 ,,	Adenine	0·01 mg.
dl-Phenylalanine	0·1 ,,	Guanine	0·01 ,,
l(+)-Arginine HCl	0·05 ,,	Uracil	0·01 ,,
l(+)-Histidine HCl	0·05 ,,	Xanthine	0·01 ,,
l(−)-Tyrosine†	0·04 ,,	Sodium chloride	5·0 ,,
dl-Tryptophan	0·08 ,,	Ammonium sulphate	3·0 ,,
		Inorganic salt solution A, B	See text

* The amino acid being assayed is omitted from the medium.

† In the assay of cystine, *dl*-tyrosine (0·08 mg./ml.) should be used in place of the natural product, which is difficult to purify from all traces of cystine and results in high "blanks."

Stock Solutions—The same stock solutions are used as in the assay of tryptophan.

PREPARATION OF INOCULUM—This is prepared in the same way as for the tryptophan assay.

PREPARATION OF HYDROLYSATES—Finely ground material, usually a 1 g. sample is sufficient, is hydrolysed by autoclaving for 5–6 hours at 15 pounds pressure with 25 ml. of

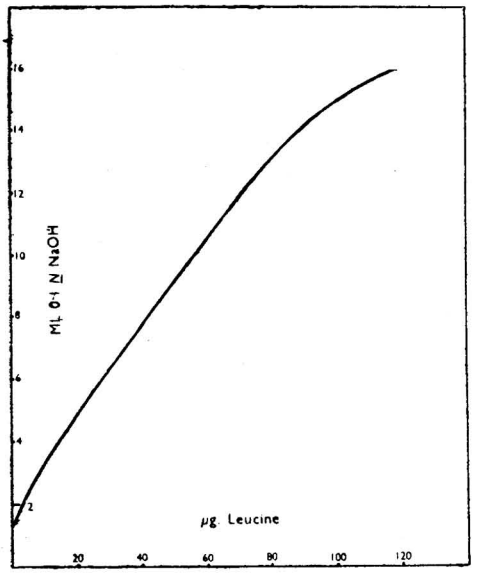


Fig. 2. Leucine.

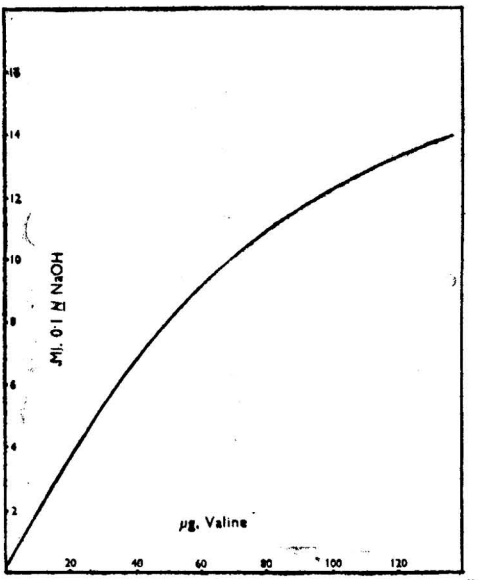


Fig. 3. Valine.

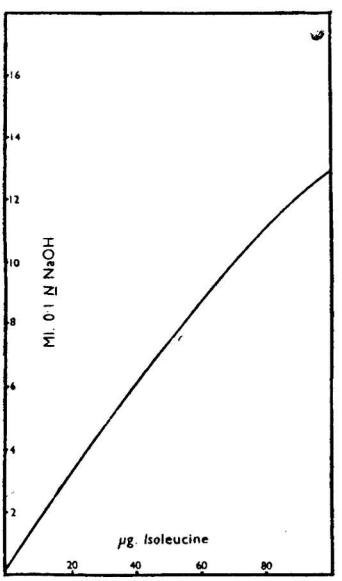


Fig. 4. *Iso*leucine.

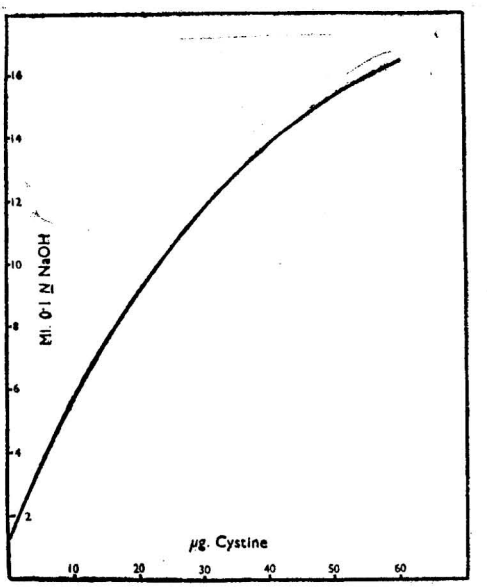


Fig. 5. Cystine.

2·5 *N* HCl. Hydrolysis is incomplete in less than 5 hours. (See also Schweigert *et al.*[7] and Stokes *et al.*[13]) The hydrolysate is cooled, 2 ml. of 2·5 *M* sodium acetate solution are added, the *p*H is adjusted to 4·5 with sodium hydroxide and the mixture made up to volume. An aliquot is filtered and the filtrate is re-adjusted to *p*H 6·8 with more sodium hydroxide and made up to suitable volume. For the assay of leucine, *iso*leucine and valine, the concentration of these amino acids in the final hydrolysate solution should be as nearly as possible

10 μg./ml., whereas for the assay of cystine the concentration should be approximately 5 μg./ml.

ASSAY PROCEDURE—The following amounts of amino acids, vitamin supplements, purine and pyrimidine bases and mineral salts will give sufficient basal medium for 100 tubes.

Amino acids		Vitamin supplements, etc.	
l(+)-Glutamic acid	400 mg.	Aneurine solution	2·0 ml.
dl-Aspartic acid	800 ,,	Calcium *d*-pantothenate solution	2·0 ,,
dl-Lysine HCl	400 ,,	Pyridoxine solution	2·0 ,,
dl-Threonine	200 ,,	Riboflavine solution	16·0 ,,
dl-Valine*	200 ,,	Nicotinic acid solution	4·0 ,,
*dl-Iso*leucine*	200 ,,	*p*-Aminobenzoic acid solution	1·0 ,,
dl-α-Alanine	200 ,,	Biotin solution	5·0 ,,
l(−)-Cystine*	100 ,,	Adenine+guanine+uracil solution	10·0 ,,
l(−)-Leucine*	100 ,,	Xanthine solution	10·0 ,,
dl-Methionine	100 ,,	Glucose	20 g.
dl-Phenylalanine	100 ,,	Sodium acetate (anhydrous)	20 ,,
l(+)-Arginine HCl	50 ,,	Sodium chloride	5·0 g.
l(+)-Histidine HCl	50 ,,	Ammonium sulphate	3·0 ,,
l(−)-Tyrosine	40 ,,	Inorganic salt solution A	5·0 ml.
dl-Tryptophan	80 ,,	,, ,, ,, B	5·0 ,,

* The amino acid to be assayed is omitted from the medium.

Note—In the assay of amino acids the synthetic *dl*-compound should be used wherever possible rather than the natural acid. A natural amino acid is always liable to be contaminated with minute traces of other acids, which is a frequent cause of high "blanks" and vitiated assays. For example, in the assay of *iso*leucine, *l*-leucine must be free from all traces of *iso*leucine, while in the assay of cystine, *dl*-tyrosine must be used in place of natural *l*-tyrosine because it is very difficult to free the latter from all traces of cystine.

The amino acids are weighed out and dissolved in 100 ml. of water. Addition of two or three drops of concentrated hydrochloric acid and gentle warming will be found necessary to bring about complete solution. Tryptophan, cystine and tyrosine should, however, be dissolved separately, tryptophan and cystine by warming in presence of a few drops of concentrated hydrochloric acid and tyrosine by warming with 0·1 *N* NaOH solution. The remaining components are now added to the amino acid mixture, the *p*H is adjusted to 6·8 with sodium hydroxide and the whole made up to a volume of 500 ml. with water. This gives a mixture having twice the concentration of the final assay medium. Five ml. of the mixture are transferred to each tube. Addition of *dl*-serine, *l*-proline, glycine and hydroxyproline was found to have no apparent influence on growth.

STANDARD SOLUTIONS OF LEUCINE, VALINE, *Iso*LEUCINE AND CYSTINE—For the preparation of the standard solution, the particular amino acid to be assayed is weighed out (0·1 g. of *l*-leucine or *l*-cystine or 0·2 g. of *dl-iso*leucine or *dl*-valine) and dissolved in 100 ml. of water. The *d*-enantiomorphs are inactive in the synthetic amino acids, *iso*leucine and valine, hence twice the concentration must be used in the preparation of the standard solutions. The stock standard solutions should be diluted to contain 20 μg./ml. of *l*-acid in the case of leucine, *iso*leucine or valine and 10 μg./ml. in the case of cystine. Cystine must be boiled with a few drops of concentrated HCl to effect solution.

For the set up of an assay, sixteen tubes are retained for the blanks and the determination of the standard amino acid curves. The effective part of the standard curve for the assay of leucine, *iso*leucine and valine is 10–60 μg. of *l*-acid and that for cystine 5–30 μg. Thus, to the tubes is added serially 5 ml. of water (= blank), 0·5, 1·0, 1·5, 2·0, 2·5, 3·0 and 3·5 ml. of standard solution. To each tube sufficient distilled water is added to bring the final volume to 10 ml., exactly as in the tryptophan assay. The remaining tubes are used for the assay of the hydrolysates.

Each assay should be carried out at four or five concentration levels at least. As a general rule if the hydrolysate has been diluted to contain approximately 10 μg./ml. of amino acid, suitable concentration levels will be 1·0, 2·0, 3·0, 4·0 and 5·0 ml. The total volume of liquid in each tube is made up to 10 ml. with water.

Subsequent procedure is exactly the same as in the assay of tryptophan. The tubes are plugged with cotton-wool, sterilised at 10 pounds pressure for 10 minutes, cooled, and

inoculated with one drop of dilute inoculum per tube with a sterile pipette. They are incubated for 72 hours at 37° C. or 30° C. and the lactic acid formed in fermentation is titrated against 0·1 *N* sodium hydroxide. Typical standard curves for the assay of leucine, valine, *iso*leucine and cystine are shown in Figs. 2, 3, 4 and 5.

Assay of Methionine, Lysine, Phenylalanine and Histidine

Organism—The organism used for the assay of these amino acids is *Leuconostoc mesenteroides* P-60. The vitamin and amino acid requirements of *L. mesenteroides* have been discussed by Gaines and Stahley,[8] Bohonos, Hutchings and Peterson,[9] Dunn, Camien. Shankman, Frankl and Rockland,[10] and Hac and Snell.[11] "Essential" vitamins for *L, mesenteroides* P-60 are nicotinic acid, aneurine, pantothenic acid, biotin and pyridoxine or pyridoxal. Riboflavine was found to be non-essential, although it is possible that it may act as a mild stimulant and on this account it is always added to the medium. Folic acid was found in the present investigation to be non-essential, but it does act as a very mild stimulant and therefore was always added to the medium.

Dunn *et al.*[10] claim that 17 amino acids are essential for normal growth of *L. mesenteroides* P-60, the non-essential amino acids being hydroxyproline, norvaline and norleucine. In the present investigation 16 amino acids were found to be essential but glycine was merely stimulatory. It is possible, however, that some of the amino acids used were slightly contaminated with glycine, which would account for this result.

Dunn *et al*[10,12] have described a medium for the assay of lysine and histidine, and Hac and Snell[11] a medium for the assay of aspartic acid with *L. mesenteroides* P-60. Both media were reinvestigated and rejected on the grounds that the amino acid concentrations were inadequate. Poor acid production was found with both media, particularly with that recommended by Hac and Snell. It should be mentioned, however, that Hac and Snell did not employ titration as a criterion of growth, but used turbidimetric methods of measurement. On the other hand the vitamin supplements recommended by Dunn *et al*[10] were found to be adequate.

The basal medium described below was found to give good growth and the results obtained on a specially purified sample of casein agreed very closely with the values found by chemical methods.

Table III

Composition of Basal Medium for Assay of Methionine, Lysine, Phenylalanine and Histidine

Weights given are per ml. of basal medium

Amino acids		Other components	
dl-α-Alanine	1·0 mg.	Glucose	20·0 mg.
l(+)-Arginine HCl	0·25 ,,	Sodium acetate (anhydrous)	12·0 ,,
dl-Aspartic acid	0·80 ,,	Ammonium chloride	6·0 ,,
l(−)-Cystine	0·10 ,,	Sodium chloride	5·0 ,,
l(+)-Glutamic acid	0·50 ,,	Adenine	0·012 mg.
Glycine	0·10 ,,	Guanine	0·012 ,,
l(+)-Histidine HCl*	0·10 ,,	Uracil	0·012 ,,
*dl-Iso*leucine	0·20 ,,	Xanthine	0·012 ,,
l(−)-Leucine†	0·10 ,,	Aneurine	1·0 μg.
dl-Lysine HCl*	0·25 ,,	Pyridoxine	1·6 ,,
dl-Methionine*	0·10 ,,	Calcium *d*-pantothenate	1·0 ,,
Norleucine	0·10 ,,	Riboflavine	2·0 ,,
Norvaline	0·10 ,,	Nicotinic acid	2·0 ,,
dl-Phenylalanine*	0·10 ,,	Biotin	0·005 μg.
dl-Proline§	0·20 ,,	Folic acid‖	0·001 ,,
dl-Serine	0·10 ,,	*p*-Aminobenzoic acid	0·10 ,,
dl-Threonine	0·50 ,,	Inorganic salt solution A	See text
dl-Tryptophan	0·10 ,,	,, ,, ,, B	See text
l(−)-Tyrosine‡	0·10 ,,		
dl-Valine	0·20 ,,		

* The amino acid to be assayed is omitted from the medium.
† *l*-Leucine must be free from all traces of methionine.
‡ *l*-Tyrosine must be free from traces of phenylalanine.
§ *l*-Proline, unless carefully purified, shows traces of histidine and lysine.
‖ Crystalline vitamin B_c was first used and later synthetic folic acid.

Stock Solutions—The initial stock solutions described for the assay of tryptophan are used *without* dilution, except in the case of *p*-aminobenzoic acid.

Folic acid—A sample of crystalline vitamin B_c was dissolved in 50% ethanol and preserved in a refrigerator. The concentration of vitamin in this solution was 2 μg./ml. It has maintained its full activity for over 10 months. A sample of synthetic folic acid was also made up to the same concentration in 50% ethanol. As far as could be ascertained there was no difference in the activity of these concentrates (see assay of arginine and threonine, p. 275).

PREPARATION OF INOCULUM—A transfer is made of a portion of an agar slope to a tube of the Snell and Strong medium (see above). This is incubated for 18–20 hours at 37° C. The inoculum is centrifuged three times as in the tryptophan assay (see above) and the contents are diluted to 30 ml. with sterile saline solution. This diluted inoculum is used for subsequent inoculation.

PREPARATION OF HYDROLYSATES—The same procedure is followed as in the hydrolysis of material for the assay of leucine, etc., *i.e.*, autoclaving for 6 hours with 2·5 *N* hydrochloric acid.

ASSAY PROCEDURE—The following amounts of amino acids, vitamin supplements, etc., will give sufficient medium for 100 tubes:

Amino acids		Other components	
dl-α-Alanine	1,000 mg.	Glucose	20 g.
l(+)-Arginine HCl	250 ,,	Sodium acetate (anhydrous)	12 ,,
dl-Aspartic acid	800 ,,	Ammonium chloride	6 ,,
l(−)-Cystine	100 ,,	Sodium chloride	5 ,,
l(+)-Glutamic acid	500 ,,	Adenine+guanine+uracil solution	12 ml.
Glycine	100 ,,	Xanthine solution	12 ,,
l(+)-Histidine HCl*	100 ,,	Aneurine solution	1·0 ,,
*dl-Iso*leucine	200 ,,	Pyridoxine solution	1·6 ,,
l(−)-Leucine	100 ,,	Calcium *d*-pantothenate solution	1·0 ,,
dl-Lysine HCl*	250 ,,	Nicotinic acid solution	2·0 ,,
dl-Methionine*	100 ,,	Biotin solution	50·0 ,,
Norleucine	100 ,,	Folic acid solution	0·5 ,,
Norvaline	100 ,,	*p*-Aminobenzoic acid soln. (diluted)	1·0 ,,
dl-Phenylalanine*	100 ,,	Riboflavine	2·0 mg.
dl-Proline	200 ,,	Inorganic salt solution A	5·0 ml.
dl-Serine	100 ,,	,, ,, ,, B	5·0 ,,
dl-Threonine	500 ,,		
dl-Tryptophan	100 ,,		
l(−)-Tyrosine	100 ,,		
dl-Valine	100 ,,		

* The amino acid to be assayed is omitted from the medium.

The amino acids are weighed out and dissolved in 100 ml. of water in exactly the same way as described for the leucine, valine, *iso*leucine and cystine assays. The remaining components of the medium are added, the *p*H is adjusted to 6·8 with sodium hydroxide and the whole made up to 500 ml. with water. This solution has twice the concentration of the final assay medium. Five ml. are transferred to each tube.

STANDARD SOLUTIONS OF METHIONINE, LYSINE, PHENYLALANINE AND HISTIDINE—For the preparation of the standard solutions of lysine, methionine and phenylalanine, 0·2 g. of the *dl*-amino acid is weighed out and dissolved in 100 ml. of water. The *d*-enantiomorph of each is inactive, so that the stock standard solution contains the equivalent of 1000 μg. of *l*-acid per ml. For the standard solution of histidine 0·1 g. is dissolved in 100 ml. of water. These stock standard solutions are now suitably diluted for establishing the standard curves. The effective assay range for methionine is 5–50 μg., for lysine 20–200 μg., for phenylalanine 10–60 μg. and for histidine 5–35 μg.

For the set up of an assay the necessary number of tubes are retained for the blanks and the determination of the standard amino acid curve. As in the other assays the total volume of each tube is made up to 10 ml. with water.

Each assay is carried out at four or five concentration levels at least, and the hydrolysate should be diluted to contain an appropriate concentration of the particular amino acid that is being assayed. Thus, for a methionine assay the hydrolysate should be diluted to contain approximately 5 μg./ml. of methionine and the concentration levels that could be used here are 1·0, 2·0, 3·0, 4·0 and 5·0 ml. The total volume of liquid in each tube is made up to 10 ml. with water.

After the addition of the standard amino acid solution or hydrolysates and the adjustment of the volume in the tubes to 10 ml., they are plugged with cotton-wool and sterilised

for 10 minutes at 10 pounds pressure, cooled and inoculated with one drop of inoculum per tube with a sterile pipette, incubated at 37° C. for 72 hours and the lactic acid formed in fermentation titrated against 0·1 *N* sodium hydroxide. Typical standard curves (Figs. 6, 7, 8 and 9) for the assay of methionine, lysine, phenylalanine and histidine are shown.

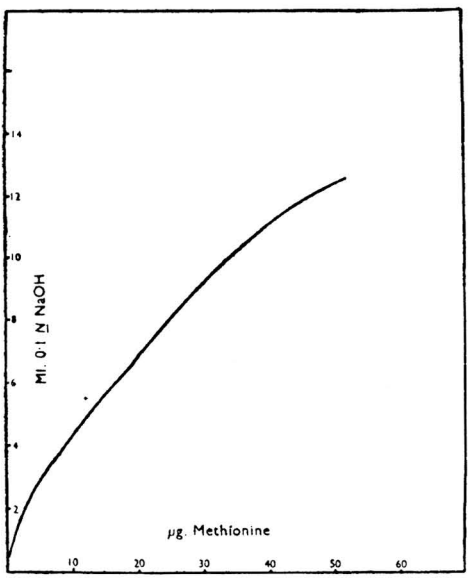


Fig. 6. Methionine.

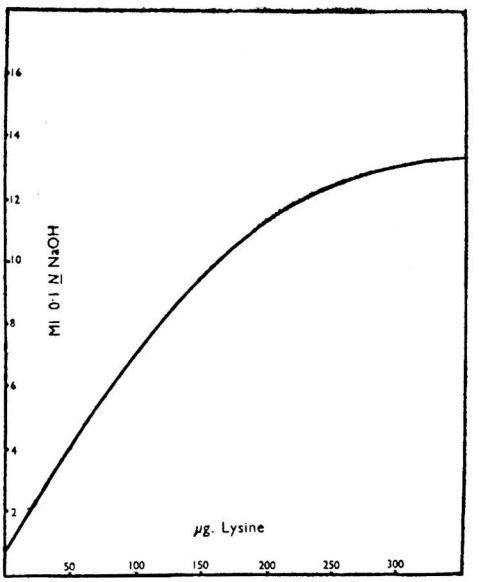


Fig. 7. Lysine.

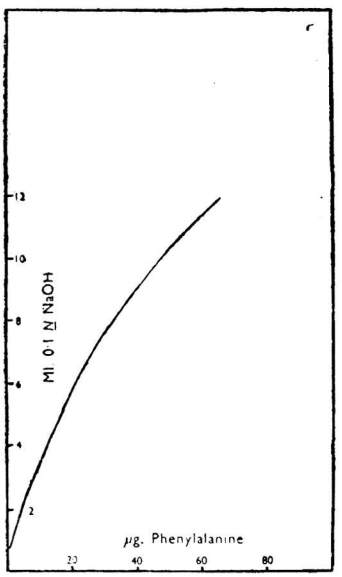


Fig. 8. Phenylalanine.

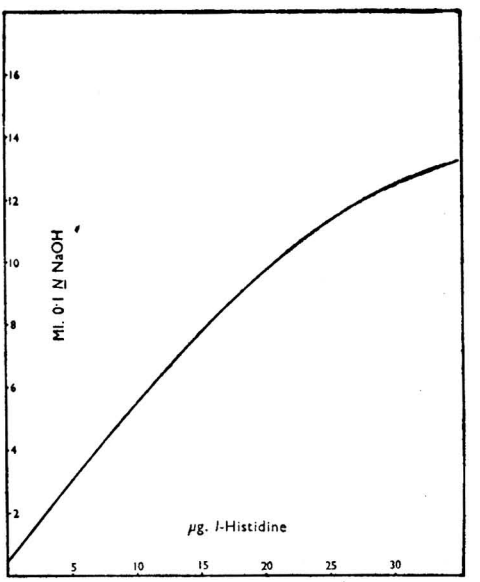


Fig. 9. Histidine.

Assay of Arginine and Threonine

Organism—The organism found to be most suitable for the assay of arginine and threonine was *Streptococcus faecalis* (*lactis*) R. *S. faecalis* is more fastidious in its vitamin requirements than either *Lactobacillus arabinosus* or *Leuconostoc mesenteroides* P-60, and folic acid is an essential nutrilite for normal growth.

COMPOSITION OF BASAL MEDIUM—The basal medium described by Stokes, Gunness, Dwyer and Caswell,[13] with some minor modifications, was found to give reliable and repeatable results.

TABLE IV

COMPOSITION OF BASAL MEDIUM FOR ASSAY OF ARGININE AND THREONINE

Weights given are per ml. of basic medium

Amino acids		Other components	
l(+)-Arginine HCl*	0·2 mg.	Riboflavine	0·2 μg.
dl-α-Alanine	0·2 ,,	Nicotinic acid	0·6 ,,
dl-Aspartic acid	0·2 ,,	Calcium *d*-pantothenate	0·2 ,,
l(−)-Cystine	0·2 ,,	Pyridoxine	1·2 ,,
l(+)-Glutamic acid	0·2 ,,	Aneurine	0·2 ,,
Glycine	0·2 ,,	Biotin	0·0004 μg.
l(+)-Histidine HCl	0·2 ,,	Folic acid	0·002 ,,
*dl-Iso*leucine	0·2 ,,	*p*-Aminobenzoic acid	0·04 μg.
l(−)-Leucine	0·2 ,,	Adenine	0·01 mg.
dl-Lysine HCl	0·2 ,,	Guanine	0·01 ,,
dl-Methionine	0·2 ,,	Uracil	0·01 ,,
Norleucine	0·2 ,,	Xanthine	0·01 ,,
Norvaline	0·2 ,,	Glucose	10·0 ,,
dl-Phenylalanine	0·2 ,,	Sodium acetate	6·0 ,,
dl-Proline	0·2 ,,	Sodium chloride	5·0 ,,
dl-Serine	0·2 ,,	Inorganic salt solution A	See text
dl-Threonine*	0·2 ,,	,, ,, ,, B	See text
dl-Tryptophan	0·6 ,,		
l(−)-Tyrosine	0·2 ,,		
dl-Valine	0·2 ,,		

* The amino acid to be assayed is omitted from the medium.

STOCK SOLUTIONS—The same stock solutions are used as in the assay of tryptophan.

PREPARATION OF INOCULUM—A transfer is made from the agar stab culture to a tube of the Snell and Strong riboflavine medium (see above) and this is incubated for 16–18 hours at 30° C. The inoculum is centrifuged three times as has already been described for the other assays and the final suspension is diluted to 100 ml. with sterile saline solution. This diluted inoculum is used for subsequent inoculation.

PREPARATION OF HYDROLYSATES—The same procedure is followed as in the hydrolysis of material for the assay of leucine, etc., *i.e.*, autoclaving for 6 hours with 2·5 *N* hydrochloric acid.

ASSAY PROCEDURE—The following amounts of amino acids, vitamin supplements, etc., will give sufficient basal medium for 100 tubes:

Amino acids		Vitamin supplements, etc.	
l(+)-Arginine HCl*	200 mg.	Riboflavine solution	8·0 ml.
dl-α-Alanine	200 ,,	Nicotinic acid solution	6·0 ,,
dl-Aspartic acid	200 ,,	Calcium *d*-pantothenate	2·0 ,,
l(−)-Cystine	200 ,,	Pyridoxine solution	12·0 ,,
l(+)-Glutamic acid	200 ,,	Aneurine solution	2·0 ,,
Glycine	200 ,,	*p*-Aminobenzoic acid solution	0·4 ,,
l(+)-Histidine HCl	200 ,,	Biotin solution	4·0 ,,
*dl-Iso*leucine	200 ,,	Folic acid solution	1·0 ,,
l(−)-Leucine	200 ,,	Adenine+guanine+uracil solution	10·0 ,,
dl-Lysine HCl	200 ,,	Xanthine solution	10·0 ,,
dl-Methionine	200 ,,	Glucose	10 g.
Norleucine	200 ,,	Sodium acetate (anhydrous)	6 ,,
Norvaline	200 ,,	Sodium chloride	5 ,,
dl-Phenylalanine	200 ,,	Inorganic salt solution A	5·0 ml.
dl-Proline†	200 ,,	,, ,, ,, B	5·0 ,,
dl-Serine	200 ,,		
dl-Threonine*	200 ,,		
l(−)-Tyrosine	200 ,,		
dl-Tryptophan	600 ,,		
dl-Valine	200 ,,		

* The amino acid to be assayed is omitted from the medium.
† If *l*-proline is used it must be free from all traces of arginine.

The amino acids are weighed out and dissolved in 100 ml. of water and the remainder of the medium is prepared in exactly the same way as for the assay of leucine, etc. The *p*H of

the medium is adjusted to 6·8 with sodium hydroxide solution and the total volume made up to 500 ml. with water. This gives a mixture having twice the concentration of the final assay medium. Five ml. of the solution are transferred to each tube. Doubling the concentration of glucose and sodium acetate made no significant difference to lactic acid production.

STANDARD SOLUTIONS OF ARGININE AND THREONINE—0·1 g. of *l*-arginine or 0·2 g. of *dl*-threonine is weighed out and dissolved in 100 ml. of water. The stock standard solutions are diluted to contain 20 μg./ml. of arginine and 40 μg./ml. of *dl*-threonine.

The assay is set up in the same way as has already been described. The effective portion of the standard curve for each acid covers 10–60 μg. of *l*-acid. Thus, to the tubes for establishing the standard curve is added serially 5 ml. of water (=blank), 0·5, 1·0, 2·0, 2·5 and 3·0 ml. of standard solution. Sufficient distilled water is added to each tube to bring the final volume in each to 10 ml. The remaining tubes are used for the assay of the hydrolysates.

Each assay is carried out at four or five concentration levels and the hydrolysate should be diluted to contain approximately 10 μg./ml. of the amino acid. After the addition of suitable amounts of hydrolysate the volume of liquid in the tubes is adjusted to 10 ml. with water. The tubes are plugged with cotton wool, sterilised at 15 pounds pressure for 15 minutes, cooled and inoculated with one drop of dilute inoculum per tube with a sterile pipette. They are

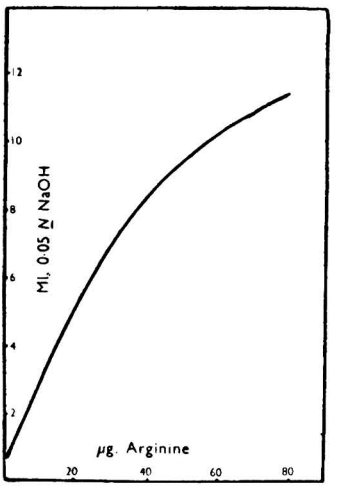


Fig. 10. Arginine.

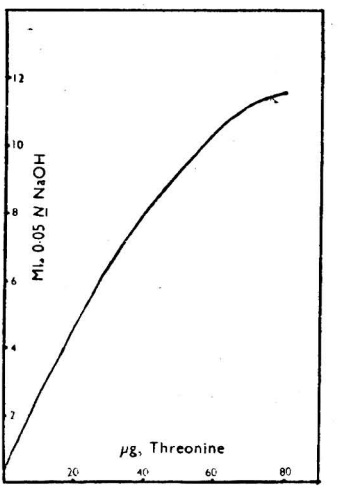


Fig. 11. Threonine.

then incubated for 72 hours at 30° C. At the end of the incubation period the tubes are titrated with 0·05 *N* sodium hydroxide. *S. faecalis* is a poor acid former and on this account 0·05 *N* alkali should be used in place of 0·1 *N* alkali. It is usual with this organism to use nephelometric or turbidimetric methods for growth measurement, but these have disadvantages when strongly coloured solutions are being used. Typical standard curves for the assay of arginine and threonine are shown in Figs. 10 and 11.

DISCUSSION

The quantitative estimation of individual amino acids by microbiological means has obvious advantages over chemical methods of analysis. The question therefore arises as to the accuracy and precision of microbiological assays. The conventional tests, *e.g.*, recovery values of added amino acids, indicate that with suitable precautions these methods do yield accurate and precise values. Moreover, direct comparison of determinations of individual amino acids in proteins by microbiological and chemical assays have on the whole given close and concordant results. In the present investigation, the amino acids tryptophan, lysine, methionine, phenylalanine and threonine were estimated microbiologically in a specially purified sample of casein which contained moisture 11·99%, ash 9·36% and total nitrogen 12·58%, corresponding to 15·99% of nitrogen on true protein. The only amino acid estimated chemically on this particular sample of protein was methionine, but in Table V a comparison is made of the values given in the literature with those found here.

TABLE V

AMINO ACIDS FOUND IN CASEIN, PER CENT.

Results calculated to 16% N on ash- and moisture-free basis

Amino acid	Microbiological assay	Chemical assay	Values in literature
Tryptophan	1·3	—	1·2
Lysine	8·1	—	6·9 ± 0·7
Phenylalanine	4·7	—	5·2 ± 0·5
Methionine	3·0	2·86	3·5 ± 0·3
Threonine	4·1	—	3·9 ± 0·1

As far as the methionine assay is concerned, for which direct comparison is possible, the agreement between the two methods is good (difference 4·7%) and lends confidence in the use of microbiological methods.

It is essential for microbiological assays of amino acids that the acids themselves are not contaminated with traces of other acids. It is on this account that synthetic *dl*-acids should be used wherever possible in place of the natural compounds. One exception to this rule is *dl*-leucine which is usually contaminated with *iso*leucine, so that it should not be used in the assay of *iso*leucine.

SUMMARY

Microbiological assay methods using the lactic acid-forming bacteria *Lactobacillus arabinosus* 17/5, *Leuconostoc mesenteroides* P-60 and *Streptococcus faecalis R* are described for the eleven essential amino acids arginine, cystine, histidine, *iso*leucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine.

The author is indebted to Dr. W. E. Gaunt (Ashe Laboratories Ltd.) and Mr. F. A. Robinson (Glaxo Laboratories Ltd.) for generous supplies of amino acids and vitamin supplements, and to Dr. A. Neuberger (National Institute for Medical Research) for samples of specially purified arginine, histidine, leucine and proline.

REFERENCES

1. Rose, W. C., *Science*, 1937, **86**, 298.
2. Barton-Wright, E. C., *Biochem. J.*, 1944, **38**, 314.
3. Greene, R. D., and Black, A., *J. Biol. Chem.*, 1944, **155**, 1.
4. Barton-Wright, E. C., and Booth, R. G., *Biochem. J.*, 1943, **37**, 25.
5. Snell, E. E., *Archiv. Biochem.*, 1943, **2**, 389.
6. Wood, E. C., *Nature*, 1945, **155**, 632; ANALYST, 1946, **71**, 1.
7. Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, 1944, **155**,183.
8. Gaines, S., and Stahley, G. L., *J. Bact.*, 1943, **46**, 441.
9. Bohonos, N., Hutchings, B. L., and Peterson, W. H., *Id.*, 1942, **44**, 479.
10. Dunn, M. S., Camien, M. N., Frankl, W., Shankman, S., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 703.
11. Hac, L. R., and Snell, E. E., *Id.*, 1945, **159**, 273, 291.
12. Dunn, M. S., Camien, M. N., Shankman, S., and Rockland, L. B., *Id.*, 1945, **159**, 653.
13. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *Id.*, 1945, **160**, 35.

RESEARCH ASSOCIATION OF BRITISH FLOUR MILLERS
CEREALS RESEARCH STATION
ST. ALBANS, HERTS

The Microbiological Assay of Amino Acids. II. The Distribution of Amino Acids in the Wheat Grain

BY E. C. BARTON-WRIGHT AND T. MORAN

(*Read at the Meeting of the Society on Wednesday, May* 1, 1946)

WHEAT and its major parts have been examined for the "essential" amino acids by the microbiological methods of determination (*cf.* Barton-Wright[1]) in order to see whether there were any marked differences in the distribution of these acids in various parts of the grain.

EXPERIMENTAL—A mixed grist composed of 60% of Manitoba and 40% of English wheat was specially milled so as to give a flour typical of a short patent flour (A roll flour, 10% extraction from the milling of 80% flour) which will hereafter be called "inner endosperm", the layer of endosperm immediately abutting on the aleurone layer of the bran ("outer endosperm"), clean bran (essentially pericarp and aleurone layer) and germ. A recent paper (Moran and Drummond[2]) describes these parts in some detail.

The moisture, ash and nitrogen figures for these fractions are given below:

	Moisture %	Ash %	Nitrogen %
Inner endosperm ..	12·3	0·41	1·91
Outer endosperm ..	11·9	3·07	2·30
Bran	10·8	5·94	1·96
Germ	9·9	4·74	5·22
Whole wheat	11·5	1·50	2·15

The "inner endosperm" is sufficiently typical. The bran is also reasonably typical of the combined pericarp and aleurone layers, the amount of attached endosperm being of the order of 5%. The germ is largely a mixture of the embryo and scutellum fractions, the former predominating. The outer endosperm is the least satisfactory fraction from the standpoint of purity, since inevitably it is contaminated with some powdered whole bran and aleurone layer.

MICROBIOLOGICAL ASSAYS—The amino acids were determined by the methods described previously (Barton-Wright[1]). The percentages of the eleven "essential" amino acids in whole wheat and its fractions, recalculated to a moisture-free basis, are given in Table I, while in Table II the same values are given recalculated on a moisture- and ash-free basis to 16% nitrogen, together with a comparison wherever possible of comparable figures quoted in the literature.

TABLE I

DISTRIBUTION OF ESSENTIAL AMINO ACIDS OF WHOLE WHEAT AND ITS FRACTIONS

Values recalculated to moisture-free basis

Amino acid	Inner endosperm %	Outer endosperm %	Bran %	Germ %	Whole wheat %
Arginine	0·46	0·86	0·99	2·62	0·66
Cystine	0·24	0·36	0·25	0·58	0·31
Histidine	0·26	0·34	0·28	0·81	0·29
*Iso*leucine	1·10	1·26	0·74	2·22	1·22
Leucine	1·42	2·43	1·08	3·10	1·45
Lysine	0·30	0·50	0·64	2·31	0·43
Methionine	0·19	0·27	0·18	0·54	0·23
Phenylalanine	0·61	0·66	0·46	1·05	0·64
Threonine	0·40	0·52	0·47	2·66	0·49
Tryptophan	0·15	0·22	0·30	0·36	0·18
Valine	0·57	0·77	0·67	1·77	0·71

DISCUSSION—It is clear from the figures in Table I that the essential amino acids are not evenly distributed throughout the wheat grain, and that apart from germ, there is a rising gradient from the centre to the outside of the grain. In most instances, with the notable exceptions of arginine, lysine and tryptophan, the concentration in the bran is, however, less than that in the outer endosperm, suggesting a diluting effect of the outer fibrous pericarp layer. Other work in these laboratories by Dr. Hinton, which will shortly be published, has in fact shown that the pericarp has a very low protein content. On the other hand the aleurone layer is rich in protein and thus tends to counterbalance the effects of the pericarp.

With regard to the concentrations of essential amino acids in the different deposits of wheat protein (Table II), the values obtained microbiologically, in some cases are very different from those found chemically. It must, however, be borne in mind that such a comparison can only be a rough one, because of the differences in the wheat varieties and the composition of the samples. Nevertheless, the difference in the methionine figure for patent flour is too great to be accounted for solely by difference of composition of sample. Chemical determinations of amino acids on material containing a high percentage of carbohydrate is difficult and it is possible that this would help to account for the discrepancy. Stokes *et al.*, on the other hand, have found microbiologically values for methionine in patent flour and wheat

very similar to those given here (patent flour 0·96%, whole wheat 1·20%). In the present investigation the methionine content of casein was determined chemically and microbiologically and the agreement was satisfactory (see Barton-Wright[1]), so that it would appear that the values given for wheat and wheaten products in the literature are too high. The values found

TABLE II

DISTRIBUTION OF ESSENTIAL AMINO ACIDS IN WHEAT PROTEIN

Calculated to 16% N on moisture- and ash-free basis

Amino acid	Inner endosperm %	Values in literature* %	Outer endosperm %	Bran %	Germ %	Values in literature* %	Whole wheat %	Values in literature* %
Arginine ..	2·92	*3·9*	4·50	7·53	6·20	*6·0*	3·81	*2·8 ∓ 0·5*
Cystine ..	1·55	*1·9*	1·90	1·50	1·37	*0·6*	1·74	*1·3 ∓ 0·3*
Histidine ..	1·65	*2·2*	1·74	1·68	3·03	*2·5*	1·65	*1–2*
*Iso*leucine ..	7·02	*3·7 ∓ 0·2*	6·56	4·50	5·23	*3·0 ∓ 0·5*	6·97	*3·3*
Leucine ..	9·14	*12·0 ∓ 2·6*	7·98	6·52	7·33	*7·4 ∓ 2·3*	8·27	*5·8*
Lysine ..	1·92	*1·9*	2·60	3·87	5·44	*5·5*	2·80	*2·7*
Methionine ..	1·12	*3·0*	1·40	1·09	1·28	*2·0*	1·32	—
Phenylalanine	3·95	*5·5*	3·43	2·45	2·47	*4·2*	3·68	*5·7*
Threonine ..	2·56	*2·7*	2·72	2·85	6·28	*3·8*	2·78	*3·3*
Tryptophan ..	0·93	*0·8*	1·12	1·83	0·90	*1·0*	1·03	*1·0*
Valine ..	3·65	*3·4 ∓ 0·5*	4·02	4·10	4·20	*4·1 ∓ 1·0*	4·00	*3·6*

* Figures from Block and Bolling.[3]

for *iso*leucine and leucine by microbiological assay and chemical methods also show poor agreement. *Iso*leucine and leucine are difficult to estimate by chemical methods and no really satisfactory method of separation has yet been found, whereas it is believed that the microbiological method specifically differentiates between these acids. It is of interest to note in this connection that the value for *iso*leucine + leucine, for example in inner endosperm, by microbiological assay is 16·6% and by chemical determination 15·7%, while in germ the values are 12·56% and 12·7%, if the higher value for leucine be taken. The combined figures for whole wheat, microbiologically 15·24% and chemically 9·1% are not in good agreement.

The outstanding difference in the concentrations of essential amino acids in wheat proteins compared with a protein from an animal source, *e.g.*, casein, is the low percentage of lysine, 2·70%, in whole wheat protein or 1·9% in patent flour compared with 7·7–8·0% in casein. On the other hand in germ protein the percentage amino acid content is generally not much below that in first class protein, even in lysine content (5·44%); in some amino acids, *e.g.*, arginine, cystine and threonine, germ protein is superior.

SUMMARY—The quantitative determination by microbiological methods of the eleven "essential" amino acids, arginine, cystine, histidine, *iso*leucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, has been made on whole wheat, patent flour (inner endosperm), outer endosperm (portion abutting on aleurone layer), bran and germ. It was found that apart from germ, the highest concentration of essential amino acids occurs in the outer part of the grain.

REFERENCES

1. Barton-Wright, E. C., ANALYST, 1946, **71**, 267.
2. Moran, T., and Drummond, J. C., *Lancet*, 1945, 698.
3. Block, R. J., and Bolling, D., "*The Amino Acid Composition of Proteins and Food*," U.S.A., 1945, Charles C. Thomas.
4. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

RESEARCH ASSOCIATION OF BRITISH FLOUR MILLERS
CEREALS RESEARCH STATION
ST. ALBANS, HERTS

DISCUSSION ON THE PRECEDING TWO PAPERS

Mr. ERIC WOOD said that while it was of course true that the slope-ratio method could not be used for computing the results of microbiological assays having non-linear response laws, and that the direct-reading method must therefore, *faute de mieux*, be resorted to, this still remained an empirical and statistically faulty procedure. A sound means of dealing with non-linear assays—perhaps by a suitable transformation of response—would doubtless sooner or later be devised, and should certainly then be adopted. In the meantime, certain general principles of design must hold for all such assays. For example, it was not logical to have more dosage-levels of the standard than of the test preparation; indeed, some of the observations made on the standard curves in amino acid assays were redundant for routine assay purposes, since

they were not used in computing the results, and therefore represented an unnecessary expenditure of labour.

Dr. W. BRADLEY remarked that the chemist engaged in chemical analysis was always certain of the constancy of behaviour of his reagents. Was the analyst employing microbiological methods equally certain of the constant behaviour of the micro-organisms used?

Mr. H. PRITCHARD, in view of the use of more than one micro-organism in the methods proposed, asked Dr. Barton-Wright's opinion of the value of the method proposed by Stokes *et al.*, in which one medium and one organism were used for the estimation of the ten essential amino acids.

Mr. R. H. HORROCKS asked (1) if the *Str. faecalis* used was of the same type as that referred to in the paper by Stokes *et al.*, (2) what was the number of standard curves, were they in duplicate, triplicate, etc.? and (3) how critical was the incubation temperature?

Dr. T. H. MEAD asked what was the origin of the anthranilic acid and indole which must be elimated so carefully from samples to be analysed for tryptophan.

Mr. D. M. FREELAND asked if the *p*H value of the hydrolysate was readily adjusted, and was this performed by colorimetric or electrometric means upon a portion of it? Was it necessary to eliminate traces of the solvents for anthranilic acid and indole to prevent any inhibition of the organism subsequently employed in the method?

Mr. A. L. BACHARACH asked if it were known whether micro-organisms would respond to the di- and polypeptides of amino acids that they could use as such. If so, micro-biological methods might be available for studying the structure of proteins through their partial degradation products following enzymatic, acid or alkaline hydrolysis. He thought that further light on the accuracy of micro-biological methods for estimating amino acids might be thrown through a comparison of the results got by these means and those got by Gale's manometric procedure, involving the use of the specific amino acid decarboxyglases produced by selected bacterial strains.

Miss M. MEIKLEJOHN asked if there was any possibility of mutation of the organisms used, in view of the paper published in January mentioning three strains of *Lactobacillus helveticus*.

Dr. A. J. AMOS said they were indebted to Dr. Moran and Dr. Barton-Wright for a wealth of useful information, and also to Dr. Barton-Wright for the manner in which it had been presented. The subject matter of the second paper was of particular interest to him and suggested some important lines of research. At first sight it might appear to concern only the nutritionist, but it went far beyound that. The miller and baker were naturally interested in the quantity of protein in a wheat, but they were perhaps even more concerned with what they term its quality, *i.e.*, its physical properties. These differ according to the type of wheat—the protein of Manitoba being strong and tough and that of English weak and distensible—and according to the portion of the wheat grain from which the endosperm originates. Indeed, each wheat sent to the cereal laboratory is not only analysed chemically but is tested on specially designed instruments in order that the physical properties of its protein may be evaluated. Difference in these physical properties are well known to be related to certain colloidal phenomena, but they may possibly be connected with the amino acid make up of the protein. It might, therefore, be a fruitful line of research to extend these amino acid analyses to the proteins of different types of wheats and to ascertain whether there appears to be any real relationship between the amino-acid constitution of a protein and its physical properties. Were this found to be so, the data thus obtained might serve as a guide to the wheat breeder and enable him to produce crosses with improved baking quality or which, alternatively, exhibit in the flour those properties particularly needed in some non-bread flours such as, for example, the flour intended for the production of certain types of biscuits. He hoped, therefore, that they might have another paper later from Drs. Moran and Barton-Wright in which they had extended their work in the direction indicated.

Dr. ALBERT GREEN suggested that the application of the term "Patent Flour" to one which represented only 10% of the wheat grain might cause confusion. "Patent Flours" were commonly of 35 to 50% extraction. The flour discussed in the second paper would be better described as a top machine flour of 10% extraction.

Mr. D. C. M. ADAMSON asked whether, as folic acid was essential in the metabolism of *Str. faecalis*, any attempt had been made to assay that factor by microbiological methods. Although bread was not normally considered as a source of protein, did the new method add any evidence to the "wholemeal versus white" controversy?

Dr. J. G. A. GRIFFITHS said it had been mentioned that wheat endosperm protein contained only 2% of lysine and that high-grade animal proteins contained 7%. A knowledge of the lysine content of a mixture of bread and meat proteins should enable the relative proportions of the two proteins to be calculated. Would the methods described be applicable to the determination of the lysine content of sausages consisting of bread and meat? Could the methods described be used to determine the ratios of the amino acids in meat? If, for example, certain ratios were found to be different in horse flesh as compared with beef, discrimination between these meats would be assisted, and it might be possible to calculate, from the relative proportions of the amino acids, the proportions of different meats in comminuted mixtures.

Dr. BARTON-WRIGHT, in reply to the various points raised in the discussion, said that as far as the calculation of amino assay results was concerned the statisticians had not solved the problem, and it was scarcely fair for them to pass it back to the microbiologist. Even if direct reading from the curve was statistically unsound, nevertheless in practice the method gave very good agreement with the slope-ratio procedure in those cases in which there was a linear portion to the standard curve, provided that the extraction of samples was correctly carried out and the assay successfully conducted. He did not anticipate, therefore, that wide differences would be found between the values of amino acid assays calculated by the present method of directly reading from the standard curve and those obtained by a statistical method, when this had been successfully developed.

The lactic organisms appeared to be perfectly stable mutants and had shown constant behaviour over a number of years. The *Str. faecalis* used in this investigation was the same strain as that used by Stokes *et al.* and could be used for the assay of nine and not ten essential amino acids. Phenylalanine could not be assayed with *Str. faecalis* as it synthesises this amino acid after an initial lag period.

The *p*H of the hydrolysate is adjusted with the aid of external indicators and traces of solvent left after removal of indole and anthranilic acid are eliminated when the medium is autoclaved.

Dr. Barton-Wright said he had no information on the subject of whether these lactic organisms could utilise polypeptides in place of individual amino acids, but he presumed that this was so because peptone can be substituted for casein hydrolysate as a source of nitrogen for the lactic bacteria.

In reply to Mr. D. C. M. Adamson, he said that it was now the practice to use *Str. faecalis* for the microbiological assay of folic acid. He thought that it would be possible to estimate whether sausages had been grossly diluted with bread by estimating the lysine content, but there was one practical difficulty in the application of the method,—soya flour might be used as a diluent. The lysine content of soya bean protein compared with other plant proteins is high (5·4%) and approaches that of an animal protein.

Dr. MORAN, in reply to Dr. Green, said that the flour used had an ash content of 0·41% and approximated to a peace-time patent flour of higher extraction. The flour was described clearly in the text, so that there should be no confusion.

The Determination of Metallics in Spontaneously Inflammable Magnesium Dust

BY J. A. ANDERSON

THE magnesium dust which is an intermediate product of the carbothermic process for the production of magnesium is so finely divided as to be spontaneously inflammable in air; the difficulties involved in the handling of such material will be fairly obvious. The apparatus and procedure described below were devised to expedite the rapid routine determination of metallics in such dust as produced in the plant of the Magnesium Metal Corporation, Ltd., Swansea.

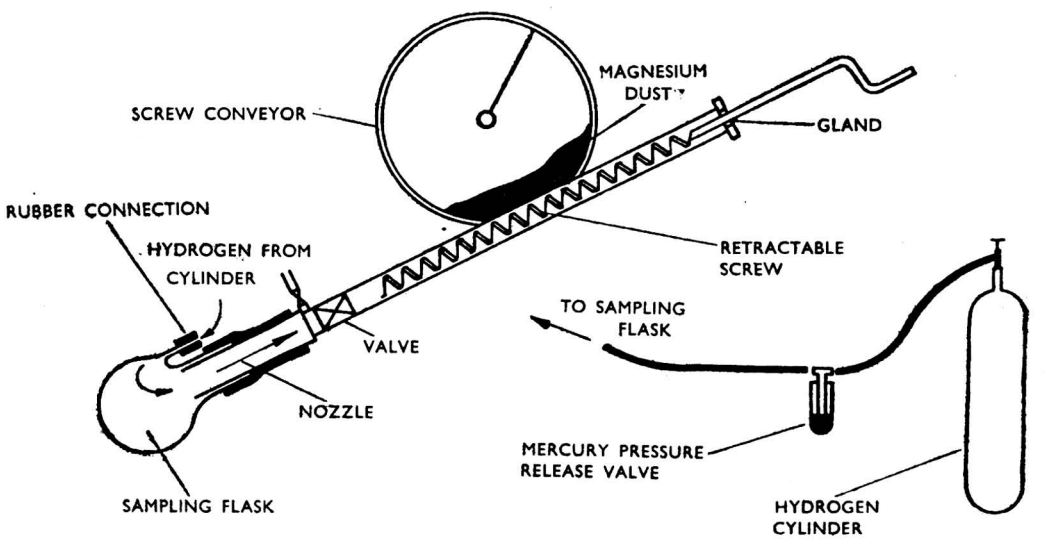


Fig. 1.

SAMPLING DEVICE—In the plant the dust is carried through screw conveyors under an atmosphere of hydrogen. The basis of the device for extracting dust samples is a small hand-operated screw conveyor mounted on the main conveyor at an angle corresponding with the natural position of the dust being conveyed, as illustrated in Fig. 1, so that the port between the two conveyors is in the position where the conveyed dust is deepest. To reduce the possibility of dust bridging between the two screws the gap has been reduced to a minimum. The movement of dust between the main screw and the sampling flask, which is attached to the exit end of the small conveyor, is facilitated by making its path as streamlined as possible, the small conveyor, valve and nozzle all being of the same internal diameter. By these means good control of the sampling operation is secured and the size of the sample can be easily regulated as the dust is carried positively into the sampling flask. In earlier apparatus the flow of dust depended on gravity, so that it was difficult to control the size of sample. Often a sample would be obtained which was too large for the capacity of the gasometric apparatus and had therefore to be discarded.

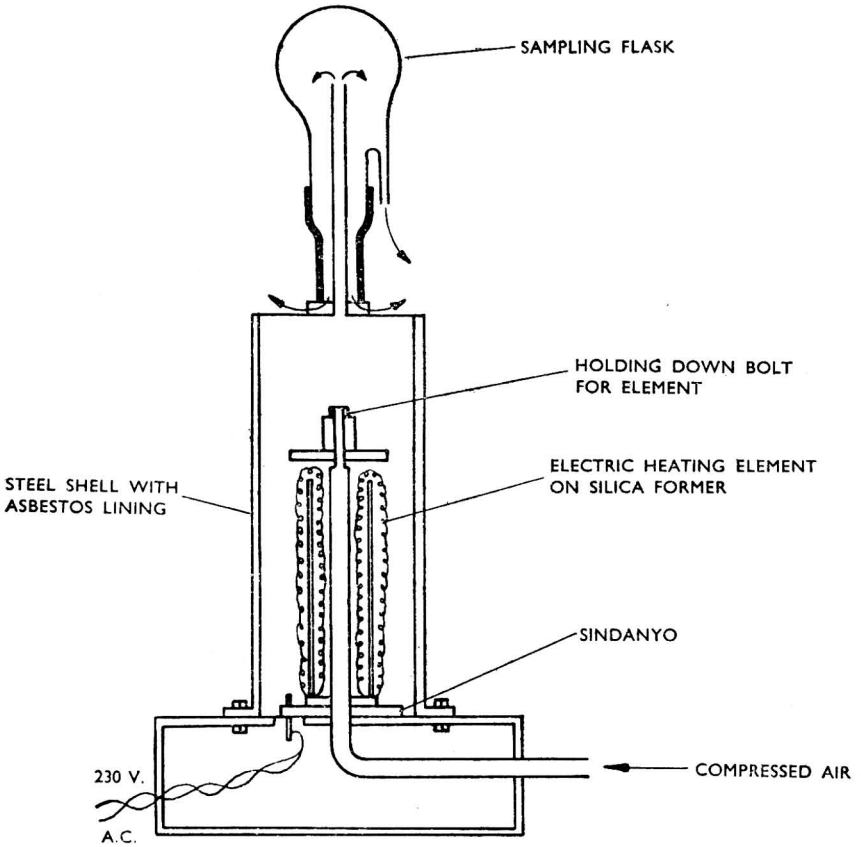

SAMPLING FLASK
HOLDING DOWN BOLT FOR ELEMENT
ELECTRIC HEATING ELEMENT ON SILICA FORMER
STEEL SHELL WITH ASBESTOS LINING
SINDANYO
230 V. A.C.
COMPRESSED AIR

Fig. 2.

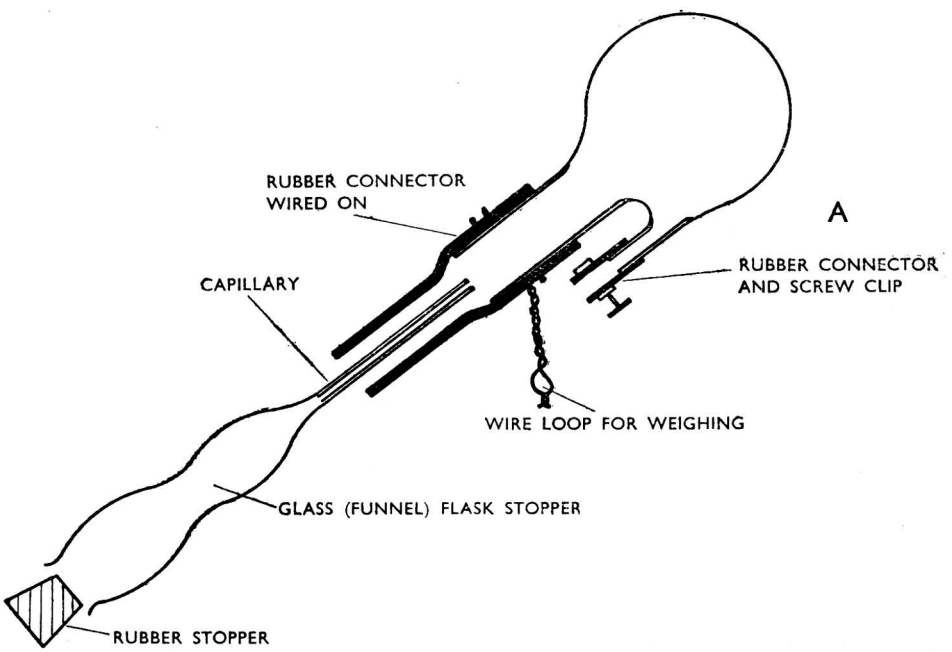

RUBBER CONNECTOR WIRED ON
A
CAPILLARY
RUBBER CONNECTOR AND SCREW CLIP
WIRE LOOP FOR WEIGHING
GLASS (FUNNEL) FLASK STOPPER
RUBBER STOPPER

Fig. 3.

The sampling flask is attached to the dust exit nozzle by a rubber connector. Consequently vibrations and shocks are not transmitted to the flask. Previously, brass male and glass female joints were used, with the result that many breakages occurred.

The total capacity of the device is small so that any residual dust in the screw can be run off quickly before a fresh sample is taken.

The small screw is retractable. In the sampling position it projects to the end of the nozzle, but it can be withdrawn when not in use and the valve closed, as shown in Fig. 1.

A side tube is fitted to the sampling flasks, which are of approximately 150-ml. capacity, so that the flask itself, and the air pocket in the nozzle at the beginning of the sampling operation, can be purged of air. The hydrogen used for this purpose is introduced *via* a mercury blow-off valve which prevents the blowing of joints and automatically allows hydrogen to flow out of the flask when this is not attached to the sampling point. Sampling can therefore be carried out in an atmosphere of hydrogen as described later.

GASOMETRIC APPARATUS—The gasometric apparatus is illustrated in Fig. 4. It is suitable for samples containing approximately 0·5 g. of metallic magnesium. Because of the nature of the material and the possibility of explosions the operator is protected by armour plate glass screens, shown in Fig. 5, which, however, have never been found essential. The screens also serve to protect the apparatus.

The reservoir, with taps A and B and the rubber bellows, eliminates the necessity of having a movable reservoir as with the Lunge nitrometer and makes enclosure of the apparatus conveniently possible.

The burette has a volume of about 600 ml. and may be graduated in steps of 1 ml., the graduated part being approximately 500 mm. long and 40 mm. in diameter, and the total height of the apparatus is about one metre to the top of the levelling tube.

The acid for attacking the sample is placed in a 15-ml. bulb which is part of a small tip-up adaptor, the object being to reduce the number of parts and joints to a minimum. The acid is conveniently introduced through the 10 mm. diameter connecting tube.

The advantages claimed for this apparatus are: its large capacity, which increases the accuracy of gas measurements; the gas is evolved under reduced pressure; a minimum of moving parts are used, making for ease of use and a reduction in the risk of breakages; a minimum number of joints are connected with the gas space; ease of testing for leaks and observing when contraction of the gas on cooling to room temperature has ceased; convenient form for enclosure. Many of the above features contribute to rapidity in use.

METHOD OF SAMPLING AND DETERMINATION OF METALLICS—The sampling flask must be perfectly dry for weighing, because the dust reacts readily with water. An electric dryer has been designed and constructed as illustrated in Fig. 2, and enables the operator to dry the flask quickly and without damaging the rubber connectors.

Purge the dry flask and stopper (Fig. 3) with hydrogen, first purging the flask in an inverted position and then inserting the glass funnel-stopper, which is allowed to purge before the rubber stopper is inserted; finally close the screw clip on the side connection, and remove and weigh the flask. Hydrogen from a cylinder *via* a blow-off valve, as illustrated in Fig. 1, is used for this purpose.

Remove any dust remaining in the sampling screw from previous operations, by opening the valve, pushing the screw forward and rotating it (clockwise) several times; then withdraw it and close the valve. Before proceeding further see that the nozzle is clean inside and out.

Next, adjust the hydrogen flow and connect the flask, without its glass stopper, with the screw clip open. When the flask is purged connect it with the nozzle and open the hydrogen exit tap (just below the main valve, Fig. 1) to purge the nozzle. Close the exit tap, open the main valve, push the screw forward and rotate to obtain a sample (0·7–2·5 g., depending on the quality of the dust); withdraw the screw, close the main valve and disconnect the flask; purging will then automatically recommence. Insert the glass stopper (without rubber stopper), and when this is purged insert the rubber stopper, close the screw clip and shut off the hydrogen.

Reweigh the flask to find the weight of sample.

Remove the rubber stopper from the glass funnel-stopper and pour in sufficient heptane to wet the sample thoroughly—10 to 15 ml. usually suffice. The heptane inhibits oxidation of the finely divided magnesium, and from this stage onwards the sample can be handled normally.

Attach the flask with the screw clip open to the gasometric apparatus. Close tap B

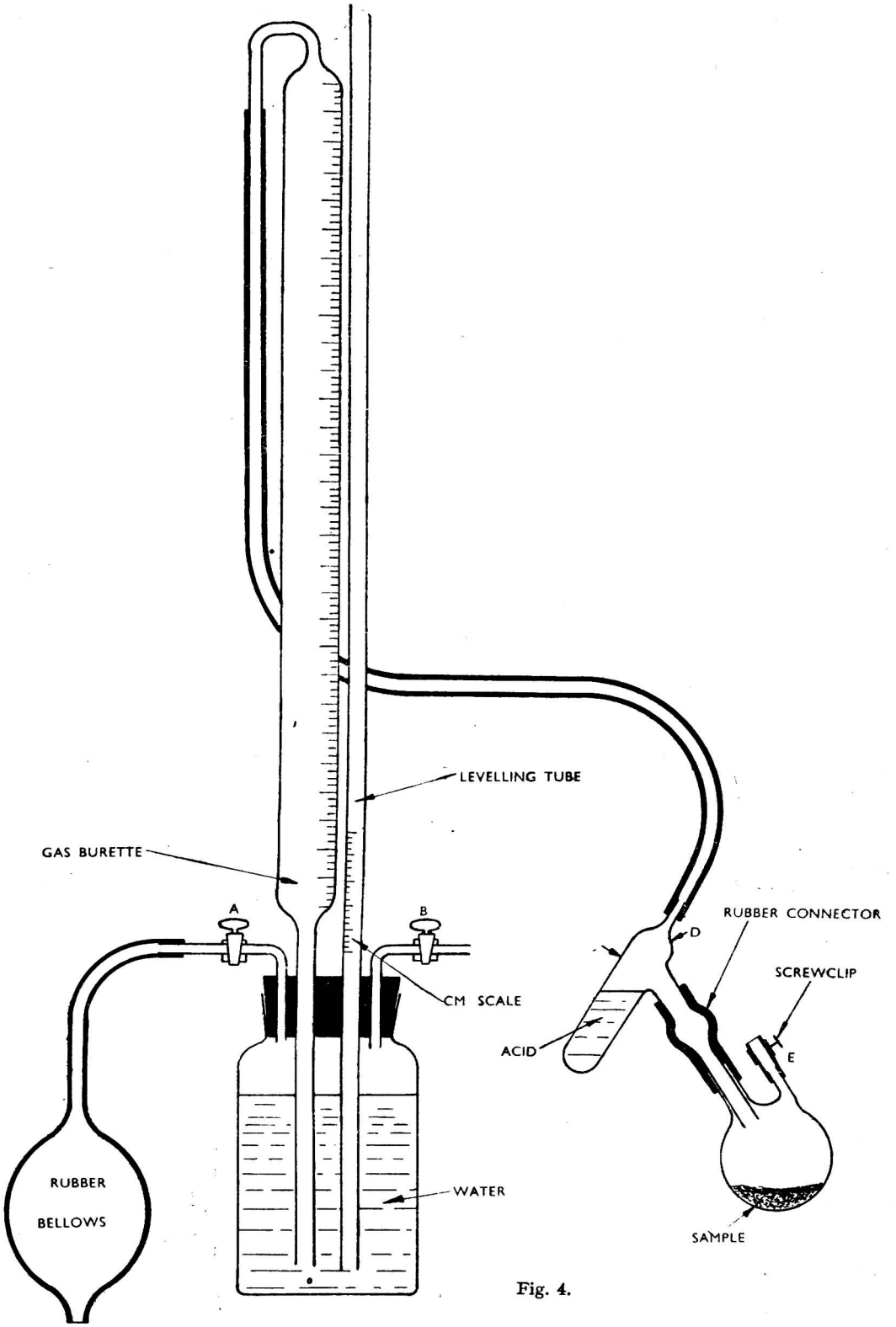

LEVELLING TUBE
GAS BURETTE
A
B
CM SCALE
RUBBER CONNECTOR
D
SCREWCLIP
ACID
E
RUBBER
BELLOWS
WATER
SAMPLE

Fig. 4.

(Fig. 4), open tap A and work the bellows to fill the burette to slightly above the zero mark. Adjust to zero by allowing air to escape through tap A. The bellows usually leak slightly, which allows the level to sink slowly; otherwise use tap B.

Charge the container C, through the neck D, with 12–15 ml. of 25% sulphuric acid. (The addition of a little "Teepol" to assist wetting is advantageous. Use 3–4 drops per 100 ml. of 25% acid.)

Close the screw clip and open tap B, allowing the level of water in the levelling tube to fall to the cm. scale. Close tap B and observe the liquid level against the cm. scale; if there is a leak the level will rise and it will be necessary to open the screw clip, pump up the liquid in the burette, adjust to zero again and, after remedying the leak, retest for leaks, and if there are none, proceed.

Fully open tap B and tilt the acid container carefully so that acid flows onto the sample. Sometimes the reaction is delayed and the flask should be gently shaken before pouring in more acid. When all the acid has been added and action has ceased, allow the flask to cool to room temperature. Cooling may be hastened by immersing the flask in a beaker of water.

Close tap B, open tap A and raise the level of liquid in the levelling tube to approximately that of the liquid in the burette. By observing this level the operator can see when contraction has ceased.

When equilibrium has been reached pump up the liquid and adjust the levels in burette and levelling tube to equality. Read off the volume of gas.

CALCULATION OF METALLIC CONTENT

24·32 g. of Mg ≡ 22·4 litres of hydrogen at N.T.P.

∴ 1 ml. of hydrogen at N.T.P. = 1·085 mg. of Magnesium.

If V = volume of hydrogen evolved at room temperature (t) and pressure (p), and S = mg. of sample taken

$$\text{Mg}\% = \frac{\text{V}\{p - (w + h)\}273 \times 1{\cdot}085 \times 100}{760(273 + t)\text{S}} \quad \ldots \quad (1)$$

where w = the vapour pressure of water at temperature t, and
h = " " " " heptane at temperature t.

Part of equation (1) remains constant, *viz.*,

$$\frac{273 \times 1{\cdot}085 \times 100}{760}$$

and may be condensed to constant K = 39·0.

The part $\frac{p - (w + h)}{273 + t}$, which depends on room temperature and pressure, has been reduced to a variable factor F which can be read from a Table.

The equation then becomes: $\text{Magnesium } \% = \frac{\text{KFV}}{\text{S}}$ .. .. (2)

CORRECTION FOR IMPURITIES—Analysis of the dust has shown the presence of calcium carbide and carbonates which produce acetylene and carbon dioxide respectively on treatment with acid. These normally account for approximately 5% of the evolved gas, and this has been taken as the standard correction. The constant K then becomes

$$\frac{273 \times 1{\cdot}085 \times 95}{760} = 37{\cdot}0$$

and equation (2) can be expressed as

$$\text{Magnesium } \% = \frac{37 \times \text{F} \times \text{ml. of evolved gas}}{\text{mg. of sample}}$$

On pure magnesium drillings results accurate to within ±0·5% have been obtained.

Acknowledgments are made to the Directors of the Magnesium Metal Corporation, Ltd., for permission to publish this paper.

MAGNESIUM METAL CORPORATION, LTD.
SWANSEA

November, 1945

Errata—May issue: p. 225, line 25: *for* "0·004 g." *read* "0·0004 g." of added NaOH
" 26: " "0·008 g." " "0·0008 g." " " "

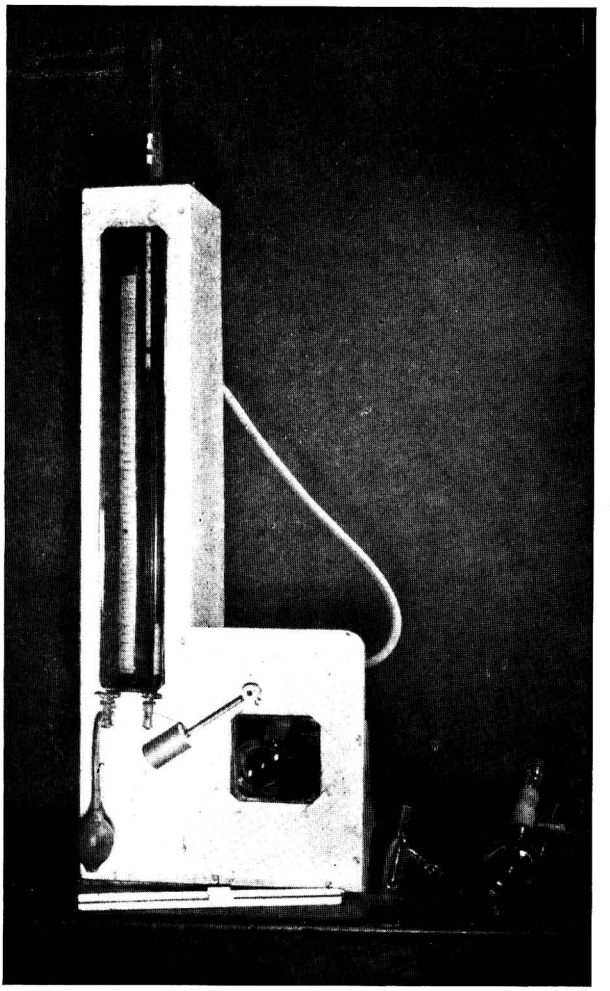
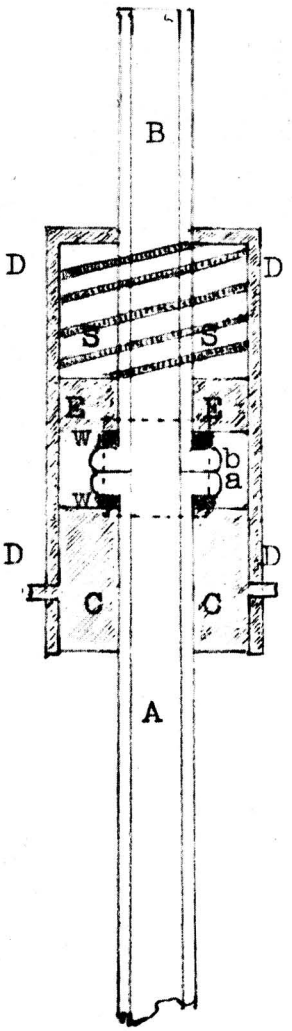
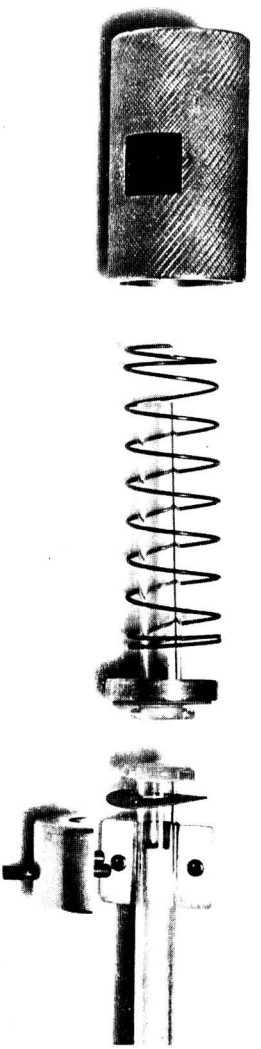
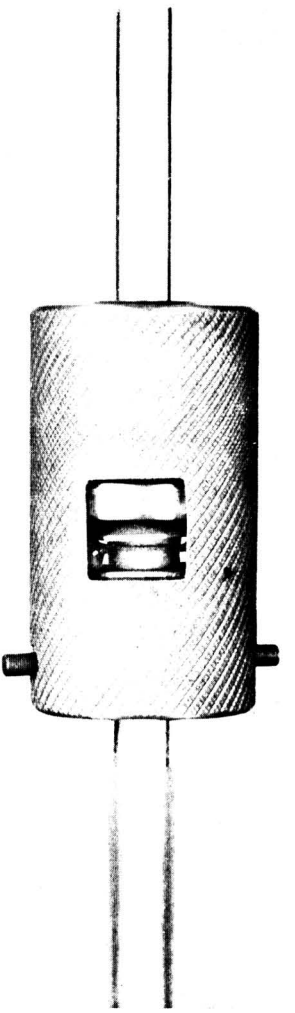


Fig. 5.
Gasometric apparatus encased







Gutzeit Test-paper Holder

Notes

A NOTE ON A NEW GUTZEIT TEST-PAPER HOLDER

In reviewing the modified Gutzeit test for arsenic with a view to adapting it for forensic work it was found that whilst the consensus of opinion held that the "diffusion through the test-paper" forms of the test were preferable, a general dissatisfaction with the current types of paper holders was indicated by the numerous "improvements" published in the literature. The following criteria appeared to me to be essential, and various published types were examined to see how far they fulfilled them:

(1) That all portions of the apparatus coming in contact with the test-paper, the reaction chamber or the evolved gases prior to their passing through the papers must be capable of being thoroughly cleaned with hot chemical solutions if necessary.
(2) That the paper is secured over the gas escape in such a manner that all the gas is forced to pass through the paper and loss by lateral diffusion reduced to a minimum.
(3) That the stains are of uniform density with sharp edges.
(4) That nothing comes in contact with the test-papers, in the neighbourhood of the stains, which may contaminate them.

It will be seen that condition (1) is probably best fulfilled by all-glass parts, whilst (2) and (3) can only be fulfilled if the paper is held between two sections in which the upper has a hole equal to the gas exit and this hole and the exit are in exact register, and if the grip on the paper is really tight and the pressure evenly distributed around the hole. As a secondary consideration it would appear to be necessary to obtain this grip without torsion due to any screwing action None of the holders fulfil all these criteria entirely. Thus:

ANALYST, 1927, **52**, 6. This holder, described by J. R. Stubbs, was discarded as rubber is in contact with the test-paper; also the elasticity of the rubber bungs occasionally spoilt the register.
ANALYST, 1927, **52**, 700. Holder described by J. White, proved very good, but bakelite plate not wholly satisfactory.
ANALYST, 1927, **52**, 701. The application of gum to the test-paper, as described by C. H. Cribb, is obviously open to criticism.
ANALYST, 1928, **53**, 152. This holder, described by A. Scott-Dodd, is made of metal, and the pressure is not necessarily evenly distributed.
ANALYST, 1930, **55**, 503. A. J. Linsey. This holder has the advantage of being all glass and was found after prolonged use to be very good, but the register can obviously vary considerably.
ANALYST, 1930, **55**, 630. T. J. Ward. The paper is in contact with rubber and lateral diffusion must be considerable; nor are all the gases forced to pass through the paper.
ANALYST, 1931, **56**, 30. G. H. Davis. The paper is in contact with rubber bungs.
ANALYST, 1938, **63**, 728. This holder, described by the Sub-Committee of the Institute of Brewing, is probably the best we have tried, but it was formed of bakelite and the possibility of torsion in the paper cannot be excluded.

Consequently, the holder illustrated in the Figures (opposite) was devised and has proved entirely satisfactory over a considerable period. The parts are as follows.

A, the gas exit, is formed of glass tubing of 6·5 mm. internal and at least 8 mm. external diameter, flattened to a button *a* which is ground flat at right angles to the axis of the tube.
B, a length of tubing exactly similar to A and similarly flattened and ground to button *b*. These flanged flat glass joints are now obtainable commercially.
C, a collar of metal or plastic, centrally bored, the boring being a sleeve fit on the tube A and carrying two flanges to lock as a bayonet fitting into D. The collar C is split into two interlocking halves for convenience of assembly.
D, a metal or plastic tube closed at the top end except for centrally bored hole through which B passes as a sleeve fit.
E, a plunger consisting of a metal or plastic washer at least ¼ in. thick that fits inside D, so as to move smoothly but without play and through which B passes through a centrally bored hole, again as a sleeve fitting.
S, a spring of sufficient strength to hold B very tightly on A.
W, leather or rubber washers.

The dotted line represents one of two ½ in. square windows in D, exactly opposite to each other, through which the strip of test-paper can be passed.

When the holder is assembled all that is necessary to put the test-paper in position is to raise B, put the paper in through the window and release B, when a tight fit with even pressure all round the edge of the hole is immediately obtained.

E. B. PARKES

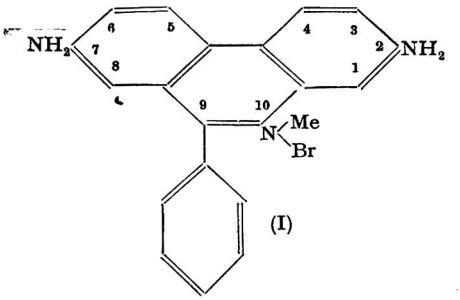
BRISTOL FORENSIC SCIENCE LABORATORY
BRIDEWELL STREET, BRISTOL, 1

January, 1946

NOTE ON THE ASSAY OF 2: 7-DIAMINO-9-PHENYL-10-METHYLPHENANTHRIDINIUM BROMIDE

The discovery that certain amino-substituted quaternary salts of the phenanthridine series possess trypanocidal activity[1] has led to an extensive investigation[2] of these compounds in the treatment of *T. congolense* and *T. brucei* infections in mice and cattle. In particular, 2: 7-diamino-9-phenyl-10-methylphenanthridinium bromide (more commonly known as Dimidium Bromide or Phenanthridinium 1553) has

been used in veterinary trials and, on this account, it became necessary to develop an assay process for the compound manufactured on a pilot-plant scale.



(I)

Examination of the structural formula (I) of dimidium bromide suggests a similarity with the series of acridine derivatives used as antiseptics, for which standard methods of analysis, depending upon the formation of insoluble ferricyanides, are available. Application of the same principle in the assay of dimidium bromide proved successful, and in conjunction with a halogen determination formed a satisfactory basis for standardising the product, which occurs as purple-black plates yielding a permanganate-coloured solution in water. Details of the analytical processes are as follows:—

Assay for Bromide—Dissolve about 0·25 g., accurately weighed, in 50 ml. of water and, after addition of a few ml. of dilute nitric acid, titrate electrometrically against $N/20$ silver nitrate, using a silver/silver bromide half cell as reference electrode. Each ml. of $N/20$ $AgNO_3$ solution is equivalent to 0·003996 g. of Br or 0·01901 g. of $C_{20}H_{18}N_3Br$.

Assay for Phenanthridinium Radical ($C_{20}H_{18}N_3$)—Dissolve about 1 g., accurately weighed, in 200 ml. of water, warming if necessary and making sure that solution is complete. Add 5 g. of sodium acetate and 20 ml. of $M/10$ potassium ferricyanide solution with vigorous shaking. Allow to stand for 5 minutes, filter through a sintered glass suction funnel and wash the precipitate with two portions (50 ml.) of water. To the combined filtrate and washings add 5 ml. of concentrated hydrochloric acid, 5 g. of sodium chloride, 2 g. of potassium iodide and 3 g. of zinc sulphate, allowing each salt to dissolve before adding the next. Allow to stand for 5 minutes and then titrate against $N/10$ sodium thiosulphate, using mucilage of starch as indicator. When the titration is nearly complete allow to stand for a further 3 minutes before finishing the determination. Determine by means of a blank experiment the number of ml. of $N/10$ sodium thiosulphate equivalent to 20 ml. of $M/10$ potassium ferricyanide solution and calculate the volume of $M/10$ potassium ferricyanide solution required by the phenanthridinium compound. Each ml. of $M/10$ potassium ferricyanide solution is equivalent to 0·1141 g. of $C_{20}H_{18}N_3Br$.

Owing to the colour of the titration solution, due to the slight solubility of 2: 7-diamino-9-phenyl-10-methylphenanthridinium ferricyanide in water, it is essential that the titration should be carried out in a good light. Samples of dimidium bromide which we have examined have contained more than 98% $C_{20}H_{18}N_3Br$, calculated with respect to the material dried at 110° C.

Attempts to apply the dichromate process, developed by Pedley[3] for the assay of 5-aminoacridine hydrochloride, to dimidium bromide afforded, in our hands, results which were 5 to 10% low.

We wish to thank the Directors of the Wellcome Foundation for permission to publish this information.

WELLCOME CHEMICAL WORKS,
DARTFORD, KENT.

G. E. FOSTER
W. F. GROVE

March, 1946.

REFERENCES

1. Browning, C. H., Morgan, G. T., Robb, J. V. M., and Walls, L. P., *J. Path. Bact.*, 1938, **46**, 203.
2. Walls, L. P., *J. Chem. Soc.*, 1945, 294.
3. Pedley, E., *Pharm. J.*, 1945, **155**, 148.

Ministry of Food

STATUTORY RULES AND ORDERS*

1946—No. 338. Order, dated March 8, 1946, amending the Feeding Stuffs (Regulation of Manufacture) Order, 1944. Price 2d.

This Order, which *came* into force on March 18, 1946, alters the composition of National Compounds by slightly increasing the maximum fibre content, by reducing the minimum wheat by-products content of the National Compounds and by increasing the minimum cereal content of all National Cattle Foods and National Poultry Foods other than National Baby Chick Food. The Order also alters the composition of National Cereal Mixture by reducing the wheat by-products and increasing the cereal content.

— **No. 386. Order, dated March 18, 1946, amending the Edible Oils and Fats (Control of Sales) Order, 1944.** Price 1d.

This Order amends the definition of Edible Oil in the main Order (*S.R. & O.*, 1944, *No.* 672) *to include* "any oil or fat used or capable of being used for the greasing of tins, trays or other utensils used in the cooking or preparation of food for human consumption."

— **No. 588. Order, dated April 18th, 1946, amending the Cheese (Control and Maximum Prices) Order, 1943.** Price 1d.

The purpose of this Order is to specify maximum wholesale and retail prices for Camembert cheese. *It substitutes the following definitions for the corresponding ones in Article I of the main Order* (*S.R. & O.*, 1943, *No.* 1766, ANALYST 1944, **69**, 151) *and its amending Order*, 1945, *No.* 1515.

* Italics signify altered wording.

"Soft Cheese" or "Curd Cheese" means cheese the moisture content of which exceeds 55% and includes the product commonly known as "Curds," but shall not include Camembert cheese imported from France.

"Specified Cheese" means any description of cheese except (*a*) blue-vein cheese not being cheese imported from Denmark; (*b*) soft cheese or curd cheese; and (*c*) cheese made from milk other than cow's milk.

Maximum prices are given for the following varieties of specified cheese: cheese imported from Denmark; Camembert cheese imported from France; Wensleydale; processed cheese and any other variety of cheese not specified above.

1946—No. 662. Order, dated May 8th, 1946, amending the Flour Order, 1945. Price 1d. This amending Order provides for the rate of extraction of national flour to be increased from 85% to 90% and of "W" flour from over 85% to over 90%, as from Sunday, May 12, 1946. (*Cf. this vol., pp.* 184 *and* 144.)

British Standards Institution

MICROCHEMICAL CARBON AND HYDROGEN COMBUSTION TRAINS

A FEW copies of the Second Draft of the British Standard Specification for Microchemical Carbon and Hydrogen Combustion Trains, CH(C)4204, issued for comment only, are available to interested members of the Society and may be obtained on application to the Secretary, J. H. Lane, 7–8, Idol Lane, London,E.C.3.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

Food and Drugs

Factors Influencing Estimation of Free Fatty Acids in Dried Egg Powders. L. Kline and C. M. Johnson (*Ind. Eng. Chem., Anal. Ed.*, **1946, 18**, 35–38)—The acidity of the ethereal extract of dried egg powder as determined by the method of the A.O.A.C. ("Methods of Analysis," 1940, 314) is frequently used as a measure of glyceride hydrolysis. Briefly, the method consists in drying the egg powder to constant wt. at 55° C. under reduced pressure, extracting 2-g. portions with anhydrous ether, removing the solvent, weighing the residue and titrating it in neutral benzene with 0·05 *N* sodium ethylate to the phenolphthalein end point. The indiscriminate use of the results of this method to measure either lipolysis or free fatty acid content may be misleading, since it is possible that at the *p*H of freshly dried egg (8·5 to 9) fatty acids liberated during storage will react with buffer constituents to form salts which would not contribute to the acidity of the extract. To determine the recovery of fatty acids as a function of the *p*H of dried egg, known amounts of oleic acid in ethereal soln. were added to aliquots of whole fresh eggs, the *p*H values were adjusted by addition of 3*N* hydrochloric acid and the samples were "lyophilised," *i.e.*, dried in the frozen state under highly reduced pressure. Controls containing no oleic acid were prepared from the same batch of egg to cover the same *p*H range. The preparations were extracted with anhydrous ether in Soxhlet extractors for 4 hr. and the acidities of the extracts were determined by the A.O.A.C. method. Only 55 to 60% of the added oleic acid was recovered at the *p*H of freshly dried egg, and complete recovery was attained only in preparations the *p*H value of which had been reduced by addition of acid to below 4·5. It was evident that the rate of increase of recovery of oleic acid with decreasing *p*H was considerably greater than the rate of increase of acidity of the ethereal extract of the controls. This indicates that the acidity value for freshly dried egg is, at least partly, due to some constituent other than free fatty acid and further expts. pointed to cephalin as this constituent. The cephalins act as monobasic acids which are completely titrated in solvents of low dielectric capacity with phenolphthalein as indicator. Jukes (*J. Biol. Chem.*, 1937, **107**, 783) found the alkali-binding capacity of egg phospholipid dissolved in 98% alcohol to be equivalent to the cephalin content as estimated by amino nitrogen determinations. Lecithin, under similar titrating conditions, has no alkali-binding capacity, the difference being due to the nature of the bases, *viz.*, choline, a strong base, in lecithin and ethanolamine, a weaker base, in cephalin. To determine the extent to which egg cephalin contributes to the acidity, extracts prepared from four egg powders were analysed. Two were commercial spray-dried products, one was prepared in the laboratory by lyophilising several dozen Grade A fresh eggs and the fourth was a commercial spray-dried powder containing 5% of moisture and which had been stored at 98° F. for 9 months. Extracts were prepared from the moist samples and after preliminary drying. To a 20-g. portion of each extract were added 180 ml. of ice-cold acetone and the mixture was cooled for 2 hr. in an ice bath. It was then centrifuged and the portion insoluble in acetone was twice kneaded with 10-ml. portions of cold acetone and centrifuged. The combined supernatant acetone solns. were freed from solvent and the residue was dried in a vacuum oven. With the oils obtained from the three unstored samples the acetone-insol. fraction represented about 13% of the original extract and contained about 70% of the acidity. The nitrogen and phosphorus contents and ratios showed this fraction to be essentially phospholipid. With the stored sample the acetone-insol. fraction represented 11% of the total extract but contained only 17% of the acidity. The acetone-sol. fractions from the unstored eggs contained 26 to 32% of the original acidity and from the stored sample 83%, indicating a large amount of free fatty acid in the last sample. Part of the acetone-sol. acidity was still apparently due to phospholipid, since about 10% of the nitrogen and phosphorus of the extracts from the unstored samples and 23% for the stored sample remained in this fraction. Drying the samples before extraction caused lower acidity of the ethereal extracts correlated with lower amounts of phospholipid in these extracts. By determinations of amino nitrogen by the method of Van Slyke applied to weighed portions of the extract in glacial acetic acid and to the hydrolysates left after hydrolysing the extracts with 2 *N* sulphuric acid for 48 hr. and removing the fatty acids with ether and also by formol titration of the hydrolysates it was established that the

alkali-binding capacity of the fatty acid-free portion of the hydrolysate is equivalent to the amino nitrogen constituent. Thus for the acetone-insol. fractions from the unstored samples the alkali-binding and amino nitrogen equivalents indicate that about 30% of the total nitrogen existed as cephalin. For the lyophilised sample the corresponding figure was 38%. The Van Slyke values obtained with the unhydrolysed phospholipids were lower, especially with the stored egg powder, the disappearance of the amino group being probably due to formation of an amide easily hydrolysed by alkali. Cephalin was found to represent about 23% of the acidity of the acetone-sol. fraction of the extract from fresh eggs, leaving the bulk of the acidity to be accounted for by fatty acids. The corresponding acetone-sol. fraction from the extract from stored egg powder contained less than 3% of its acidity as cephalin, the free fatty acid content being about 10 times that of the corresponding fraction from unstored powder. Expts. with vacuum-dried samples showed that with increasing moisture content the acidity of the ethereal extract increases, and this was correlated with increased phospholipid extraction. The acetone-sol. fraction was not affected by changes of moisture content.

The error caused by incomplete extraction of fatty acids from egg powder can thus be eliminated by reconstituting the powder, acidifying to *p*H 4·5 and drying the frozen mixture under reduced pressure before extracting with ether. In the measurement of the free fatty acid content of egg oil, the alkali-binding capacity of cephalin must be taken into account since it may constitute the greater part of the total acidity. Precipitation with acetone removes most of the cephalin, and for complete removal alcoholic magnesium chloride soln. may be used in conjunction with acetone. In egg oil the cephalin contributes 60 to 70% of the acidity of the total ethereal extract, thus obscuring significant increases in free fatty acidity. Interpretation of the acidity of the ethereal extract is further complicated by variable ethereal extraction of cephalin at different moisture levels.

A. O. J.

Determination of Sulphur Dioxide in Fruits. J. D. Ponting and G. Johnson (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 682–686)—The method combines some of the features of the methods of Iok'helson and Nevstrueva (*Voprosy Pitaniya*, 1940, **9**, 25), Jensen (ANALYST, 1928, **53**, 133), Bennett and Donovan (*Id.*, 1943, **68**, 140) and Prater *et al.* (*Ind. Eng. Chem., Anal. Ed.*, 1944, **16**, 153; ANALYST, 1944, **69**, 247). Distillation is avoided, errors due to enzymic oxidation of sulphur dioxide are eliminated or greatly reduced, extraction and clarification are improved by "blending" and filtration respectively, formaldehyde is used to obtain a blank value for reducing substances other than sulphur dioxide and combined sulphur dioxide is liberated by action of alkali. The method for dried fruit differs slightly from that for fresh or frozen fruit.

To 100 g. of fresh or frozen fruit in a Waring Blendor add 10 ml. of 0·5 *M* tartrate buffer at *p*H 4·5 (tartaric acid and sodium hydroxide) and 490 ml. of 20% w/w sodium chloride soln. and "blend" the mixture for 3 to 5 min. Filter at least 100 ml. of blended material through coarse filter paper with suction or through a cotton milk-filter disc. A filter aid is useful with apricots and other gummy fruits. To each of two 50-ml. portions of the filtrate add 2 ml. of *N* sodium hydroxide and, after 30 sec. acidify each with about 2 ml. of 6 *N* hydrochloric acid. Titrate one portion immediately with 0·02 *N* iodine, using 1 ml. of 1% starch soln. as indicator. To the second portion add about 1 ml. of 40% formaldehyde and, after 10 min., titrate with iodine. The difference between the two titres is a measure of the sulphur dioxide content of the sample. If w is the wt. of the sample originally taken, v the vol. of liquid in this portion and n the number of ml. of 0·02 *N* iodine required for titration of the sulphur dioxide in a 50-ml. aliquot, the sulphur dioxide content in p.p.m. is $640\,n\,(500 + v)/50\,w$. The value of v may be taken as 94 ml. per 100 g. for fresh and frozen fruits.

With dried fruits blend a 20-g. or 40-g. sample (according to the amount of sulphur dioxide present) with 10 ml. of tartrate buffer and 490 ml. of water for 10 min. Tough fruits should be cut into strips or ground, but soaking is not recommended. After filtering, treat 50 ml. aliquots of the filtrate as described for fresh fruit but use 4 ml. of *N* sodium hydroxide to liberate the combined sulphur dioxide. Calculate the sulphur dioxide content as before, taking the vol. of liquid in the sample as the wt. of sample × % moisture/100. The moisture content need be estimated only to 5 to 10%.

Investigation of the method established that bisulphite ion is the most stable of the molecular species concerned and that loss of sulphur dioxide in the Blendor is negligible in presence of a buffer that maintains it as that ion. Since the texture of many fruits prevents penetration of sulphur dioxide below the surface before or during frozen storage, oxidising enzymes remain potentially active. When the fruit is thawed, the active enzyme catalyses first the oxidation of the sulphur dioxide and afterwards the oxidative darkening of the fruit. Fruits with high phenol oxidase activity require a higher content of sulphur dioxide to protect their colour after thawing. In the proposed method the enzyme is inactivated by moderately strong brine. Formaldehyde was found preferable to acetone for forming the addition compound stable in presence of iodine, and the somewhat slow reaction is complete within 8 min. Expts. showed that formaldehyde does not bind any other constituents titratable with iodine (*e.g.*, ascorbic acid). A. O. J.

A Rapid Test for Distinguishing Human from Cow's Milk Based upon a Difference in their Xanthine Oxidase Content. F. L. Rodkey and E. G. Ball (*J. Lab. Clin. Med.*, 1946, **31**, 354–356)—Adulteration of human with cow's milk becomes profitable where the former is purchased for institutional distribution. Human milk is almost devoid of xanthine oxidase whereas cow's milk, even after commercial pasteurisation, is rich in this enzyme. *Method*—Transfer 2 ml. of the milk to a Thunberg tube and add 0·5 ml. of a 0·005% soln. of methylene blue. Into the side arm or hollow stopper of the tube place 0·3 ml. of a 0·5 *M* soln. of xanthine in 0·05 *M* sodium hydroxide or 0·2 ml. of a similar soln. of hypoxanthine. Evacuate the tube, adjust the temperature if appreciable cooling has occurred and mix the substrate with the milk by inverting the tube. Note the time for the conversion of the methylene blue into its leuco form. Perform a blank on each sample in the same manner except that the substrate is omitted. The optimum temperature is 40° C., but the reaction takes place satisfactorily at room temperature, so that a water bath need only be employed when a rapid result is required. The test takes only half as long if hypoxanthine is available in place of xanthine.

Figures are quoted which indicate that the procedure will detect admixture of 9% of cow's milk with human milk. It is noted that the presence of the oxidase in cow's milk is dependent upon the duration of pasteurisation and this should be checked for the milk available in any particular district; milk that has been boiled is devoid of xanthine oxidase. J. A.

Chemical Examination of the Seeds of *Amaranthus Gangeticus*. I. Fatty Oil from the Seeds. N. Chidambaram and R. R. Iyer (*J. Indian Chem. Soc.*, 1945, **22**, 117–118)—Seeds of *Amaranthus Gangeticus*, from South Travancore, on repeated extraction with petroleum spirit (b.p. 50–60° C.), yielded 6% of light yellow oil having the following characteristics:—sp.gr. 0·9021 at 29° C.; n 1·4733 at 28° C.; Wijs iodine val. 76·3; sap. val., 175·6; acid val. 12·4; acetyl val. 19·7; R.M. val. 0·71; Polenske val. 0·50; unsap. matter 2·6%. The composition of the fatty acids was:—unsaturated (basis of oil) 71·12% (oleic 43·7%; linolic 27·3%); saturated (basis of oil) 22·88% (palmitic 20·84%; stearic 2·16%). Other constants were:—mean mol. wt. of unsaturated acids 283·0; of saturated acids 264·5; iodine val. of unsaturated acids 106·4; of saturated acids 2·6. E. B. D.

Component Fatty Acids of Oil of *Citrullus Vulgaris*, Schrad (Water-melon) Seeds. D. R. Dhingra and A. K. Biswas (*J. Indian Chem. Soc.*, 1945, **22**, 119–122)—Oil obtained by cold pressing of water-melon seeds of red shell and not of black shell, from North West Punjab, had the following characteristics: sap. val. 196·3; sap. equivalent 285·4; iodine val., 124·2%; acid val., 5·2; unsap. matter, 0·3%. These results differ from those obtained on an American sample by Power and Salway (*J. Amer. Chem. Soc.*, 1910, **32**, 360) and the fatty acids obtained from this Indian oil also differ from those obtained by Pierserts (*Bull. Soc., Pharmacol.*, 1917, **24**, 204); it is considered that the other oils must be for different species of water-melon. Nolte and Loesecke (*J. Amer. Chem. Soc.*, 1939, **61**, 889) did not fractionate the liquid acids from their Cuban Queen variety of water-melon seed oil and therefore could not find out lower saturated acids which are dissolved in the alcohol-soluble portion during the lead-salt separation of mixed acids; their results are thus considered incorrect. The liquid and solid fractions obtained from the Indian oil were each methylated and the methyl esters separately fractionated under high vacuum. The percentage composition of the acids was calculated as: caprylic 0·2; capric 1·1; lauric 0·8; myristic 0·2; palmitic 7·6; stearic 6·1; oleic 35·3; linolic 48·7. Unsap. matter is 0·2%. Fully saturated glycerides are absent and trilinolein is only 0·9%, although linolic acid is 48·7% of the mixed acids. This result agrees with the Hilditch theory of "even distribution of the fatty acids in the seeds oil" (*Biochem. J.*, 1929, **23**, 1273). The association ratio of saturated to unsaturated acids (1 : 4·68) also indicates absence of fully saturated glycerides in the oil. E. B. D.

Component Fatty Acids of Melon (*Cucumis Melo*, Linn.) Seed Oil. D. R. Dhingra and P. Narain (*J. Indian Chem. Soc.*, 1945, **22**, 123–126)—The component fatty acids of oil of seeds of melon or sweet melon (*Cucumis melo*, Linn.), grown abundantly all over India, are oleic, 43·2%; linolic, 45·2%; caproic, 1·0%; caprylic, 2·0%; myristic, 1·1%; palmitic, 7·3% and stearic, 0·2%. Unsaponifiable matter, 0·9%. These results differ somewhat from those for Californian melon seed (Baughman and Jamieson, *J. Amer. Chem. Soc.*, 1920, **42**, 152), but the total of unsaturated acids is almost the same. This botanical family is characterised by the high percentage of oleic and linolic acids. Fully saturated glycerides are absent and tri-linolein does not exceed 1·0%. The constants for the present sample (A) are compared with those for Indian *Cucumis Sativus* (B) examined by Hooper (*Ann. Rept., IndianMuseum*, 1907–1908, 13), as follows: sp.gr.: (A)—, (B) 0·923–0·924; sap. val.: (A) 207·4; (B) 195·2–196·9; sap. equivalent: (A) 270·0, (B) 284·7–286·8; acid val.: (A) 0·9, (B) 11·5; iodine val.: (A) 117·1, (B) 117·7–118·5; unsap. matter: (A) 0·79, (B) —. Results for African *Cucumis Melo*, L. (*Bull. Imp. Inst.*, 1913, 56) and for a South Russian sample (Lewkowitsch, "Chemical Technology and Analysis of Oils, Fats and Waxes," 1922, Vol. II, p. 171) are also quoted. These oils are different from the Indian. The kernels of the Indian seeds contained 40% of oil, 22·7% of proteins and 0·75% of P_2O_5. The kernels, which are nutritive and medicinal, are used as substitutes for almond and pistachio kernels; the oil can also replace almond oil medicinally. E. B. D.

Chemical Estimation of Vitamin E in Vegetable Oils. J. Tošić and T. Moore (*Biochem. J.*, 1945, **39**, 498–507)—A method is described of preparing suitable extracts to which the α:α′-dipyridyl reaction can be applied, to give results consistent with those obtained biologically.

To 1 g. of oil, add 2 ml. of 5% alcoholic pyrogallol solution and heat to 86° C. Add 0·44 ml. of a solution of 4 g. of potassium hydroxide in 2·7 ml. of water and shake for 2 mins. Add 8 ml. of water, and extract the unsaponifiable matter with 10- and 5-ml. portions of ether. Wash the combined ethereal extracts with two 5-ml. portions of 0·5% aqueous potassium hydroxide solution and then with several 10-ml. portions of water. Evaporate the ethereal solution under reduced pressure and dissolve the residue in a suitable volume of ethanol. Evaporate under reduced pressure 1–2 ml. of the solution, equivalent to 1 mg. of ferric-chloride-reducing substances, taking care to remove all the ethanol, and dissolve the residue in 5 ml. of light petroleum containing 1% of ethanol. Run the solution through a column, 50 × 10 mm., of activated alumina which has been washed with 10 ml. of the same solvent mixture and develop the column with a further 15 ml. of the solvent. Biologically inactive ferric-chloride-reducing substances are retained on the column, whilst vitamin E passes quantitatively into the filtrate. Evaporate the filtrate under reduced pressure, dissolve the residue in ethanol and make up to 25 ml. Estimate carotenoid pigments in one portion of the solution colorimetrically in a Zeiss-Pulfrich photometer with the S47 filter. Dilute another portion, containing 100–160 μg. of ferric-chloride-reducing substances, to 20 ml. with ethanol, and add 1 ml. of freshly prepared 0·2% alcoholic ferric chloride solution, followed by 1 ml. of freshly prepared 0·5% alcoholic α:α′-dipyridyl solution. Make up to 25 ml. with ethanol and prepare a blank in the same way, but omitting the unsaponifiable matter. Leave for 15 mins. in a dark place and compare the colours using the S53 filter; use diffused daylight or low artificial illumination. To convert the extinction value into μg. of α-tocopherol, multiply by the factor 166·6. From the result, subtract the

α-tocopherol equivalent of the carotenoids, 1 μg of which has a reducing power equivalent to 2·5 μg. of α-tocopherol. In the recovery of α-tocopherol added to wheat germ oil unsaponifiable matter, the error was ±2·5%. F. A. R.

Biochemical

The Use of Charcoal-treated Peptone in Microbiological Assays. H. Isbell (*Science*, 1945, **102**, 671–672)—The chief source of nitrogen in the media used for the microbiological determination of nicotinic acid, pantothenic acid and biotin with *Lactobacillus arabinosus* is a charcoal-treated casein hydrolysate. The preparation of this material is laborious and it is expensive to purchase. It is suggested that it may be replaced by a charcoal-treated peptone which gives satisfactory blanks and maximal acid production and is easy to prepare. *Preparation of charcoal-treated peptone*—Dissolve 100 g. of Bacto-Difco peptone in 800 ml. of distilled water, adjust the reaction of the soln. to *p*H 3·0 with conc. hydrochloric acid (a faint cloud forms at this point), add 20 g. of activated charcoal (Darco G60 is recommended) and stir mechanically for 1 hr. Filter the mixture by suction, readjust the reaction to *p*H 3·0 and repeat the treatment with charcoal, using 10 g. of the adsorbent. After stirring for 1 hr., filter and dilute the almost colourless soln. to 1 litre with water. This soln. is used in place of the casein hydrolysate, 10 ml. being substituted for 5 ml. of the latter in the medium of Krehl, Strong and Elvehjem (*Ind. Eng. Chem. Anal. Ed.*, 1943, **15**, 471). It is stated that the peptone should be tested before being used for microbiological assays; if properly prepared, less than than 2·0 ml. of 0·1 *N* acid should be produced by *L. arabinosus* incubated in a medium from which one of the growth factors has been omitted, whereas at least 17 ml. of 0·1 *N* acid will be produced in presence of 2·0 μg. of calcium pantothenate, 2·0 μg. of nicotinic acid and 0·01 μg. of biotin after 66 hours' incubation at 37° C. There is no significant difference between assays conducted with media prepared with peptone and those prepared with casein hydrolysate. J. A.

Microbiological Estimation of Amino Acids in Animal Proteins. S. W. Hier, C. E. Graham, R. Freides and D. Klein (*J. Biol. Chem.*, 1945, **161**, 705–716)—Glutamic acid, leucine, *iso*leucine, valine and threonine were estimated by means of *Lactobacillus arabinosus* 17-5, using a basal medium of the following composition: glucose 20, sodium acetate 20, ammonium sulphate 6 g. per litre; adenine sulphate 10, guanine hydrochloride 10, uracil 10, aneurine hydrochloride 0·2, nicotinic acid 0·2, calcium pantothenate 0·2, pyridoxine hydrochloride 0·2, *p*-aminobenzoic acid 0·1, riboflavine 0·2 mg. per litre; biotin 0·4 μg. per litre; *l*(−)-tryptophan 40, *l*(−)-tyrosine 40, *dl*-phenylalanine 100, *l*(+)-lysine hydrochloride 200, *dl*-alanine 200, *l*(+)-arginine hydrochloride 50, *l*(−)-histidine hydrochloride hydrate 50, asparagine 400, *l*(−)-proline 100, *dl*-serine 50, *dl*-norleucine 100, *l*(−)-hydroxyproline 100, glycine 100, *dl-iso*leucine 200, *dl*-leucine 200, *dl*-threonine 200, *l*(+)-glutamic acid 400, *dl*-valine 200, *l*(−)-cystine 100, *dl*-methionine 100 mg. per litre; Salts A* 5, Salts B* 5 ml. per litre. The amino acid being assayed was omitted from the basal medium and the normal procedure was followed for inoculation and incubation. The final titration was carried out with 0·1 *N*-sodium hydroxide. The time of autoclaving was found to be important, as excessive autoclaving gave a low maximum growth and, with threonine, high blanks, possibly owing to formation of pyridoxal and pyridoxamine, enabling the organism to synthesise threonine. Arginine, phenylalanine and tyrosine were estimated by means of *Lactobacillus helveticus*, using the medium of McMahan and Snell (*J. Biol. Chem.*, 1944, **152**, 83; ANALYST, 1944, **69**, 188). Histidine and lysine were estimated by means of *Leuconostoc mesenteroides* P-60 with Medium D of Dunn *et al.* (*J. Biol. Chem.*, 1944, **156**, 703, 715; ANALYST, 1945, **70**, 182). The following method was used for the preparation of the samples: Reflux 2 g. of protein with 80 ml. of 8 *N* hydrochloric acid for 8 hr. in an all-glass apparatus, dilute to 500 ml. and neutralise to *p*H 6·8 with 8 *N* sodium hydroxide. Dilute to 1 litre, filter and take a suitable aliquot of the filtrate for the assay of each amino acid. The maximum deviation from the mean was less than 10%. Recoveries of all the amino acids from lactalbumin and beef muscle ranged from 88 to 114% of the theoretical, with an average of 100%. The microbiological results agreed satisfactorily with the chemical results for all amino acids except phenylalanine, for which it is believed that the chemical values are in error. F. A. R.

Microbiological Determination of Free Leucine, *Iso*leucine, Valine and Threonine in Dog Plasma. S. W. Hier and O. Bergeim (*J. Biol. Chem.*, 1945, **161**, 717–722)—Collect 40 to 50 ml. of the blood, add heparin to prevent clotting, and centrifuge within 1 hour of collection. Put 16·0 ml. of the plasma into a flint glass 125-ml. bottle and add 22·4 ml. of water. Shake and slowly add 5·6 ml. of (exactly) 0·60 *N* sulphuric acid and 4·0 ml. of (exactly) 10% sodium tungstate solution. Shake for 2–3 mins. and filter through a Whatman No. 40 paper. Adjust the *p*H to 6·8 with 0·7 *N* sodium hydroxide. Each ml. of tungstic acid filtrate is thus equivalent to 0·33 ml. of original plasma. Put 1·0-ml. quantities of the basal medium described by Hier *et al.* (see preceding abstract) into small tubes, add the standard and test solutions and dilute the contents of each tube to 2·0 ml. Carry out the remainder of the procedure as previously described and titrate with 0·02 *N* sodium hydroxide. Agreement between the values for all four amino-acids at different levels were within 10% of the mean, and recoveries of known amounts of the *dl*-amino acids added to plasma prior to precipitation of protein were satisfactory, their averages and standard deviations being 98·5 ±8·4, 100 ±13·7, 102 ±10·8 and 93·5 ±11·9% for leucine, *iso*leucine, valine and threonine respectively. F. A. R.

Colorimetric Assay of Urinary Corticosteroid-like Substances. N. B. Talbot, A. H. Saltzman, R. L. Wixom and J. K. Wolfe (*J. Biol. Chem.*, 1945, **160**, 535–546)—The procedure used in this method resembles that of Venning *et al.* (*Endocrinology*, 1944, **35**, 49) with the exception of the final colorimetric estimation.

Collect a 24-hr. sample of urine without preservative and, within 1–2 days, extract with 4 portions of chloroform each equal to 15% of the volume of urine. Record the total amount, and make a correction for any chloroform lost as emulsion. Evaporate the extract to dryness at below 50° C.;

See footnote, ANALYST, 1944, **69**, 157.

the dry residue may be stored in the refrigerator for 24 hrs., if necessary. Transfer the residue with the aid of 100 ml. of chloroform to a separating funnel and wash with three 10-ml. portions of cold 0·1 *N* sodium hydroxide and then with three 10-ml. portions of water. Extract each washing with 10 ml. of chloroform and add the extract to the original chloroform solution before carrying out the next washing. Discard the washings and evaporate the chloroform solution to dryness. Transfer the residue to a small separating funnel with the aid of 30 ml. of benzene, extract with ten 30-ml. portions of water and discard the benzene. Extract the combined aqueous solutions with four 45-ml. portions of chloroform and evaporate to dryness at below 50° C. Add to the dry residue 200 mg. of Girard's reagent T and 0·5 ml. of glacial acetic acid, stopper with tin- or aluminium-foil and heat for 2 mins. in a boiling water-bath. Cool in an ice-bath, transfer to a small separating funnel with 40 ml. of cold water and add 3 ml. of 10% sodium hydroxide solution. Extract with three 20-ml. portions of chloroform, wash the combined extracts with 60 ml. of water and discard the chloroform. Add 1 ml. of conc. sulphuric acid to the water washings and add the mixture to the aqueous phase. Add 20 ml. of chloroform, leave for 2 hrs. at room temperature and extract with the chloroform and three further 20-ml. portions. Wash the combined extracts, which contain the ketonic fraction, with 10 ml. of 0·1 *N* sodium hydroxide and with three 10-ml. portions of water, extracting each wash in turn with an equal volume of chloroform and adding this to the soln. before carrying out the next washing. Evaporate the washed extract to dryness below 50° C., free the residue from chloroform by addition and evaporation of three 5-ml. portions of methanol. Dissolve the residue in a measured volume of methanol and assay a portion according to the following procedure. With normal urines it is convenient to use 2 ml. of methanol and 0·5 ml of the solution for assay. Add equal volumes of the methanol solution and water to a Folin-Wu macro blood-sugar tube calibrated at 7·0 ml., and add 1 ml. of copper reagent (freshly prepared from 25 parts of Copper Reagent A and 1 part of Copper Reagent B according to the method of Nelson, *J. Biol. Chem.*, 1944, **153**, 375; ANALYST, 1944, **69**, 313). Heat in a boiling water-bath for 20 mins., cool and add 1 ml. of arsenomolybdate reagent (Nelson, *loc. cit.*). Dilute to 7 ml., transfer to a colorimeter tube and evaluate the colour, using a filter transmitting maximally at 660 *mμ*. Set the galvanometer at 100 with a blank carried through the same procedure. Calculate the "corticosteroid" content from a calibration curve prepared from pure solutions of 17-hydroxycorticosterone, corticosterone or dehydrocorticosterone; for each of these the value of the constant K in the expression:

$$C = \frac{1}{K}(2 - \log G)$$

(where C is the concentration of "corticosteroid" in the solution and G the galvanometer reading) is 5·4 ± 0·1 when the amount is between 0·03 and 0·08 mg. The recoveries of 17-hydroxycorticosterone, 17-hydroxydehydrocorticosterone, corticosterone and dehydrocorticosterone were 100, 100, 55 and 38% respectively when the purification with Girard reagent T was omitted and 90, 90, 45 and 35% respectively when this step was included. Desoxycorticosterone was not extracted in this procedure. F. A. R.

Artificial Standard for use in the Estimation of Haemoglobin. Q. H. Gibson and D. C. Harrison (*Biochem. J.*, 1945, **39**, 490–497)—Colorimetric methods of estimating haemoglobin suffer from the disadvantage that they require to be standardised by oxygen capacity or by the estimation of iron in blood. The use of artificial standards has been proposed but those hitherto suggested have only been suitable for visual colorimetry. The proposal to use haemin as a standard (Rimington, *Brit. Med. J.*, 1941, **1**, 177; Clegg and King, *ibid.*, 1942, **2**, 329) appeared to solve these difficulties, since haemin has an absorption spectrum similar to that of blood and is therefore suitable for use with absorptiometers. It has now been found that haemin does not give uniform or stable colours, and a solution of inorganic salts is proposed for this purpose; it gives satisfactory results, both in colorimetric and in absorptiometric measurements. The salts comprise chromium sulphate in its green modification, which gives a band similar to the α-band of alkaline haematin, cobaltous sulphate to provide bands in the yellow and blue, and a small quantity of potassium dichromate to increase the absorption in the blue-green. For accurate work the following method of preparation is used; Dry about 5 g. of pure potassium dichromate at 100° C., weigh out 0·690 g. and dissolve in about 80 ml. of water. Weigh out another 3·42 g. of potassium dichromate, dissolve in about 100 ml. of water, add from a pipette 25 ml. of (exactly) *N* sulphuric acid and then add slowly from a measuring cylinder a freshly prepared saturated solution of sulphur dioxide, until reduction of the dichromate is complete, followed by a slight excess. (A total of 50–60 ml. is usually sufficient.) Add a few glass beads and boil for 45 mins. to expel sulphur dioxide. Dilute to 200 ml. Heat about 30 g. of pure cobaltous sulphate heptahydrate for 2 hrs. at about 96° C., with frequent stirring, and then heat overnight at 400° C. Transfer whilst hot to a stoppered bottle and, when it is cool, quickly weigh out of 13·10 g. Dissolve in about 80 ml. of water, and add the solution, together with the potassium dichromate solution, to the flask containing the chromium. Make up to 1 litre with water. The solution should always be heated for 4 mins. in a boiling water-bath before use to obtain the correct equilibrium between the red and green forms of chromium sulphate. A simplified method of preparing the standard is as follows: Weigh out 11·61 g. of pure potassium chromium alum, 13·10 g. of anhydrous cobaltous sulphate, prepared as described above, and 0·690 g. of potassium dichromate. Dissolve in about 500 ml. of water, add 1·8 ml. of *N* sulphuric acid, boil for 1 min., cool and make up to 1 litre with water.

Estimation of blood haemoglobin—Pipette 10 ml. of 0·1 *N* sodium hydroxide (pure) into a dry boiling-tube, add 0·1 ml. of blood, stopper with a loosely-fitting ground-glass stopper (corks must not be used) and mix. Treat the same volume of standard in a similar way. Immerse the two tubes in a boiling water-bath for exactly 4 min. and cool. Evaluate the colour visually, preferably with a green filter over the eye-piece, setting the standard at 10 mm. Haemoglobin value of standard = 16·0 g. per 100 ml. using filters and 16·2 g. per 100 ml., using unfiltered light (both calculated as iron). Alternatively, compare the colours of standard and blood solution in a photoelectric absorptiometer with a suitable filter, *e.g.*, Chance O Gr., 2 mm., Hilger H455, No. 5 (maximum at 530 *mμ*) and (with Hilger H503, 2 mm., infra-red absorbing glass) Ilford Spectrum filters Nos.

603–606 inclusive (maxima at 490, 520, 550 and 580 $m\mu$ respectively). F. A. R.

Modification of the Gutman Method of Estimating "Acid" Phosphatase Activity. J. Benotti, L. Rosenberg and B. Dewey (*J. Lab. Clin. Med.*, 1946, **31**, 357–360)—The procedure developed by Gutman and Gutman for the determination of the "acid" phosphatase activity of blood in cases of prostatic carcinoma (*J. Biol. Chem.*, 1940, **136**, 204: *J. Clin. Investigation*, 1938, **17**, 473) gives high results because of appreciable hydrolysis of the substrate in absence of "acid" phosphatase. It is pointed out that glycerophosphate is not a satisfactory substitute for phenyl phosphate as substrate since with the former the degree of hydrolysis must be assessed by means of a phosphate determination instead of the simpler and more accurate phenol determination. The original procedure has been modified by incorporating a "substrate blank," and spontaneous hydrolysis of the buffered disodium phenylphosphate substrate is prevented by storage as two separate solutions.

Method—Mix 9 ml. of a citrate buffer soln. of *p*H 4·9 (prepared by dissolving 18·9 g. of citric acid in 500 ml. of water, adding 180 ml. of *N* sodium hydroxide and 100 ml. of 0·1 *N* hydrochloric acid and diluting to 1 litre with water), with 1 ml. of a 1% soln. of disodium phenylphosphate, add 0·5 ml. of serum and incubate at 37° C. for 1 hr. Cool, add 4·5 ml. of Folin and Ciocalteu's phenol reagent previously diluted threefold with water and filter. A substrate blank should be prepared in precisely the same manner as the test except that the serum is replaced by 0·5 ml. of water, and the serum blank is conducted by mixing 10 ml. of water with 0·5 ml. of serum, immediately adding 4·5 ml. of the diluted phenol reagent and filtering. The intensity of the blue colour produced is determined by means of a photoelectric colorimeter calibrated to give mg. of phenol per 100 ml. of serum. The amounts of phenol in mg. per 100 ml. found in the two blanks are added together and substracted from that found in the test, to obtain the phenol hydrolysed by acid phosphatase in mg. per 100 ml. of serum; this number is by definition the number of "King units" of activity (King and Armstrong, *Can. Med. Assoc., J.*, 1934, **31**, 376). It is stated that results by this improved procedure have proved more reliable than those obtained by other methods.

J. A.

Inorganic

Nephelometric Determination of Small Amounts of Sodium. F. K. Lindsay, D. G. Braithwaite and J. S. D'Amico (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 101–102)—The reagent used is a soln. of magnesium uranyl acetate in acetic acid, water and alcohol. Various solvents for the reagent have been tried and the results show that ethyl alcohol with water gives a sensitive and consistent reagent. As part of the ethyl alcohol may be replaced by methyl alcohol with negligible effect, alcohol denatured by the addition of methyl alcohol is a satisfactory substitute.

Reagent—To 30 g. of uranyl acetate dihydrate, 150 g. of magnesium acetate tetrahydrate and 20 ml. of glacial acetic acid add 500 ml. of alcohol and water to make 1 litre. Heat on a steam bath, with stirring, until the salts dissolve, taking care to lose as little solvent as possible. Stir until cool and filter into a brown bottle.

Method—(A). To 2 ml. of the soln. to be tested (*e.g.*, a water) add 1 drop of conc. hydrochloric acid and 15 ml. of reagent. Mix by inverting 5 times, leave for 5 min., invert 5 times, leave for 5 min. and measure the transmittance, using a blue filter. Interpret the result from a prepared calibration curve. The accuracy is about 1 grain of sodium chloride per gallon (0·015 mg./ml.). Addition of 20 grains per gallon of lithium or potassium chloride to a solution containing 10 grains per gallon of sodium chloride does not affect the result. (B). Method was primarily developed for work on fluid cracking catalysts for the petroleum industry. This siliceous material is treated as follows: To 10 g. of catalyst add 20 ml. of diluted hydrochloric acid (1 + 1) and boil for exactly 2 min. Filter, take 2 ml. of the filtrate, add 10 ml. of the reagent and mix and proceed as above. The following results are given, the figures in parentheses being the corresponding values obtained by the standard gravimetric procedure. Sodium oxide, %: sample 1, 0·017 (0·0195); sample 2, 0·008 (0·010); sample 3, 0·021 (0·019); sample 4, 0·009 (0·012).

[A few particulars of the components and some dimensions are given for the photometer used in the development of this method. These suggest that it is "home-made" and very simple, but no information is given as to how it is operated and calibrated. However, a blue filter and a cell with 2 mm. walls and a 16 mm. light-path through the soln. are mentioned. ABSTRACTOR.] L. A. D.

Determination of Cuprous Chloride. L. F. Hatch and R. R. Estes (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 136–137)—Methods for the analysis of essentially pure solid cuprous chloride and of dilute solns. are given and discussed. In all the following methods allowance must be made for the "blank" of the reagents.

The pure cuprous chloride used as a standard for the following methods was a reagent grade product, further purified by known methods and stored in small glass-stoppered vials in a desiccator over magnesium perchlorate. It showed no change in composition over a period of several months. Determination of chloride ion by the Volhard method, and of copper by electrodeposition after oxidation to cupric sulphate, indicated 99·8% of cuprous chloride and 0·2% of cupric chloride.

(A). Dissolve 0·5 g. in the cold in 25 ml. of ferric ammonium sulphate soln. made by dissolving 10 g. of ferric ammonium sulphate in 100 ml. of diluted hydrochloric acid (1 + 1). Add 5 ml. of phosphoric acid and 200 ml. of water and titrate with permanganate.

(B). Weigh 0·3 g. into a dry 500 ml. conical flask, add 25 ml. of ferric ammonium sulphate soln. made by dissolving 10 g. in sufficient 3 *M* sulphuric acid to make 100 ml. and swirl gently until dissolved. Add 200 ml. of water and 5 ml. of phosphoric acid and titrate with 0·1 *N* permanganate until the pink colour persists for 15 sec.

(C). Weigh as in (B) and add 25 ml. of soln. made by dissolving 10 g. of ferric ammonium alum in enough *N* hydrochloric acid to make 100 ml. When dissolved, add 300 ml. of a soln. of 80 ml. of sulphuric acid and 25 ml. of phosphoric acid per litre, add 5 drops of 0·2% barium diphenylamine sulphonate soln., and titrate with 0·1 *N* dichromate.

(D). Weigh and dissolve as in (B). Add 1 drop of ferrous-phenanthroline indicator soln. and titrate with a 0·1 *N* soln. of ceric ammonium sulphate in 0·5 *M* sulphuric acid.

(E). For the determination of cuprous chloride in dilute solutions pipette 25 ml. of sample soln. into

25 ml. of soln. made by dissolving 33 g. of ferric ammonium sulphate in enough 3 *M* sulphuric acid to make 1 litre. Add 250 ml. of water and 1 drop of ferrous-phenanthroline indicator soln. and titrate as in (D).

Method (A) is the method approved by the Amer. Chem. Soc. Committee on Analytical Reagents and is considered unsatisfactory by the authors. (B), (C) and (D) are all superior to (A), (C) being recommended for general use and (D) when the highest accuracy is required. It is then preferable to use oxygen-free ferric ammonium sulphate solns. (E) is applicable to solns. containing up to 0·6% of cuprous chloride, although a modification of (C) (as (E) is modified from (D)) may be used also. In general it is recommended that the chloride ion concentration should be kept low and that the amount of ferric iron used should be at least 1·5 times that theoretically necessary. L. A. D.

Determination of Magnesium in Aluminium Alloys. H. C. Deterding and R. G. Taylor (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 127–129)—The sample is dissolved completely in acid and the magnesium is precipitated as hydroxide; other elements are kept in solution as complexes formed under controlled *p*H conditions. The analysis may be concluded by absorptiometric, volumetric or gravimetric means.

Reagents—Sodium hydroxide soln., 200 g./litre. Potassium cyanide soln., 300 g./litre. Sodium tartrate soln., 300 g./litre. Tartaric acid soln., 300 g./litre. Bromocresol purple indicator soln., 40 mg. in 100 ml. of ethyl alcohol. 8-Hydroxyquinoline soln., 20 g. in 1 litre of *iso*propyl alcohol. Alcohol-ammonia wash soln., 500 ml. of *iso*propyl alcohol, 480 ml. of water and 20 ml. of ammonia soln. Sodium hydroxide wash soln., 20 g./litre. Pyridine wash soln., 15 ml. of pyridine, 40 ml. of ammonia soln. and 1 litre of water. Ammonium benzoate soln., 100 g./litre. Alizarin Red S soln., 0·10 g. in 100 ml. of glacial acetic acid. Dilute acetic acid, 200 ml. of glacial acid per litre.

Method—I. For the method with *absorptiometric* finish take 0·6 g. of sample if the alloy contains 0·2 to 2·0% of magnesium, and 1·2 g. if the magnesium content is less than 0·2%. Dissolve in 20 ml. of hydrochloric acid (1 + 1) in a 500 ml. conical flask, adding 10 ml. of nitric acid when the first reaction is over. When silicon is present add hydrofluoric acid in 1 to 2 ml. portions, warming after each addition, until the soln. is clear. Evaporate to about 10 ml., add 30 ml. of hot water and boil to dissolve all salts quickly. Add 20 ml. of sodium tartrate soln., boil vigorously, add 20 ml. of tartaric acid soln. and again boil vigorously. Add 8 to 10 drops of bromocresol purple indicator and then sodium hydroxide soln. until a definite purple colour is produced, but avoid excess. Keep the soln. at or near boiling point throughout. Add 10 ml. of potassium cyanide soln., boil for one min., add 30 ml. of sodium hydroxide soln. and boil vigorously until the soln. is perfectly clear. Slowly add 100 ml. of hot water and maintain at the boiling point for 1 to 2 min. Remove the flask from the heat and swirl. After slight cooling the magnesium hydroxide usually appears as fine particles. Heat just below the boiling point for 3 to 5 min. When the ppt. coagulates filter through a Whatman No. 30 or 40 or equivalent paper. Wash the flask and paper thoroughly with hot sodium hydroxide wash soln. Purify the magnesium concentrate by one of the three following methods.

(*a*) Dissolve the ppt. into the flask with 30 ml. of hot dilute acetic acid. Wash the paper with hot water and adjust the soln. to a vol. of 60 to 75 ml. Add 10 ml. of the ammonium benzoate soln., bring just to boiling and leave for 2 min. Filter, and wash with warm water. Bring the volume of the filtrate, which contains the magnesium, to 100 to 125 ml. (*b*) Dissolve the ppt. in 30 ml. of hot diluted hydrochloric acid (1 + 1) and wash with hot water. To the soln. (60–75 ml.) add 2 drops of bromocresol purple soln. and neutralise carefully with ammonia soln. until a faint purple colour is produced. Add 1 ml. of Alizarin Red S soln. and 2 ml. of ammonia soln. Heat to 90° C., remove from the heat and leave until a red ppt. appears. Filter, and wash the precipitate with warm water. (*c*) Proceed as in (*b*) until the purple colour is produced. Add 2 ml. of pyridine and warm to 45° to 50° C. Remove from the heat, shake, and leave for a few min. Filter, and wash the ppt. with pyridine wash soln.

To the magnesium soln., purified by one of the methods (*a*) to (*c*) described above, add 5 ml. of 8-hydroxyquinoline soln. and 1 ml. for each 0·1% of magnesium above 0·5%. (If method (*a*) has been used neutralise the soln. first with ammonia soln. If the magnesium content is small add 2 or 3 ml. of tartaric acid soln. before neutralising.) Add 10 ml. of ammonia soln., boil vigorously, keep hot for 5 to 10 min., leave for a further 5 min., filter on a No. 40 paper and wash the ppt. with alcohol-ammonia wash soln. Dissolve the ppt. in 40 ml. of diluted hydrochloric acid (1 + 1), wash the paper and dilute the soln. to 200 ml. exactly. Take 20 ml., add 10 ml. of diluted hydrochloric acid (1 + 1), dilute to 200 ml. exactly and measure the optical transmission. (A mercury vapour lamp and a narrow band 365 mμ filter are recommended). If the magnesium content is small, omit the second dilution and calculate accordingly. Prepare a calibration curve as follows. Dissolve 0·5 g. of pure magnesium in 50 ml. of diluted hydrochloric acid (1 + 1) and dilute to 1 litre. Take 30 ml. of this soln., dilute to 125 ml., add 20 ml. of 8-hydroxyquinoline soln. and 12 ml. of ammonia soln., precipitate, filter and wash as above. Dissolve the ppt. in 40 ml. of hydrochloric acid (1 + 1) and dilute to 500 ml. Take 2, 4, 6, etc. ml. portions (equivalent to 0·2, 0·4, 0·6%, etc. of Mg on 0·6 g. samples), add 10 ml. of diluted hydrochloric acid (1 + 1), dilute to 100 ml. and measure transmission or optical density.

II. For the method with *volumetric* finish take 0·5 or 1 g. of sample and proceed as in I. up to the oxine pptn. Dissolve the oxine ppt. in 30 ml. of diluted hydrochloric acid (1 + 1), cool, dilute to 125–150 ml. and add 5 drops of carbon tetrachloride. Titrate with standard potassium bromate soln. until the carbon tetrachloride becomes faintly red. Add 10 ml. of potassium iodide soln. and titrate with standard sodium thiosulphate soln. using starch soln. as indicator.

III. For the method with *gravimetric* finish take 0·5–1·0 g. of sample and proceed as in I up to the purification of the magnesium concentrate, using method (*c*) for this. Make the resulting soln. just acid to methyl red with diluted hydrochloric acid (1 + 1). Add 10 to 20 ml. of saturated diammonium phosphate soln. with constant stirring. Add ammonia soln. slowly with continued stirring until no further pptn. is observed and then an excess of about 5% of the original volume. Leave overnight (preferably in an ice-box), filter, and wash with cold water containing 2% of ammonia soln. Ignite finally at 1050° to 1150° C. L. A. D.

Rapid Determination of Iron in Aluminium Alloys. M. S. Pepi (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 111–112)—Absorptiometric measurements are made on the orange coloured soln. produced by the reaction of ferrous iron with 1: 10-phenanthroline. The colour is fully developed 15 min. after addition of the reagent, and is stable for at least 48 hr. The absorption peak is near 490 mμ, and the system obeys Beer's law, for iron contents up to 5%, when the method given below is followed. Most interfering elements are removed in the preparation of the soln., but not more than 10 p.p.m. of zinc should be present.

Reagents—Hydroxylamine hydrochloride soln.: dissolve 10 g. of the pure compound in 100 ml. of water and store in a refrigerator; do not use if the soln. is brown. 1: 10-Phenanthroline soln.: dissolve 0·5 g. of the pure monohydrate in 150 ml. of boiling water, cool, and dilute to 100 ml.; store in a refrigerator and discard if the soln. has a brown colour, which indicates decomposition.

Method—Dissolve 0·5 g. of sample in 30 ml. of diluted hydrochloric acid (1 + 1). Filter through a Whatman No. 41 paper into a 500 ml. graduated flask, wash the paper 5 times with hot water, and dilute the filtrate to 500 ml. Pipette a fraction of the soln. into a 100 ml. graduated flask (10 ml. if the sample contains up to 0·5% of iron, 5 ml. if more iron is present). Add about 70 ml. of water, mix, add 10 ml. of 1: 10-phenanthroline soln., mix, dilute to the mark, mix thoroughly and leave for 15 min. Measure the absorption at 490 mμ and refer to a calibration curve. L. A. D.

Absorptiometric Determination of Copper in Corrosion-Resistant Steel. O. I. Milner (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 94–96)—The blue colour of the cuprammonium ion has been used for many years for the determination of copper, but the method has the disadvantage that the colour, for a given amount of copper, depends on the concentration of ammonia used. It is found, by spectrophotometric measurements, that the amount of ammonia in the soln. influences the wavelength (about 620 mμ) at which absorption is a maximum: but if the measurements are made at a wavelength of 580 mμ the absorption gives an accurate indication of the copper concentration and is not influenced by the amount of ammonia present.

The following rapid method for determining copper in steels is based on this observation. Dissolve 5 g. of sample by warming gently with 100 ml. of acid (600 ml. of water, 150 ml. of conc. sulphuric acid and 250 ml. of conc. hydrochloric acid). Add a few drops of hydrofluoric acid (48%) and boil for several min. Dilute to 300 ml., boil gently, and add carefully 20 ml. of sodium thiosulphate soln. (500 g. in 500 ml. of water) in 3 to 5 ml. portions. Boil until the ppt. coagulates (15 to 20 min.) filter through a coarse paper and wash with hot water. Place the paper and the ppt. (impure copper sulphide) in the flask in which pptn. took place and add 35 ml. of a mixture of 250 ml. of conc. nitric acid and 80 ml. of perchloric acid (70 to 72%). Shake well, and heat gently until the paper and sulphides are decomposed and yellow beads of sulphur remain. Continue heating until dense fumes of perchloric acid are evolved, and then cool. Add 35 ml. of water, neutralise with ammonia soln., add an excess of 2 to 3 ml., and boil for a minute or two, testing with litmus paper to ensure that the soln. remains ammoniacal. (If the steel contains more than 1% of manganese, use a 10 ml. excess of ammonia soln. and add 2 g. of ammonium persulphate to the boiling soln.) Cool to room temp. and filter through a coarse paper into a 100 ml. graduated flask. Add 20 ml. of ammonia soln. to the filtrate, and wash the precipitation flask and ppt. with ammonium nitrate soln. (1%) until the graduated flask is full to the mark. Stopper, and mix the filtrate and washings well. Transfer a portion of the soln. to the absorptiometer and read, using diluted ammonia soln. (1 to 4) to set the instrument. (The apparatus mentioned is a Klett-Summerson Photoelectric Colorimeter, Research model, with a 4 cm. glass cell and a glass filter with maximum transmission at 580 mμ.) Refer to a calibration curve prepared from figures obtained with portions of standard copper nitrate soln. The accuracy and precision of the results are good. The average deviations for National Bureau of Standards sample 73a (14% Cr, 0·080% Cu) and sample 101b (18% Cr, 9% Ni, 0·16% Cu) are about 0·002%. Chromium and nickel, as much as 25% of each in the sample, are not pptd. with the copper sulphide. Some chromium may be present as chromate in the final soln. if some insoluble carbides collected with the copper sulphide are decomposed in the perchloric acid treatment. It is found that 100 mg. of chromium, as chromate, in 100 ml. of ammoniacal copper soln. cause an error less than 0·002% of copper. If desired, the chromium may be removed by reducing it with a few drops of hydrogen peroxide soln. after the perchloric acid treatment, and removing the hydroxide when the soln. is made ammoniacal and filtered. Tests show that if the steel contains 1% of molybdenum and it is assumed that all of it is precipitated with the copper sulphide, no error in the copper value results. Tungsten is mostly removed in the course of the analysis; traces that may remain do not interfere significantly. Under the conditions used, the final solutions obey Beer's law when the copper concentration is not more than 60 mg. per litre. L. A. D.

Physical Methods, Apparatus, etc.

Improvements in the Potentiometric Titration of Chlorides. R. P. Yeck and G. H. Kissin (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 692–3)—The electrode system, which is mounted in a compact unit with the stirrer, consists of a ribbon or wire of high-purity silver and a silver chloride electrode. The latter is constructed of 7 mm. outside-diameter Pyrex tubing and is about half filled with either 0·1 *N* potassium chloride or preferably 1 *N* potassium nitrate saturated with silver chloride. A ground-glass sleeve at the lower end prevents leakage whilst permitting electrical contact with the soln. to be titrated. A silver wire coated anodically with silver chloride extends to near the bottom of the tube. The electrodes are connected to an electronic titrimeter (see, for example, Garman and Droz, *Id.*, 1939, **11**, 398).

Use about 150 ml. of soln. for silver nitrate titration, which is carried out at room temp. The soln. should contain sulphuric, acetic or nitric acid. To avoid dissolution of the silver electrode the concentration of nitric acid should be very low (*p*H 1 to 2), but up to 10% by vol. of sulphuric or acetic acid has been used. Iodides, bromides and silver-reducing substances must be absent, but heavy metal ions need not be removed. Up to the end point the potential of the silver electrode is determined by the solubility of silver chloride, which is approximately constant. A slight excess of silver nitrate causes an abrupt potential change which is indicated by the titrimeter. Standardise

by adding known amounts of chloride to chloride-free material as nearly identical with the sample as possible. J. T. S.

Polarographic Determination of Nickel in Steel and Nickel Ore. P. W. West and J. F. Dean (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 686–8)—The polarographic determination of nickel in steels and nickel ores is rapid and compares favourably in accuracy with gravimetric methods. Use of sodium fluoride as supporting electrolyte prevents interference of iron by pptg. large amounts of the latter and by forming a complex with small amounts. Unless present in disproportionate amounts, cobalt does not interfere. The height of the nickel wave depends upon the *p*H, which should be maintained within ± 0·2 of that used for standardisation. Gelatin soln. is used for suppression of maxima and should be fresh; otherwise the nickel wave may be masked.

Method for steels—To 1 g. of the sample add 50 ml. of diluted hydrochloric acid (1 + 1) and warm until dissolved. Add 5 ml. of conc. nitric acid, boil off oxides of nitrogen, evaporate off most of the free acid and dilute to 50 ml. To a 5-ml. aliquot add 25 ml. of 1 *M* sodium fluoride (the *p*H should then be 4·5 or slightly above) and 1 ml. of 0·2% gelatin soln. Dilute to 50 ml. and filter through a medium-texture paper, discarding the first 10 ml. Deoxygenate a suitable portion of the filtrate by bubbling through it oxygen-free nitrogen and polarograph. Evaluate either by constructing a wave height/concentration curve from known weights of nickel or by adding to a second aliquot of soln. a known weight of nickel and observing the increase in the wave height.

Method for nickel ores—To 0·5 g. of the sample add 25 ml. of diluted hydrochloric acid (1 + 1) and digest for 20 min. (If difficult to dissolve, attack with potassium perchlorate and nitric acid, or fusion with potassium bisulphate may be used.) Evaporate off excess acid, extract with hot water, filter off any silica and dilute to 50 ml. To a 10-ml. aliquot, add 25 ml of 1 *M* sodium fluoride and 0·5 ml. of 0·2% gelatin soln. Dilute to 50 ml., filter and polarograph as above. J. T. S.

Polarographic Analysis of Aluminium Alloys. I. M. Kolthoff and G. Matsuyama (*Ind. Eng Chem., Anal. Ed.*, 1945, **17**, 615–20)—Polarographic determination of iron, copper, lead, nickel and zinc in aluminium alloys is rapid and reliable. In routine analysis the five elements may be determined in less than 45 min. The diffusion current constant of the various metals depends upon the concn. of aluminium and must be determined in a supporting electrolyte containing approximately the same amount of aluminium as the unknown. In the determination of iron, chloride interferes and, since ferric iron oxidises mercury, a polarographic cell with an external reference electrode should be used. In aluminium alloys, iron and copper generally greatly exceed lead in amount; to prevent interference in the determination of lead, iron may be reduced to the ferrous state and copper pptd. as the thiocyanate. Alternatively, lead may be determined along with zinc after dithizone extraction. Nickel and zinc may be determined rapidly if citrate is added to the soln. after eliminating interference due to iron and copper. Alternatively, nickel may be determined after electrolysis, whereby copper is removed and ferric iron is reduced. When the ratio of nickel to zinc is large, separation is necessary. This may be effected by dithizone extraction followed by re-extraction with hydrochloric acid, whereby lead and zinc are separated from other metals.

Method:—To 1 g. of drillings add slowly 11 ml. of 15% sodium hydroxide soln., completing the reaction by heating. Add 20 ml. of diluted nitric acid (1 + 1), stir, boil off oxides of nitrogen and dilute to 50 ml. (Solution A). Using aluminium in place of the alloy, prepare Solution B_g in the same way.

(1) *Iron and Copper*—(*a*) To 10 ml. of Soln. A add 0·5 ml. of 0·5% gelatin soln. and dilute to 25 ml. Deoxygenate by bubbling with nitrogen, and polarograph or measure the diffusion currents at + 0·15 and − 0·15 volt (all potentials are versus the saturated calomel electrode). If the total current exceeds about 20 microamp., use less Soln. A, making up to 10 ml. with Soln. B. Correct for the residual current by replacing Soln. A by Soln. B and compare the corrected diffusion current at + 0·15 volt with that of a standard, to obtain the amount of iron. Subtract the corrected diffusion current at + 0·15 volt from that at − 0·15 volt and similarly obtain the amount of copper. (*b*) If the concn. of iron is more than about ten times that of copper, determine the latter as follows. To 10 ml. of Soln. A add 0·3 ml. of 2 *M* hydroxylamine hydrochloride, boil, cool, add 0·5 ml. of 0·5% gelatin soln. and dilute to 25 ml. Deoxygenate for 5 to 10 min., measure the current at + 0·15 and − 0·15 volt respectively and obtain the amount of copper as above.

(2) *Lead in presence of much copper and iron*—(*a*) To 10 ml. of Soln. A add 1 drop of 0·1% thymol blue and 1 *M* sodium hydroxide until orange, and then add 1 ml. of the alkali in excess. Add 0·5 ml. of 2 *M* hydroxylamine hydrochloride and 0·5 ml. of 2 *M* potassium thiocyanate. Shake gently until the red colour disappears, wash down the walls of the flask, add 0·5 ml. of 0·5% gelatin soln. and dilute to 25 ml. Deoxygenate and polarograph from − 0·2 to − 0·6 volt or measure the current at − 0·3 and − 0·5 volt. Correct for the residual current and obtain the amount of lead by comparison with a standard. (*b*) Alternatively, proceed as in (5) below.

(3) *Rapid method for nickel and zinc*—To 15 ml. of Soln. A add 5 ml. of 1 *M* sodium hydroxide and mix to redissolve the ppt. Add 0·70 ml. of 2 *M* hydroxylamine hydrochloride and 0·50 ml. of 2 *M* potassium thiocyanate and rinse down the neck of the flask. Shake until the red colour disappears and then add 5 ml. of 1·25 *M* sodium citrate. Mix thoroughly, add 0·50 ml. of pyridine and 2 drops of 0·1% bromocresol green and then 15% sodium hydroxide soln. until the colour is a distinct green (*p*H 4·5). Add 1 ml. of 0·5% gelatin and dilute to 50 ml. Deoxygenate, polarograph from − 0·4 to − 1·4 volt and determine nickel and zinc by comparing the diffusion currents with those of standards.

(4) *Separation method for nickel*—Electrolyse a suitable aliquot of Soln. A to remove copper and to reduce iron. Add 10 ml. of pyridine, dilute to 50 ml. and shake vigorously. Filter through a dry, coarse paper (*e.g.*, S. & S., No. 589 "Black Band"). To 20 ml. of the filtrate add 0·5 ml. of 0·5% gelatin soln. and dilute to 25 ml. Deoxygenate, polarograph between − 0·6 and − 1·0 volt and determine nickel by comparing the diffusion current with that of a standard.

(5) *Separation method for lead and zinc*—For most alloys treat 15 ml. of Soln. A with 0·5 ml. of 2 *M* hydroxylamine hydrochloride and 0·5 ml. of 2 *M* potassium thiocyanate (the pptd. copper thiocyanate need not be removed). For alloys low in zinc, dissolve 1 g. in 25 ml. of hydrochloric acid of

constant b.pt. and filter. Add thymol blue and 10 ml. of saturated sodium citrate soln. and then ammonia until greenish-yellow. Transfer to a separator with an ungreased stopcock, add 10 ml. of 0·05% dithizone in carbon tetrachloride and shake for 1 to 2 min. (A bright cherry red carbon tetrachloride layer shows appreciable amounts of lead and zinc.) Run off the latter layer and extract the aqueous layer with further 10-ml. portions of dithizone soln. until the latter remains green or becomes brownish purple; then extract once more. (A brownish-purple scum of nickel dithizonate may form; do not run this off.) Shake the aqueous layer with 2 small portions of carbon tetrachloride and add these to the combined extracts. To the latter add 25 ml. of water containing 1 drop of ammonia, shake and run off the carbon tetrachloride layer. Shake the ammonia soln. with 2 small portions of carbon tetrachloride and add these to the main extract. Shake the latter for 2 to 3 min. with 20 ml. of 0·1 *M* hydrochloric acid and run off the carbon tetrachloride layer (which will have turned green if no copper is present). Rinse the hydrochloric acid layer with 2 small portions of carbon tetrachloride and add these to the main carbon tetrachloride layer, leaving any scum and droplets of carbon tetrachloride in the separator. Shake the carbon tetrachloride layer for 2 to 3 min. with 10 ml. of 0·1 *M* hydrochloric acid, rinse the latter with 2 small portions of carbon tetrachloride and then add it to the previous 20 ml. of hydrochloric acid soln. Discard the carbon tetrachloride phase. Shake the combined hydrochloric acid soln. with several small portions of chloroform until the latter remains colourless. Boil the soln. to expel chloroform, cool, add 1 ml. of 0·5% gelatin soln. and 1 ml. of pyridine and dilute to 50 ml. De-oxygenate and polarograph between −0·2 and −0·6 volt to determine lead and between −0·8 and −1·2 volt to determine zinc. Compare the diffusion currents with standards prepared by diluting known amounts of lead and zinc solns., 30 ml. of 0·1 *M* hydrochloric acid 1 ml. of pyridine and 1 ml. of 0·5% gelatin soln. to 50 ml. J. T. S.

Reviews

AN INTRODUCTION TO THE CHEMISTRY OF CELLULOSE. By J. T. MARSH and F. C. WOOD. 3rd Edition. Pp. 525. London: Chapman & Hall, Ltd. 1945. Price 32s. net.

The Preface to the new edition of this now well-known work emphasises the growing importance of its subject. However, the authors do not need to justify this continuation of their work, because the standard set by the previous editions was a high one and it is well maintained in the present instance. The Preface also emphasises the abnormal conditions of the four years elapsing since the last edition. These have made themselves felt in two ways. There has been the inevitable effect on the availability of the world's scientific literature on the subject. This is to some extent a disadvantage, but a more beneficial result is the widening of the background of knowledge behind the book. By way of example, in a review of the last edition the present writer commented on the fact that the subject was treated almost exclusively from the viewpoint of the textile chemist, whereas other aspects, and particularly that of paper manufacture, are of equal importance, even though the literature of this branch of the subject is less abundant. This limitation has to a great degree been remedied in the present edition. Thus, the usual commercial methods for pulping and bleaching wood for paper manufacture are described briefly, and there is also a short section on beating. Five pages are devoted to nylon, although it is not a cellulosic material; this is interesting, because not only is nylon a possible rival to rayon for use in fabrics, but also recent researches on its structure have an important bearing on the general problems of chain length and molecular weights. These points are mentioned to illustrate the breadth of treatment and up-to-date character of the new edition.

The general scope of the book is indicated by the headings of the five main sections. After two chapters on the occurrence and general properties of cellulose, its constitution, molecular weight and molecular structure are dealt with at length. Subsequent sections are concerned with dispersed and modified celluloses, and the commercial and other derivatives of cellulose provide the subject of the last ten chapters, the longest section of the book.

Analysts will be interested in the testing methods described. The book does not of course pretend to be a treatise on the analytical side of the subject, but where such treatment is called for it is adequate. A good deal of space is given to viscosity determinations and their applications. The classical method of Clibbens and Geake receives due prominence, but the recent cupriethylene-diamine method, which offers the great advantage of rapidity for routine testing, is also dealt with. A number of useful qualitative tests, which as a rule are not easy to find in the voluminous literature of the subject, are also described. These include tests for mercerisation (based principally on dyestuff affinity tests), for oxycellulose and for hydrocellulose. Applications of X-ray methods are also dealt with. Two useful tables show the effects of degradation and of the swelling or "activation" of cellulose on its physical properties and chemical reactions.

The book is well illustrated and documented, with adequate indexes and a list of patent specifications. It can be recommended thoroughly. JULIUS GRANT

MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY. By E. J. KING, M.A., Ph.D. Pp. vii + 169. London: J. & A. Churchill, Ltd. 1946. Price 10s. 6d.

Micro-methods of chemical analysis have gained much ground in recent years, and in many laboratories they have ousted macro-methods almost completely. Nor is this surprising, for the resulting economy in time and materials—to name but two of the advantages—is considerable. In general, however, pathologists and medical biochemists (not "pure" biochemists, who indeed have been pioneers in the scaling-down process) have lagged behind and tended to retain the older techniques. This is a Bad Thing, for in their case there is a further special benefit to be gained by using micro-methods, in blood analysis at any rate,—one which would undoubtedly have led to their adoption long ere this if only it were compulsory for pathologists and their assistants, as part of their training, to undergo as temporary "patients" the procedures they will in due course carry out on others. The results would be salutary in many ways; and in particular, it would be realised that any patient would prefer to be pricked with a needle three times than to have a venipuncture once!

Because the ability to look at experimental techniques from the guinea-pig's angle is rare, Dr. King deserves credit for making this very point, amongst others, in the preface to his admirable book, based on a series of papers from his laboratory which have been published during the last fifteen years. The methods advocated are largely colorimetric (or more precisely, absorptiometric) and as such are well suited to the needs of the pathological laboratory, giving adequate accuracy for diagnostic purposes with a minimum of trouble. One method (there are no alternatives) is given for each of the estimations usually called for on whole blood, plasma, serum, cerebro-spinal fluid, faeces and urine, and procedures are included for the identification of calculi, gastric analysis, function tests, determination of pH, and spectroscopic tests. Each method is preceded by brief notes on the chemical principles involved and the use of the results obtained in diagnosis. A chapter on colorimeters includes full constructional details of a simple but reliable photo-electric instrument of the one-cell type designed by the author (*Lancet*, 1942, i, 511). All the techniques are described very clearly and succinctly; no misprints were detected, and the only point of detail noticed at all open to criticism is the use, in the determination of non-protein nitrogen in whole blood, of the same standards as in the determination of urea, since these contain 1 ml., instead of 3 ml., of Nessler's solution (and may contain gum ghatti) and are not therefore strictly comparable with the test solution.

Since it is a well-established practice for reviews to be made an excuse for the riding of personal hobby-horses, the present reviewer may perhaps be permitted to bring out one of his own particular pets and parade him for a few lines. The first chapter of Dr. King's book is entitled "Normal Values," and lists the figures to be expected in health for each of the estimations described in the succeeding chapters. In judging the diagnostic significance of a result, the normal value is clearly crucial. Yet—and it is a sad reflection on the present state of knowledge in this branch of science—the best that can apparently be done is to list the range within which the normal values usually lie. Perhaps in some future edition Dr. King may be able to replace this inefficient, misleading, and anachronistic method of expressing information by a table of means and standard deviations. Before this can be done, however, three conditions must be satisfied. First, all pathologists must agree to use the methods detailed in this book and then carry out many series of estimations on large numbers of healthy subjects. Second, the results obtained must be collected and submitted to proper statistical analysis. Third, the pathologists and others who will use the book must learn as an essential part of their education sufficient statistics to understand the meaning and use of the data in their revised form. In other words, the same process must be gone through in this branch of biochemistry as has already taken place in many other branches. Until then, one can only break a lance in the cause of statistics and then return one's steed to the stable.

These musings must not be allowed to detract in any way from the excellence of Dr. King's book. It deserves to become an essential part of the equipment of any laboratory which carries out either so many biochemical estimations that it needs procedures which are standardised, simple, speedy, and unqualified-assistant-proof, or so few that it needs clear and detailed descriptions of methods which do not call for special reagents or unusual apparatus and can be relied upon to work first time. Seldom in these days of vanishing money-values can half-a-guinea be so well spent.

ERIC C. WOOD

COMPILATION OF A BIBLIOGRAPHY OF PUBLISHED APPROVED AND STANDARD METHODS OF ANALYSIS

IN THE ANALYST of April, 1945, the formation by the Analytical Methods Committee of a Standard Methods Sub-Committee under the chairmanship of Mr. G. Taylor was announced and those interested were requested to communicate with the Hon. Secretary, Dr. D. W. Kent-Jones, 88, Madeley Road, Ealing, London, W.5.

The Sub-Committee, working under the general direction of the Analytical Methods Committee, has undertaken as its first task the compilation of a Bibliography of published approved and standard methods, and for this purpose a list of subjects broadly based on the classification as used in *British Abstracts* has been adopted. The Sub-Committee is therefore dealing with some fifty subjects, and of these about one-third are already being surveyed by various helpers. These cover:

I.A.i. Elements, in order of periodic table.
I.A.ii. Metals, commercial, ferrous, non-ferrous.
I.B.iii. Coal, coke and other solid fuels.
I.B.iv. Coal gas, tar and tar products.
I.B.v. Petroleum, lubricants, asphalte and natural gas.
I.B.vii. Feeding stuffs, soils and fertilisers.
II.B.i. Cellulose, cotton and paper.
II.B.ii. Leather and glue.
II.B.iii. Rubber and allied substances.
II.B.iv. Fatty oils and soaps.
II.B.viii. Sugars, starches and gums.
II.B.ix. Cereals and confectionery.
II.B.xi. Dairy products.

Tentative arrangements with respect to another eight subjects are being negotiated:

I.B.ii. Cement, clay and building materials.
I.B.ix. Water, sewage and sanitation.
II.B.v. Essential oils and spices.
II.B.x. Meat and meat products.
II.B.xii. Fermentation products.
II.B.xv. Vitamins.
Dyes.
Wood.

The Sub-Committee appeals for help with the other subjects, and especially with respect to:

I.B.i. Acids, alkalis, salts and heavy chemicals.
I.B.viii. Antiseptics, disinfectants and insecticides.
I.B.xi. Industrial gases.
I.B.xii. Glass enamels, ceramics and refractories.
III.A.i. General physico-chemical methods.
III.A.ii. Thermal methods.
III.A.iii. Optical methods.
III.A.iv. Electric methods.
III.A.v. Sub-atomic methods.

Anyone who is prepared to help, or wishes to have more information, is invited to communicate with the Hon. Secretary of the Sub-Committee, Dr. D. W. Kent-Jones, who will be pleased to forward an example of the way the arrangement of information should be set out.

It is realised that the task undertaken is a difficult one, but its value to analysts generally will be considerable. It is hoped therefore that this appeal will meet with response.

Photograph by courtesy of I.C.I. (Explosives) Ltd

The advance of micro-chemical technique has been facilitated by the reliability and handiness of Oertling micro and semi-micro balances

For example: The No. 55 PR Micro Chemical Balance shown gives direct readings in thousandths of a milligram up to 0·1 mg., plus or minus, without the use of riders or fractional weights.

L. OERTLING, LTD.,
110, GLOUCESTER PLACE, LONDON, W.1
(Near Baker Street Station)
'Phone: WELbeck 2273.

Oertling
FOUNDED IN LONDON BEFORE 1847

TAS/OR.242

Volume 71 June, 1946

No. 843, Pages 251—300

Contents

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

Printed for the Society of Public Analysts and other Analytical Chemists by W. Heffer & Sons Ltd., Hills Road, Cambridge. **Communications to be addressed to the Editor, J. H. Lane, 7-8, Idol Lane, London, E.C.3.**