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THE ANALYST

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

DEATH

WE deeply regret to have to record the death of Ernest Gabriel Jones.

Obituary

GEORGE DAVIDSON ELSDON

ELSDON died very suddenly at his home in Manchester on January 18th, 1945, at the age of 56. He had complained to his wife earlier in the day of feeling unwell and he passed away very shortly afterwards, before a doctor could be summoned.

George Davidson Elsdon graduated as B.Sc., with honours in Chemistry, at Birmingham University, where he continued under Professor Frankland as a Priestley Research Scholar until 1910. He passed the Institute of Chemistry examination in Branch E in 1912, and was elected a Fellow in 1915. Subsequently he proceeded to the D.Sc. of his University.

He began his professional career as assistant to Mr. F. H. Alcock, of Birmingham, and was then for a short time an assistant to Mr. J. F. Liverseege, Public Analyst for Birmingham. From 1912 to 1914 he was lecturer in chemistry at the Walsall Technical School. In 1914 he was appointed Public Analyst and Agricultural Analyst for the County Borough of Salford, from 1926 to 1937 he was Public Analyst for the County of Lancaster, and in 1937 he became Chief Inspector to the Lancashire Rivers Board, which post he held at the time of his death.

The foundations of Elsdon's success were undoubtedly laid when he was assistant to Liverseege and later as Public Analyst for Salford, but his best work was done when Analyst for the County of Lancaster. Here he carried out his most valuable work on the freezing-point of milk, work with which his name will always be associated. From his laboratory came also several other excellent and practical papers of which he was the inspiration.

His book *"The Chemistry and Examination of Edible Oils and Fats"* is well known, and he was joint author with Mr. Norman Evers of *"The Analysis of Drugs and Chemicals."* He also collaborated with Dr. G. H. Walker in revising and bringing up to date that classic work *"Richmond's Dairy Chemistry."*

Elsdon was an enthusiastic and active member of the North of England Section of our Society, particularly at its inception, and was an original member of the Committee. He was a most popular Chairman of the Section in 1930 and a staunch supporter of its very successful summer meetings.

He also gave his loyal support to the Institute of Chemistry, serving two periods as a Member of Council, and in 1937 he became Examiner in Branch E.

Elsdon had very many friends but perhaps not many intimate friends, for he was somewhat difficult to get to know intimately; but all with whom he came into contact had the highest regard for him and for the excellence and integrity of his work. An outstanding characteristic was his charming personality and his diffidence. He need have had no such diffidence, for all knew the value of his work. His friends will greatly deplore his early death.

W. GORDON CAREY

Rapid Methods for the Determination of Phosphine and Hydrogen Sulphide as Impurities in Acetylene Generated from Commercial Calcium Carbide

BY V. BRAMELD

THE impurities in acetylene from commercial calcium carbide scheduled for determination in British Standard Specification for Carbide of Calcium (No. 642—1935) are phosphine and hydrogen sulphide. The methods detailed therein involve the oxidation of the impurities with sodium hypochlorite soln. saturated with sodium bicarbonate, followed by determination

of the phosphate volumetrically as phosphomolybdate and of the sulphate gravimetrically as barium sulphate. Both methods are very slow and a need was felt for speedy yet accurate methods, which could be used for routine analysis involving several samples at a time. With this end in view, attention was turned to the possibility of determining the phosphate colorimetrically as the blue phosphomolybdate reduction compound and the sulphate turbidimetrically as barium sulphate. A Hilger's Spekker Photoelectric Absorptiometer was used for the colour and turbidity absorption measurements.

The phosphate determination is based on Denigès'[1] original work on the blue reduction complex of phosphomolybdate. Whereas Denigès used sulphuric acid, hydrochloric acid was found more satisfactory. The ammonium molybdate and stannous chloride reagents were also modified to suit the conditions prevailing in this instance. The method of formation of a stable barium sulphate suspension is based on work by Parr and Staley[2] and by Sheen, Kahler and Ross,[3] in connection with the determination of sulphate, using different forms of turbidimeters.

The chief difficulty to be overcome before phosphate and sulphate could be determined absorptiometrically was that of removal of excess of sodium hypochlorite. Boiling the solutions down to dryness with acid was found satisfactory in the determination of sulphate but not in that of phosphate, for which widely inconsistent results were always obtained. The removal of excess of sodium hypochlorite, prior to determination of the phosphate, had thus to be accomplished in other ways.

PROCEDURE—To obtain a suitable light absorption range for the "Spekker," use a carbide sample of 70 to 80 g for the acetylene generation. Pass the impure acetylene through absorption bulbs containing 150 ml of sodium hypochlorite sclution (2·5% of available chlorine), with addition of 1 g of sodium bicarbonate for every 100 ml of soln. used. When the carbide decomposition is complete empty the absorption bulbs into conical flasks, rinsing out the bulbs with distilled water. Boil off dissolved acetylene, cool and make up to 250 ml in standard flasks. Use these solns. containing the acetylene impurities in the form of phosphate and sulphate for the subsequent determinations.

PHOSPHINE—*Reagents*—(*a*) 10% soln. of urea in water; (*b*) 2·85 g of AnalaR stannous chloride ($SnCl_2 2H_2O$) in 30 ml of conc. hydrochloric acid—this soln. must be made up weekly; (*c*) solid AnalaR potassium iodide; (*d*) approx. *N*/10 sodium thiosulphate; (*e*) approx. *N*/5 sodium hydroxide; (*f*) standardised *N* hydrochloric acid; (*g*) 2·5% soln. of AnalaR ammonium molybdate, $(NH_4)_6Mo_7O_{24}4H_2O$, in water. For use in the determination dilute 1 ml of the stock soln. with 4 ml of water. This soln. must be used almost immediately.

Method—Take by means of a precision pipette 1-ml aliquots from the 250 ml bulk of hypochlorite soln. Place in 100-ml conical flasks, add 4 drops of conc. hydrochloric acid and 5 to 10 ml of water, and boil steadily on a hot-plate for 5 to 10 min. On no account allow the solns. to boil to dryness; if there is any danger of this happening, add more water and continue the boiling for the specified time. Remove the flask from the hot-plate and cool. Add 5 ml of the 10% urea soln., mix well, add 0·5 g of solid potassium iodide, dissolve and mix well. Titrate any liberated iodine with approx. *N*/10 sodium thiosulphate, and add three drops in excess. Using phenolphthalein solution as indicator, add just sufficient approx. *N*/5 sodium hydroxide to produce a definite pink colour. Add 15 ml of *N* hydrochloric acid, make up to 50 ml with water and place the flasks containing the treated solns. in a thermostatic bath at 25° C. Allow 5 min. for the solns. to attain the bath temperature. Add 2 ml of the ammonium molybdate soln., mix well and then add immediately 0·15 ml of the diluted stannous chloride soln. Allow 5 min. for maximum colour development. Remove from the thermostatic bath and obtain a drum reading on the "Spekker," using 1-cm cells; water-water setting 0·90; Ilford Spectrum Red Filters 608. At the same time run a blank on a proportionate amount of the sodium hypochlorite soln. used for the oxidation and treat exactly as outlined above. Obtain a drum reading on the "Spekker." This gives a blank for the sodium hypochlorite soln.

Convert the difference between these drum readings, for the sample and the blank, to ml of phosphine at 15° C. and 760 mm from a graph obtained from solns. of known (AnalaR) phosphate content, containing also appropriate amounts of sodium hypochlorite and sodium bicarbonate and treated exactly as outlined above.

$$PH_3, \% \text{ by vol.} = \frac{27{\cdot}78 \times \text{ml of } PH_3 \text{ at } 15^\circ \text{ C. and } 760 \text{ mm (from graph)}}{\text{Weight of dry acetylene evolved from sample (g)}}$$

Discussion of Method—It is important that the solns. should not boil to dryness in the initial stage of the decomposition of the sodium hypochlorite, since if this happens the phosphate, originally present as orthophosphate, is converted into meta- or pyro-phosphate. In these two latter forms the phosphorus gives no colour reaction with ammonium molybdate and stannous chloride. Thus low and variable results are obtained. Prolonged boiling in acid soln. converts meta- and pyro-phosphates into orthophosphate, but this adds considerably to the time needed for a determination and, as the aim is to keep the time down to a minimum, the alternative procedure, given above, is adopted.

Addition of urea has the effect of inhibiting the decomposition of the excess of thiosulphate at the acid concn. necessary for the suppression of any blue colour due to reduction of ammonium molybdate alone. In absence of urea, decomposition of the thiosulphate results in pptn. of sulphur at this acid concn.

Quantitative tests with standard phosphate solns. showed that some latitude was permissible in the final acid, ammonium molybdate and stannous chloride concns. Concordant "Spekker" readings were obtained when these reagents were varied independently over the following concn. ranges:

Hydrochloric acid ..	$27 \times 10^{-2} N$ to $30 \times 10^{-2} N$
Ammonium molybdate	$78 \times 10^{-5} M$ to $94 \times 10^{-5} M$
Stannous chloride ..	$7 \times 10^{-5} M$ to $19 \times 10^{-5} M$

Quantitative measurements of the rate of colour development showed that under the conditions used the colour reached a maximum intensity 2·5 min. after addition of the reducing reagent and remained constant in intensity up to 10 min., after which fading set in. The rate of colour development is appreciably dependent on the temperature at which the reduction proceeds; 25° C. was arbitrarily taken as a convenient temperature.

Discussion of Results—A standard soln. of AnalaR potassium dihydrogen phosphate standardised by means of gravimetric determination of a suitable aliquot. Test solns. were prepared from this standard solution and given to several operators, of varying degrees of skill, for determination of the phosphate contents. The saving in time required was most marked and the maximum deviations in duplicate determinations were rarely more than one "Spekker" drum division (0·01 on the drum) corresponding to ±0·001% of phosphine (see Table I).

TABLE I

Phosphine, % by vol.

	Found by operator				
Present	1	2	3	4	5
0·011	0·011	0·011	0·011	0·011	0·011
0·022	0·022	0·021	0·022	0·022	0·022
0·033	0·034	0·032	0·033	0·033	0·033
0·044	0·044	0·043	0·043	0·043	0·043
0·055	0·055	0·054	0·055	0·056	0·054
0·066	0·066	0·065	0·066	0·067	0·063

Comparison with the volumetric method for the determinates of phosphate gave equally satisfactory results (see Table II).

TABLE II

Phosphine, % by vol.

Volumetric method		Absorptiometric method	
Present	Found	Present	Found
0·006	0·006	0·006	0·006
0·011	0·012	0·011	0·011
0·023	0·023	0·023	0·022
0·034	0·035	0·034	0·033
0·056	0·060	0·056	0·056
0·079	0·083	0·079	0·078

HYDROGEN SULPHIDE—*Reagents*—(*a*) AnalaR barium chloride (dihydrate), 20/60 mesh; (*b*) acid-salt soln: (1) 20 ml of AnalaR conc. hydrochloric acid and (2) 240 g. of AnalaR sodium chloride—made up to 1 litre with water; (*c*) approx. *N*/5 sodium hydroxide; (*d*) 0·5% 2:4-dinitrophenol in 50/50 water-alcohol solvent.

Method—Take by means of a precision pipette 10-ml aliquots from the 250 ml of hypochlorite soln., and put them in 100-ml conical flasks. Add 1 ml of conc. hydrochloric acid

and evaporate the solns. to dryness, taking care to avoid losses by spirting. Leave on the hot-plate for 5 min. after the disappearance of the last visible traces of liquid. Remove from the hot-plate, allow to cool, and add 10 to 20 ml of water and 4 drops of conc. hydrochloric acid and replace on the hot-plate. Boil for 5 to 10 min. but *not* to dryness this time. Remove from the hot-plate, cool, and, using 5 drops of 2:4-dinitrophenol soln. as indicator, add just sufficient approx. $N/5$ sodium hydroxide to obtain a definite gold-yellow colour. Make up to 50 ml with water and add 10 ml of the acid-salt soln. Mix well and add 0·35 g of the solid barium chloride by means of a standardised glass measuring spoon. Mix with a swirling motion for one min. exactly and leave undisturbed for 4 to 6 min. Obtain a drum reading on the "Spekker," using two 1-cm cells in series; water-water setting 1·30 and Ilford Spectrum Violet Filters 601. Obtain a blank on the appropriate amount of sodium hypochlorite soln., containing sodium bicarbonate, treated in exactly the same manner.

Convert the difference between these drum readings to ml of hydrogen sulphide at 15° C. and 760 mm from a graph obtained by using known standardised solns. of AnalaR sodium sulphate, containing the appropriate amounts of sodium hypochlorite and sodium bicarbonate, treated in exactly the same manner as described above.

$$\text{Hydrogen sulphide, \% by vol.} = \frac{2{\cdot}778 \times \text{ml of } H_2S \text{ at } 15^\circ \text{ C. and } 760 \text{ mm (from graph)}}{\text{Weight of acetylene evolved from sample (g)}}.$$

Discussion of Method—The first baking down to dryness with acid results in decomposition of the sodium hypochlorite, but at the same time the orthophosphate, also present, is converted into meta- and pyro-phosphate. In the form of metaphosphate it exerts the well-known "threshold effect"; *i.e.*, it partly inhibits the pptn. of the barium sulphate in the later stages of the determination. The second boiling with dil. acid for 5 to 10 min. sufficiently re-converts the metaphosphate into orthophosphate to overcome interference from this source.

The acid-salt soln. acts as a fairly strong acid buffer, thus providing identical acidity conditions in consecutive determinations. The ppt. formed under these specified conditions is so fine that it settles very slowly and thus no difficulty is experienced in measuring the light absorption due to the turbidity of the solutions.

TABLE III

Hydrogen sulphide, % by vol.

Operator 1		Operator 2	
Found	Present	Found	Present
0·013	0·013	0·014	0·013
0·040	0·041	0·019	0·019
0·058	0·057	0·033	0·033
0·079	0·080	0·032	0·033
0·085	0·085	0·041	0·041
0·120	0·120	0·055	0·054
		0·086	0·081
		0·111	0·111

Results—Using prepared solns. of standardised sulphate content, very good results were obtained by this procedure. The elimination of the tedious processes of weighing was of great advantage for routine purposes. As in the determination of phosphate, using the "Spekker," different operators of varying degrees of skill obtained results in duplicate determinations agreeing in most of the tests within ±1 "Spekker" drum division (0·01 on the drum) corresponding to an accuracy of ±0·001% of hydrogen sulphide (see Table III).

Comparison was also made with the gravimetric method for the determination of sulphate as laid down in British Standard Specification for Carbide of Calcium (No. 642—1935), with equally satisfactory results (see Table IV).

It is notable that up to 0·025% of hydrogen sulphide the gravimetric figures are lower than the absorptiometric figures. Above 0·025% of hydrogen sulphide the reverse holds true. This is presumably due to errors inherent in the gravimetric method, *viz.*, solubility error, due to the ppt. not being completely insoluble, and the adsorption error, due to adsorption of ions by the barium sulphate during pptn. These two errors are in opposite directions. The solubility error will obviously produce low results and be more marked when the weight of ppt. is small, whereas the adsorption error will produce high results and be more marked as the weight of ppt. becomes large. Somewhere in the region of 0·025% hydrogen sulphide, under the conditions of pptn. used in the British Standard Specification, these two errors

become equal, the solubility error being the predominant factor below this value and the adsorption error being predominant above it.

The absorptiometric method will not be affected by the adsorption error at all, and, since the instrument is primarily calibrated with solns. of standard sulphate content, under carefully standardised conditions the solubility error is automatically compensated for during calibration.

TABLE IV

Hydrogen sulphide, % by vol.

Absorptiometric	Gravimetric	Absorptiometric	Gravimetric
0·004	0·004	0·024	0·019
0·010	0·007	0·035	0·040
0·013	0·011	0·036	0·037
0·014	0·014	0·037	0·039
0·017	0·015	0·048	0·050
0·019	0·018	0·052	0·055
0·022	0·018	0·059	0·064

CONCLUSION—Both of these methods have been in daily use in these Laboratories for a considerable period. They have proved very satisfactory for the routine determination of impurities in acetylene from commercial calcium carbode. A considerable saving in time has been effected (see Table V), while correct results have been assured owing to the ease with which determinations can be carried out in duplicate or triplicate.

TABLE V

Phosphine. Time saved	
Time required for single volumetric determination	2 hr.
,, ,, ,, ,, absorptiometric ,, ..	¾ ,,
,, ,, ,, set of ten volumetric determinations ..	6 ,,
,, ,, ,, ,, ,, ,, absorptiometric ,, ..	2 ,,
Hydrogen sulphide. Time saved	
Time required for gravimetric determination. At least ..	24 ,,
,, ,, ,, absorptiometric ,, (set of 10) ..	2 ,,

The saving in consumption of chemicals, especially ammonium molybdate and nitric acid, is important. Further, it is a relatively simple matter to train unskilled operators, in these methods, for routine purposes.

I wish to thank The British Oxygen Co., Ltd., and my Chief, Mr. C. Coulson-Smith, M.Sc., F.R.I.C., for permission to publish this paper.

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The Theory and Practice of the Microbiological Assay of the Vitamin-B Complex; together with the Assay of Selected Amino Acids and Potassium

BY E. C. BARTON-WRIGHT

(*Based on a Lecture delivered on May* 16, 1945, *before the London and South Eastern Section of the Royal Institute of Chemistry, to whom the Society is indebted for permission to publish here*)

IN 1906 Dr. (now Sir) Frederick Gowland Hopkins, in a lecture to the Society of Public Analysts, said: "In the future the analyst will be asked to do more than determine the total protein of a foodstuff; he must essay the more difficult task of a discriminate analysis." Truly a remarkable prophesy, because this very thing has come to pass to-day.

I cannot attempt to review the whole field of vitamin chemistry and will therefore confine my remarks to a small and limited section of a large and ever-growing subject, *viz.*, the components of what is now generally known as the vitamin-B complex. As the chemistry and biochemistry of these substances have already been lucidly discussed by Robinson,[1] I will merely give a brief review of the vitamin and amino acid requirements of certain bacteria

and yeasts. To-day, this is a subject of great practical importance because certain of these organisms can be used for the quantitative determination of the known and characterised components of the vitamin-B complex, for the assay of about 15 or 17 amino acids, including the "essential" amino acids, and for determining potassium.

Besides the known components (*i.e.*, those compounds which have been isolated in the pure condition in the laboratory and whose structure has been confirmed by synthesis), the vitamin B complex includes the following uncharacterised components.

Folic Acid—This was first isolated from spinach leaves; hence the name. It is possible that there is more than one folic acid. It is a stimulant for certain bacteria, notably *Lactobacillus helveticus*, *Streptococcus faecalis* (*lactis*) R, *Leuconostoc mesenteroides* and *Lactobacillus fermentum* 36.

Vitamin B_c—This has now been isolated in the crystalline condition from liver and yeast. It is probably identical with folic acid and is a powerful growth stimulant for the bacteria mentioned above.

Vitamin B_{10} and B_{11}—Little is known about these substances. It was at one period considered that they might be the same as vitamin B_c, but more recent evidence seems to indicate that they are different.

*Factors HL*1, *HL*2, *HL*3, *HL*4—These factors were isolated from liver and examined by Robinson, Emery and myself.[2] They stimulate the growth of the lactic organisms *Lactobacillus helveticus* and *Streptococcus faecalis* R. It is probable that factor HL2 is identical with folic acid, but the remainder are certainly different. For example, they are soluble in chloroform, whereas folic acid is said to be insoluble in all organic solvents except glacial acetic acid. Moreover, the activity of folic acid is destroyed by acetylation, benzoylation and the action of nitrous acid, whereas these three factors are unaffected.

As regards the assay of the vitamin-B complex, there are at present only three members that need concern the analyst, *viz.*, aneurine, riboflavin and nicotinic acid or nicotinamide. Under the labelling order of the Ministry of Food (S.R. & O., 1944, No. 738) manufacturers must now disclose the amounts of these substances in their products if their presence is claimed.

These three vitamins are essential for normal human metabolism. They are all components of enzyme systems concerned in important oxidation-reduction reactions and reference may be made to Robinson's paper for details of how these enzyme systems function in cellular metabolism.

The function of pantothenic acid and pyridoxine in the human economy is not known at present. They are probably, almost certainly, essential factors, and it is equally probable that they are components of enzyme systems. Pyridoxal, which is chemically very closely related to pyridoxine, is a component of an enzyme system concerned in the decarboxylation of the amino acid tyrosine in the bacterial organism *Streptococcus faecalis* R and is very much more efficient in this respect than pyridoxine.[3] Pyridoxine is an essential factor for the pupation of certain insects,[4] notably in the genus *Tribolium*. Of all the components of the vitamin-B complex, however, biotin is probably by far the most interesting because it is required in such minute amounts and can be detected in as low a concn. as 1 in 10,000,000,000 by bacterial and yeast-growth methods.

Micro-organisms, bacteria and fungi (including yeasts) resemble animals in requiring vitamins and amino acids for normal growth and metabolism. The difference between the two groups, however, is that the list of amino acids and vitamins required by micro-organisms is longer and more formidable than it is for the higher animals. The rat, for example, requires 10 amino acids for normal nitrogen metabolism, whereas when we turn to the bacteria the list of amino acids has in some instances to be extended to as many as 15 and even 17.

The heterotrophic bacteria, and it is with the heterotrophic bacteria that we are concerned, differ greatly in their nutritional needs. For example, a large number of bacteria isolated from the soil can be cultivated in artificial media of relatively simple composition made up of inorganic salts with ammonia or ammonium salts as a source of nitrogen and carbohydrate or relatively simple organic acids as source of carbon. These organisms possess the power of synthesising the vitamins and amino acids that they need. Others require a more complex medium containing protein digests with or without added carbohydrate, and a third group is yet more exacting in its nutritional needs and will not flourish without addition of liver extract, yeast autolysate, blood, etc. In other words, these organisms do not possess the power of synthesising certain of the vitamins they require and must rely upon

outside sources. It is with such organisms that we are concerned in the devising of assay methods for vitamins of the B-complex.

Fildes[5] has put forward the hypothesis that organisms that require a large number of compounds from which to build up their cellular material have lost the power of synthesising many of these substances, as a result of prolonged culture in a medium with a rich assortment of molecules which, so to speak, are ready-made at hand.

The wide differences shown by bacteria in their vitamin requirements are best indicated by actual examples. Thus, *Proteus vulgaris* can be grown on a simple medium composed of inorganic salts with either sodium or ammonium lactate as a source of carbon, and needs but one vitamin—nicotinic acid or its amide—for vigorous growth. With another species of *Proteus*—*P. morganii*—the conditions are slightly more complex. In this organism two factors are required for normal growth to take place: nicotinic and pantothenic acids. When, however, we turn to the pathogens we find that the conditions for normal growth are still more exacting. *Corynebacterium diphtheriae* will multiply on a medium composed of casein hydrolysate plus tryptophan or, in lieu of casein hydrolysate, a medium made up of a long list of amino acids together with ethyl alcohol (as an additional source of energy), nicotinic acid and β-alanine. In the so-called *gravis* strains of *C. diphtheriae*, however, the conditions are still more complex. The *gravis* strains will not multiply on the medium described above; they are unable to synthesise pantothenic acid from β-alanine, and pantothenic acid as such must be supplied in place of the latter.

Bacterial Assay of Vitamins and Amino Acids—With regard to the utilisation of bacteria for the quantitative determination of vitamins and amino acids, it is true to say that we could use *Proteus vulgaris* for the assay of nicotinic acid[6] and *P. morganii* for the assay of pantothenic acid,[7] but there are practical difficulties in the way. With both these organisms turbidometric or nephelometric methods of growth determination must be used, and when one is dealing with extracts, which are often highly coloured, a large number of replicates have to be used, with the result that the number of samples that can be dealt with at one time is strictly limited. Certain pathogens may also be used, but it is scarcely necessary to point out that for obvious reasons they should not be employed by the inexperienced. The difficulty of growth measurements, however, does not arise with the so-called lactic fermenters, because, with one exception, the lactic acid formed by these organisms in the course of their metabolic activities is directly proportional to the concn. of vitamin in the medium, and direct titration with 0·1 *N* sodium hydroxide is sufficient to establish a standard curve.

The different lactic bacteria used for the assay of vitamins and amino acids have been isolated from a variety of sources, such as cheese, milk, silage and the soil. It was first observed by Orla-Jensen and his colleagues[8] that when milk was shaken with an activated charcoal it was unable to support the growth of many of these lactic bacteria, but if riboflavin plus the material eluted from the charcoal adsorbate were returned to the treated milk growth was resumed and almost reached the same intensity as in untreated milk. From this initial discovery have come the various methods for the microbiological assay not only of riboflavin, but also of practically all the other members of the vitamin-B complex.

The Assay of Riboflavin

It is necessary to discuss the assay of riboflavin in some detail, because it is undoubtedly the most difficult and exacting of all micro-biological assays, and once the principle of the method has been grasped, the assay of the remaining members of the B-complex is relatively simple. The organism used for the assay is *Lactobacillus casei* ϵ or *helveticus*. In 1939 Snell and Strong[9] described a medium for the quantitative assay of riboflavin, using this organism. This medium has the following composition:

Snell and Strong Basal Riboflavin Medium

Sodium-hydroxide-treated peptone (plus sodium acetate)	0·5%
Cystine hydrochloride	0·01%
Yeast supplement	0·1%
Glucose	1·0%
Inorganic salt solution A	0·5 ml per 100 ml
,, ,, ,, B	0·5 ml per 100 ml

Inorganic salt soln. A is composed of a mixture of 25 g of KH_2PO_4 and 25 g of K_2HPO_4 in 250 ml of water.

Inorganic salt soln. B consists of 10 g of $MgSO_4.7H_2O$, 0·5 g of $MnSO_4.4H_2O$, 0·5 g of $FeSO_4.7H_2O$ and 0·5 g of NaCl in 250 ml of water.

This medium works well and gives reliable results. It will, however, be found to be much better to use a modified medium of the following composition:

Modified Basal Riboflavin Medium

Sodium-hydroxide-treated peptone (plus sodium acetate)	0·5%
Cystine hydrochloride	0·01%
dl-Tryptophan	0·01%
Yeast supplement	0·2%
Glucose	2·0%
Xylose	0·1%
Sodium chloride	0·5%
Ammonium sulphate	0·3%
Adenine	10·0 p.p.m.
Guanine	10·0 p.p.m.
Uracil	10·0 p.p.m.
Xanthine	10·0 p.p.m.
p-Aminobenzoic acid	0·4 p.p.m.
Pyridoxine	0·1 p.p.m.
Nicotinic acid	0·1 p.p.m.
Ca *d*-pantothenate*	0·1 p.p.m.
Inorganic salt soln. A	0·5 ml per 100 ml
,, ,, ,, B	0·5 ml per 100 ml

Inorganic salt solution B is slightly modified to contain 0·1 g of $FeCl_3$ in place of 0·5 g of $FeSO_4.7H_2O$ and the NaCl is omitted, since it is added to the medium as such.

* If this is unobtainable, twice the amount of Ca *dl*-pantothenate may be used.

The standard curve is steeper and the assay range slightly increased with this modified medium (Fig. 1).

The assay itself is carried out as follows. The medium is made up in double concn., *i.e.*, if a litre of medium be required the components are dissolved in 500 ml of water. Five ml aliquots are pipetted into test-tubes (18 × 150 mm). The standard riboflavin medium, which contains 0·1 μg/ml of the vitamin, is pipetted into the tubes in the necessary concns. The amounts of the vitamin required to establish a standard curve are 0·0 (blank ≡ 5 ml of water), 0·05, 0·075, 0·10, 0·125, 0·15, 0·175, 0·2, 0·25 and 0·3 μg of riboflavin and the final vol. in the tubes is adjusted in every instance to 10 ml with glass-distilled water. The standard tubes are set up in triplicate for each assay. The extracts of the unknowns are treated in the same way and assayed at three or four levels. The tubes are sterilised at 10 lb. pressure for 10 min., cooled and inoculated the next day. *All these operations are carried out in a dim light.* The inoculum is prepared by taking a portion from the agar stock culture on a sterile platinum loop, adding it to a tube of liquid medium containing 0·25 μg of riboflavin/10 ml and incubating for 16 to 18 hr. at 37° C. There should be good visible growth at the end of this period. The inoculum is centrifuged under sterile conditions and the supernatant liquor is poured off. Ten ml of sterile 0·9% saline soln. are then added, 1 ml of this final suspension is added to 10 or 20 ml of sterile saline soln., and this dilute inoculum is used for subsequent inoculation. For the inoculation of standard and extract tubes, 1 drop of inoculum is added to each with a sterile pipette. A fresh inoculum must be prepared for each new set of assays by going back to the stock agar culture. *In no circumstances whatever must a drop of previously prepared inoculum be added to a tube of medium for the preparation of fresh inoculum.* If this be done, the blanks will show unrestricted growth. After inoculation the tubes are incubated for 72 hr. at 37° C., but the incubation period can be extended to 96 hr. without harmful effect.

It has been stated that erratic results are often met with at lower levels of riboflavin concn. in the standard tubes. This difficulty has been traced by Price and Graves[10] to fluctuations and variations in temperature within the incubator. This finding is quite correct, but I would strongly advise, whether the incubator shows fluctuations or not, to shake all the tubes after intervals of 24 and 48 hr. so as to mix well the contents. At the end of the incubation period the contents of the tubes are titrated with 0·1 *N* sodium hydroxide, using bromothymol blue as indicator. The titration is carried out directly into the tubes in the way described by Barton-Wright and Booth,[11] using a comparator for this purpose.

PREPARATION OF EXTRACTS—It cannot be emphasised too strongly that correct extraction of material is vital for a successful assay. There are two points to be remembered in this connection: (1) The organism is stimulated by starch, and (2) the organism is stimulated by certain free fatty acids, notably stearic and oleic acids, and either stimulated or inhibited by

others, depending upon the concn., *e.g.*, linolic acid. A further important fact to be borne in mind is that such materials as blood, malt and cooked foods, *e.g.*, bread, contain some stimulating substance or substances which must be removed before an assay is attempted, or fictitiously high results will be obtained.

The extraction of cereals presents no great difficulties if the procedure described by Strong and Carpenter[12] be followed. In this method the cereal is hydrolysed at 15 lb. pressure for 15 min. in an autoclave with 0·1 *N* hydrochloric acid and cooled, 2 ml of a 2·5 *M* soln. of sodium acetate are added, the *p*H is adjusted to 4·5–4·6 with sodium hydroxide and the whole is filtered. The *p*H of the filtrate is next adjusted to 6·8 with more sodium hydroxide and made up to the requisite volume. If a ppt. should appear after the final adjustment of the *p*H to 6·8, the extract should be refiltered before an assay is carried out.

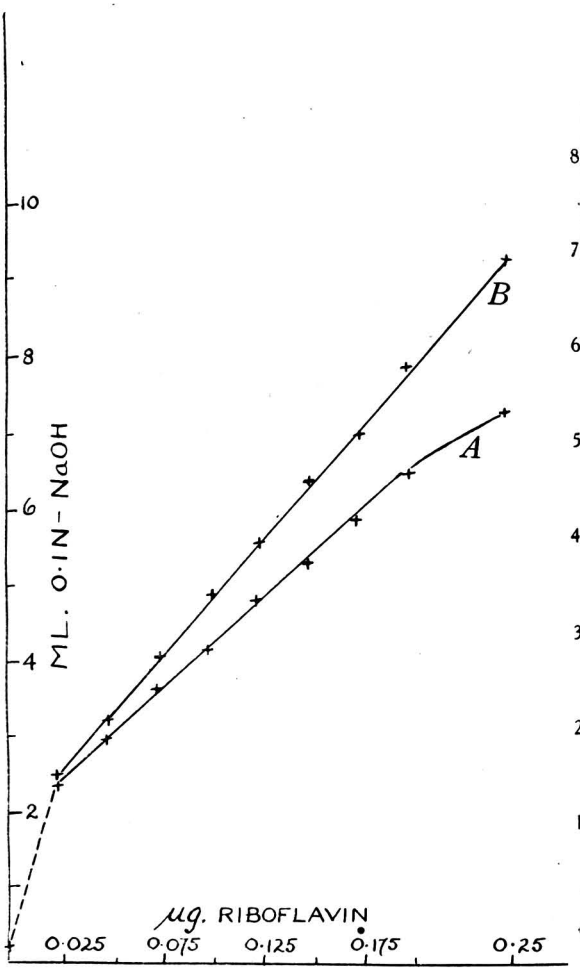

Fig. 1. A—Standard curve obtained on original Snell and Strong medium. B—Standard curve obtained on modified medium.

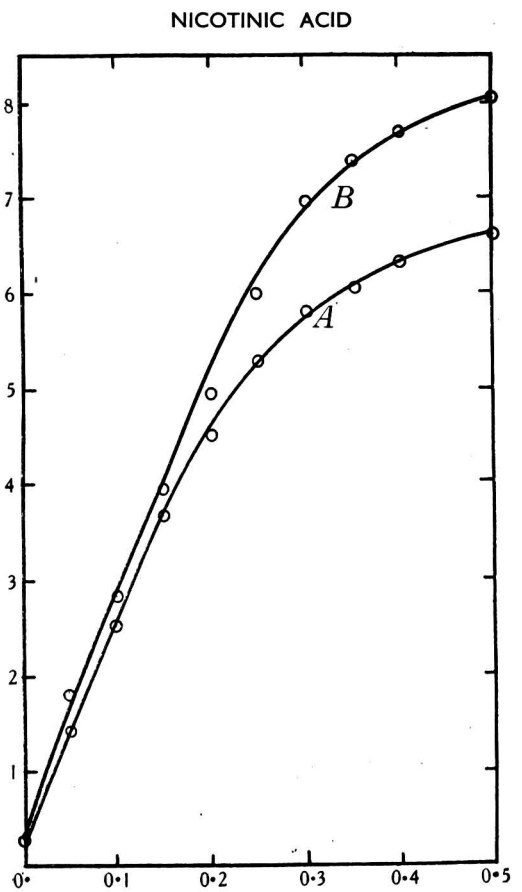

Fig. 2. A—Standard curve on original Snell and Wright medium. B—Standard curve on modified medium with 2% glucose and 2% sodium acetate. (From *Biochem. J.*)

This procedure is not sufficient for materials with a high fat content, such as wheat germ, maize, oats, meat, soya bean, etc. Such materials should be given a preliminary extraction with light petroleum in a Soxhlet apparatus before hydrolysis. After hydrolysis sodium acetate soln. is added, the *p*H is adjusted to 4·5, and the whole is shaken in a separating funnel with ethyl ether. As a general rule there is no need to filter prior to treatment with ether, as the solid material passes into the ethereal layer. The lower, aqueous layer is run off, the ether is washed two or three times with water, and the washings are added to the main aqueous layer. Finally, the *p*H of the extract is adjusted to 6·8 and made up to volume.

With bread a preliminary extraction with light petroleum is useless, but extraction with ether is necessary after hydrolysis. Malt and blood require the same treatment as bread.

The reason for using ether is to remove the fatty acids released by hydrolysis from lipins. Workers lay great stress upon removing free fatty acids from a sample, but the fact is nearly always forgotten that stearic acid, oleic acid and linolic acids, whether originally present or produced by the hydrolysis of simple glycerides or of lipins, are still stearic, oleic and linolic acids and therefore capable of stimulating or inhibiting the growth of *Lactobacillus helveticus*.

METHOD OF COMPUTATION—The usual method is to read off the results from different levels on the standard curve, and the values at three or more levels should agree within 10% of one another. It has been suggested by Wood[13] that the so-called "slope ratio" method should now be used for calculating the results. In fact, I myself now use this method in preference to reading from the curve. If, however, the extraction has been correctly made and the assay successfully carried out, the results by either method will agree very closely. Either method will indicate when an assay is unsatisfactory. If, for example, it is found that there is a drift in the figures, either in the upward or downward direction by direct reading from the curve, then it is clear that such an assay is invalid and must be rejected. Similarly, if by the slope ratio method the curve for the unknown does not cut the axis at the same point, or very nearly at the same point, as the standard curve, the assay must be looked upon as suspect. In such circumstances it is necessary to carry out a recovery experiment with added riboflavin and, if the recovery be greater or less than the theoretical by 10%, some serious error has been made in procedure.

THE ASSAY OF NICOTINIC ACID

The assay of nicotinic acid is perfectly straightforward and presents none of the difficulties found with riboflavin. The organism used is *Lactobacillus arabinosus* 17/5 and the method of assay was originally described by Snell and Wright.[14] However, in recent years a number of improvements have been made in the medium by Krehl *et al*[15] and myself.[16] *Lactobacillus arabinosus* is not stimulated by starch or free fatty acids, so that there are none of the difficulties associated with extraction in the riboflavin assay. Nevertheless, I recommend that the same treatment of extracts be used as with cereals in the assay of riboflavin. This procedure gives very clean bright solns. The actual hydrolysis is carried out by autoclaving with *N* hydrochloric acid for 15–20 min. at 15 lb. pressure. The composition of the medium is as follows.

Basal Nicotinic Acid Medium

Vitamin-free acid-hydrolysed casein ..	1·0%
dl-Tryptophan	0·01%
l-Cystine	0·02%
Glucose (anhydrous)	2·0%
Sodium acetate (anhydrous)	2·0%
Xylose	0·1%
Sodium chloride	0·5%
Ca *d*-pantothenate*	0·1 p.p.m.
Aneurine	0·1 p.p.m.
Riboflavin	0·2 p.p.m.
Pyridoxine	0·1 p.p.m.
p-Aminobenzoic acid	0·1 p.p.m.
Biotin	0·0004 p.p.m.
Adenine	10·0 p.p.m.
Guanine	10·0 p.p.m.
Uracil	10·0 p.p.m.
Xanthine	10·0 p.p.m.
Ammonium sulphate	0·3%
Inorganic salt soln. A	0·5 ml per 100 ml
,, ,, ,, B	0·5 ml per 100 ml

* If this is unobtainable, twice the amount of Ca *dl*-pantothenate may be used.

Salt solns. A and B are added at the same rate as in the riboflavin assay. Crystalline sodium acetate is to be preferred to the anhydrous salt (1 g of anhydrous acetate ≡ 1·66 g of hydrate).

The preparation of the casein hydrolysate is crucial for a successful assay, and the method of preparation has been described.[16] Messrs. Ashe Laboratories, Ltd., however, have now marketed a spray-dried vitamin-free casein hydrolysate which is a standard product and gives excellent results. If the spray-dried hydrolysate be used it should be employed in a

concn. of 0·6%. The *p*H of the medium is adjusted to 6·8 and the cultures are incubated at 37° C. for 72 hr.

The reason for increasing the concn. of glucose and sodium acetate to 2% is to increase the slope and range of the standard curve (Fig. 2, p. 287). Personally, I always grow my inoculum on the Snell and Strong riboflavin medium. Heavy growth is obtained on this medium and the inoculum should be centrifuged twice with saline so that the blanks are not abnormally high from "carry over" of traces of nicotinic acid from the medium. The inoculum should be diluted in the same way as that described for *L. helveticus*. *L. arabinosus* is a very much less exacting organism than *L. helveticus* and requires only three vitamins for normal growth: nicotinic acid, pantothenic acid and biotin. Aneurine, riboflavin and pyridoxine may be omitted from the medium without harmful results, but their presence is said to stimulate the early stages of growth.

Assay of Biotin

Lactobacillus arabinosus is by far the best organism to use for the assay of biotin. The medium described for the assay of nicotinic acid may be used with a few modifications. The concn. of glucose should be reduced to 1% and the concn. of sodium acetate to 0·6%. Biotin is omitted from the medium and nicotinic acid is added in its stead at a concn. of 0·2 p.p.m. There are one or two points of importance that should be borne in mind for a successful assay with this organism. First, the casein hydrolysate *must be free from* biotin. The correct blank is of the order of 0·4 to 0·5 ml of *N* sodium hydroxide, with an extreme limit of 1·0 ml. If higher blanks than this are obtained, then the casein hydrolysate is not biotin-free. Secondly, a very dilute inoculum must be used. The organism should be grown on a full nicotinic acid medium and not on the riboflavin medium and it must be centrifuged three times with sterile saline soln. One ml of this inoculum is then diluted with 100 ml of saline, and this highly diluted inoculum is used for inoculating the tubes. Thirdly, incubation should be carried out at 30° C. and not at 37° C.

The extraction of material for a biotin assay is carried out by autoclaving for 2 hr. with 6 *N* sulphuric acid at 15 lb. pressure.

Assay of Pantothenic Acid

There are two possible courses available here, and either *Lactobacillus helveticus* or *L. arabinosus* may be used for the assay of pantothenic acid. From the practical point of view it is far better to employ *L. arabinosus*, because the various difficulties associated with the presence of starch and fatty acids in the assay material do not then arise. Pantothenic acid is unstable to acid and alkali and extraction must be carried out enzymatically, preferably with a mixture of taka-diastase and papain. The assay medium for pantothenic acid with *L. arabinosus* has the following composition.

Basal Pantothenic Acid Medium

Sodium-hydroxide-treated peptone (plus sodium acetate)	0·5%
Glucose	2·0%
Sodium acetate	1·4%
Acid-hydrolysed casein	0·2%
Cystine hydrochloride	0·1%
Adenine	10·0 p.p.m.
Guanine	10·0 p.p.m.
Uracil	10·0 p.p.m.
Xanthine	10·0 p.p.m.
Nicotinic acid	0·2 p.p.m.
Biotin	0·0004 p.p.m.
Riboflavin	0·2 p.p.m.
Pyridoxine	0·1 p.p.m.
p-Aminobenzoic acid	0·1 p.p.m.
Sodium chloride	0·5%
Ammonium sulphate	0·3%
Inorganic salt soln. A	0·5 ml per 100 ml
" " " B	0·5 ml per 100 ml

Lactobacillus arabinosus is very sensitive to pantothenic acid and all trace of the vitamin must be removed from the inoculum. The inoculum should therefore be centrifuged twice with sterile saline soln. and 1·0 ml of the third suspension should be diluted with 100 ml of

saline in the same way as described for the biotin assay. The cultures are incubated at 30° C. The straight-line portion of the curve lies between 0·005 and 0·06 or 0·08 μg.

Assay of Aneurine

The assay of aneurine with the soil organism *Lactobacillus fermentum* 36 has recently been described by Sarett and Cheldelin.[17] Unfortunately, nephelometric methods of growth measurement must be used with this organism and not simple titration with alkali, because after 20 hr. incubation the organism responds to the pyrimidine and thiazole moieties of the aneurine molecule. The medium recommended has the following composition.

Basal Aneurine Medium

Alkali-treated peptone (plus sodium acetate)	1·0%
Acid-hydrolysed vitamin-free casein	0·2%
Glucose	2·0%
Sodium acetate	1·6%
Cystine hydrochloride	0·01%
Adenine	10·0 p.p.m.
Guanine	10·0 p.p.m.
Uracil	10·0 p.p.m.
Riboflavin	0·1 p.p.m.
Ca *d*-pantothenate*	0·1 p.p.m.
Nicotinic acid	0·1 p.p.m.
p-Aminobenzoic acid	0·1 p.p.m.
Pyridoxine	0·1 p.p.m.
Biotin	0·0004 p.p.m.
Folic acid†	0·00025 p.p.m.
Sodium chloride	0·5%
Inorganic salt soln. A	0·5 ml per 100 ml
,, ,, ,, B	0·5 ml per 100 ml

* If this is unobtainable, twice the amount of Ca *dl*-pantothenate may be used.
† (Potency = 40·000.)

Unfortunately, at the present time folic acid cannot be obtained in this country. But this difficulty can be overcome by using a yeast extract prepared in the following way. Difco yeast extract is autoclaved at 15 lb. pressure for 30 min. with 0·5 *N* sodium hydroxide. The soln. is allowed to cool, neutralised with glacial acetic acid and filtered to remove any pptd. protein. The *p*H is then adjusted to 1·5 with hydrochloric acid and the mixture is stirred for 20 min. with 10% of an activated charcoal. It is then filtered and the *p*H is readjusted to 1·5, if necessary. The process is repeated with a second lot of 10% activated charcoal. The soln. is filtered, neutralised with alkali, and stored in a refrigerator under sulphur-free toluene. The yeast extract is added to the medium at the rate of 0·01%. The *p*H of the medium is adjusted to 6·5 and not to 6·8.

Material to be assayed for aneurine is extracted with a mixture of taka-diastase and papain. The medium should not be sterilised in an autoclave, because there is always the danger of destruction of aneurine. It should be steamed in a steamer for 15 min., cooled and inoculated. The inoculum should be centrifuged twice, as described for the pantothenic acid assay, and 1 ml of the suspension diluted to 10 ml with sterile saline. The organism is very sensitive to aneurine, and the range of values for the construction of a standard curve is 0·0 (blank), 0·005, 0·01, 0·015, 0·02, 0·03, 0·04 and 0·05 μg/tube. The cultures are incubated for 16 to 18 hr. (not longer) at 37° C. The tubes are then cooled in a refrigerator to stop growth, and the turbidity is measured in a photometer.

Assay of Pyridoxine

None of the lactic bacteria can be used for the assay of pyridoxine because they respond to a very much greater degree to the two closely related vitamins that have recently been isolated, *viz.*, pyridoxal and pyridoxamine. Landy and Dicken[18] described a medium for the assay of pyridoxine, using *Lactobacillus helveticus*. It must be emphasised that this medium is quite invalid for the reason stated above. All the chemical methods that have been described from time to time for the assay of pyridoxine are without exception difficult and tedious to carry out, while with the rat-growth method (see Copping[19]) a result cannot be obtained under a month. There is, however, a microbiological method available for the assay of pyridoxine, *viz.*, to use the X-ray mutant of the fungus *Neurospora sitophila* (Mutant 299).

The method of procedure is well described in the publication by Stokes *et al.*,[20] and I have only altered the medium in one or two minor particulars. The modified medium is as follows.

Basal Pyridoxine Medium (Undiluted)

Sucrose	30·0 g
Ammonium tartrate	10·0 g
Sodium dihydrogen citrate	4·0 g
KH_2PO_4	5·0 g
$MgSO_4.7H_2O$	1·0 g
NaCl	0·2 g
$CaCl_2$	0·2 g
$FeCl_3$	10·0 mg
$ZnSO_4.7H_2O$	4·0 mg
Biotin	8·0 μg
Distilled water	1000 ml

The alterations made in the medium as recommended by Stokes *et al.* consist in adding sodium dihydrogen citrate and doubling the concn. of zinc sulphate. It is essential for a successful assay that the *p*H of the medium be maintained below 5·6 or the organism will not respond quantitatively to pyridoxine. It is on this account that sodium dihydrogen citrate is added, because, although this salt stimulates growth slightly, it does maintain the *p*H of the medium at 4·6, so that all danger from a high *p*H in the medium is avoided. Zince sulphate is added to the medium to inhibit sporulation. Sporulation does not interfere with the quantitative response of the organism, but it does interfere with the quantitative harvesting of the mycelium. The original concn. of zinc sulphate recommended by Stokes *et al.* is not sufficient to prevent sporulation at the higher levels of pyridoxine in the culture flasks for the standard curve, but by doubling this concn. sporulation is practically eliminated throughout the range. A further important point that must be kept in mind is that all aneurine present in material to be assayed must be destroyed. In presence of aneurine the organism no longer responds quantitatively to pyridoxine. The aneurine is destroyed with sodium sulphite in the way described by Stokes *et al.*, but the fact is not sufficiently emphasised that *all* sulphite in turn must be destroyed. Any sulphite left in the extract will inhibit growth of the fungus. The sulphite is destroyed with 2% hydrogen peroxide, and it is necessary to add a slight excess of hydrogen peroxide so that the starch iodide indicator shows a definite purple coloration. In the preparation of the extracts it will be found to be more convenient to use double the amounts of material recommended by Stokes *et al.*, *i.e.*, 2 to 10 g of material in place of 1 to 5 g. After hydrolysis with *N* hydrochloric acid for 2 hr. at 15 lb. pressure in an autoclave and filtration, the total vol. of the extract is made up to 100 ml in place of 50 ml. It should also be mentioned that after destruction of aneurine the *p*H of the extract should be adjusted to 4·6 with acid. This fact is unfortunately not mentioned in the original paper.

For harvesting the mycelium I employ a Gooch crucible with a pad of filter-paper cut out to fit the bottom completely. This procedure will be found more convenient than trying to harvest the mycelium with a wire, as recommended by Stokes *et al.*

The Assay of Amino Acids

Not only is it possible to assay the different members of the vitamin-B complex with the lactic bacteria, but it is also possible to assay the greater number of known amino acids, and the operation is straightforward and presents no great difficulties. The organisms that can be used for this purpose are *Lactobacillus helveticus*, *L. arabinosus*, *Streptococcus faecalis* R and *Leuconostoc mesenteroides* P.60. By means of the first three organisms it is possible to estimate about 11 amino acids, including 7 of the "essential" acids, but, according to Dunn *et al.*,[21] *L. mesenteroides* can be used for the quantitative estimation of 17 acids. I am not certain, myself, that this organism will estimate as many as 17 amino acids, but it will certainly estimate 15.

The principle underlying the assay of amino acids is simply this: Just as there are certain essential vitamins without which these lactic organisms will not multiply, so too, there are certain essential amino acids necessary for normal growth and multiplication. For the assay of these acids the casein hydrolysate in a medium is replaced by its constituent amino acids, omitting only the particular acid to be assayed.

There are, however, two important points that must be kept in mind in this connection. First, so far as the lactic organisms are concerned, the amino acids can be divided into two

classes: essential and stimulatory. If a medium be composed solely of the essential amino acids, growth will occur, but not maximum growth. Secondly, there is the phenomenon of "antagonism," which has an important bearing on the problem.

In the preparation of basal media, in which mixtures of amino acids were used in place of a casein or other protein hydrolysate, the phenomenon of antagonism was not observed by the early investigators because the amino acid mixtures that were used were modelled on the analysis of a protein such as casein or gelatin. Thus, more or less by accident, the possibility of antagonistic effects between different amino acids was avoided. It was not until 1939 that Gladstone[22] showed that it was not possible to determine optimum concn. for single amino acids, and that the need for a single amino acid was in many instances not an absolute but a relative value. For example, an amino acid X might be required to neutralise the antagonistic influence of an amino acid Y, but in absence of Y, X no longer functions as an essential amino acid.

The antagonistic effects of amino acids are shown among the lactic acid bacteria. For instance, *Lactobacillus helveticus* will grow, growth being measured by acid production, on a medium composed of glucose, sodium acetate, adenine, mineral salts, the vitamins riboflavin, nicotinic acid, folic acid, biotin, pantothenic acid and pyridoxine, plus the following ten amino acids in place of casein hydrolysate: glutamic acid, aspartic acid, leucine, valine, serine, tryptophan, cystine, arginine, phenylalanine, and tyrosine. In the table given below it will be seen that maximum acid production is not obtained on this mixture, but that it is attained when the number of amino acids is increased to twenty, the acid production then equalling that obtained with casein hydrolysate. Furthermore, it will be seen from the table that histidine and *iso*leucine show marked antagonism. This antagonism is partly removed by adding alanine and completely eliminated when alanine plus lysine is added to the mixture.

EFFECT OF STIMULATORY AMINO ACIDS ON *Lactobacillus helveticus* (AFTER HUTCHINGS AND PETERSON[23])

Amino acid added to basal medium	ml of $N/10$ acid per 10 ml of medium
Basal	3·2
Threonine	6·3
Threonine + methionine	7·4
20 Amino acids	10·8
Hydrolysed casein	10·9

ANTAGONISTIC RELATIONS AMONG AMINO ACIDS

Amino acid added to basal medium	ml of N/10 acid per 10 ml of medium
Basal*	5·9
Histidine	5·3
*Iso*leucine	5·3
Lysine	6·6
Alanine	7·6
Histidine + *iso*leucine	**4·9**
Histidine + lysine	5·9
Histidine + alanine	7·1
*Iso*leucine + lysine	5·4
*Iso*leucine + alanine	7·7
Lysine + alanine	9·6
Histidine + *iso*leucine + lysine	5·4
Histidine + *iso*leucine + alanine	**7·2**
Histidine + lysine + alanine	9·2
*Iso*leucine + lysine + alanine	11·2
Histidine + *iso*leucine + lysine + alanine	**11·5**
Hydrolysed casein	11·4

* Contained the 10 amino acids already listed plus threonine and methionine; 2 mg of each amino acid per 10 ml of medium.

It is therefore essential in an amino acid assay by microbiological methods that antagonism be avoided at all costs by increasing the number of amino acids to at least 16 and preferably to 20.

Assay of Tryptophan

The assay of tryptophan may be discussed apart from the remaining amino acids because it forms a special exception. Tryptophan is destroyed by prolonged hydrolysis with strong acids under the conditions used for the hydrolysis of casein. It is therefore possible to use *Lactobacillus arabinosus* for the assay of this amino acid with only slight modification of the basal medium employed for the assay of nicotinic acid. All that is necessary is to omit tryptophan from the medium and add excess of nicotinic acid (0·02 p.p.m.). Greene and Black[34] have recently described such a medium, but in place of the concn. of 1% of glucose and 0·6% of sodium acetate recommended by them it is preferable to increase the concn. of both these substances to 2% because the standard curve is then steeper and the titres are higher (Fig. 3).

Strong acids cannot be used for hydrolysis of material in this assay because of partial or complete destruction of the tryptophan. The best hydrolysing reagent is barium hydroxide. It must be remembered, however, that racemisation takes place with this reagent and hydrolysis must be continued until racemisation is complete. Since the *d*-enantiomorph is quite inactive, the figure found by assay will have to be doubled to give the correct value.

Although glutamic acid is an essential amino acid for both *Lactobacillus helveticus* and *L. arabinosus*, neither organism can be used directly for assaying this compound. *L. helveticus* is stimulated to a greater degree by the unnatural *d*-enantiomorph than by the *l*-form, and no response is shown by *L. arabinosus* to *l*-glutamic acid in the lower concns., *e.g.*, 10 to 50 μg, except in presence of glutamine, while in the higher concns. the organism is stimulated to a greater degree by the *d*-enantiomorph.

For the remaining 15 or 17 amino acids that can be estimated by microbiological methods the lactic organism *Leuconostoc mesenteroides* strain P.60 should be tried. The assay of amino acids with this organism has recently been described by Dunn *et al.*[21] Personally, I have not had much experience with this organism, chiefly because I have been unable to obtain a reliable supply of folic acid, but the results which I have so far obtained with a liver eluate concentrate have been very promising.

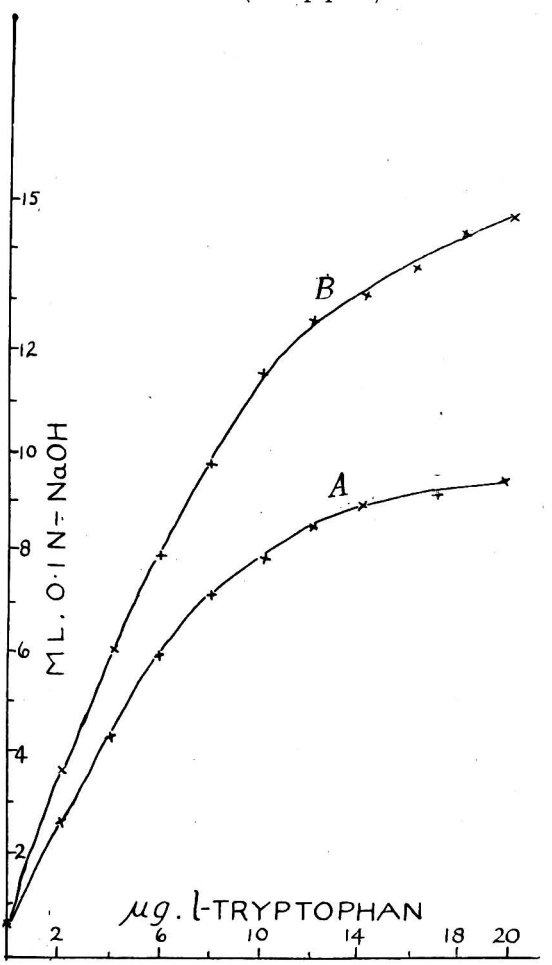

Fig. 3. A—Standard curve obtained on Black and Greene's medium. B—Standard curve obtained on medium with 2% glucose and 2% sodium acetate.

Assay of Potassium

It is possible to assay potassium with *Lactobacillus helveticus*, but it is far preferable to use *Streptococcus faecalis* R. The latter is unique among the lactic bacteria, inasmuch as it requires only one mineral element, potassium, for normal growth. Unlike the remaining lactic organisms, it does not need magnesium, manganese and iron. The nature of the potassium salt has no influence upon the standard curve, and either potassium phosphate, chloride or phthalate may be used. The range of the assay is from 0 to 1 mg and the composition of the medium is as follows.

Basal Medium for Potassium

Casein hydrolysate	0·5%
Glucose	2·0%
Sodium acetate	0·4%
l-Cystine	0·01%
dl-Tryptophan	0·06%
Adenine	10 p.p.m.
Guanine	10 p.p.m.
Uracil	10 p.p.m.
Xanthine	10 p.p.m.
Aneurine	0·2 p.p.m.
Riboflavin	0·2 p.p.m.
Nicotinic acid	0·6 p.p.m.
Pyridoxine	1·2 p.p.m.
Ca *d*-pantothenate*	0·4 p.p.m.
Biotin	0·8 p.p.m.
Folic acid	(0·5 ml/100 ml of media)

* If this is unobtainable, twice the amount of Ca *dl*-pantothenate may be used.

Here, again, the main difficulty is a supply of folic acid, but satisfactory results were obtained with factor HL2 prepared from liver and described earlier in this paper (p. 284). Although *Str. faecalis* R grows rapidly, it is but a poor acid producer. On this account, if it should prove to be inconvenient to use turbidometric methods of growth measurement, 0·05 *N* sodium hydroxide should be used in place of 0·1 *N* soln.

GENERAL REMARKS ON MICROBIOLOGICAL METHODS—In conclusion I would like to give a few words of advice and warning. Microbiological methods are still in the early stages of development, but progress has been remarkable in the last few years. The various chemicals required, such as tryptophan and biotin, so difficult to obtain at one time, are now in regular supply. The only great handicap at the present time for workers in this country is a supply of folic acid, but I am optimist enough to think that even this difficulty will be overcome in the near future.

Setbacks are bound to occur when first these methods are attempted, but with a little perseverance microbiological methods of assay will be found no more difficult than any other analytical procedure.

The advantages of microbiological methods of assay over either biological or chemical methods of determination of the various members of the vitamin-B complex, as well as of amino acids, are manifest and many. First, biological methods of assay are expensive and laborious. Secondly, chemical methods for the quantitative determination of the members of the vitamin-B complex, with the exception of vitamin B_1 (aneurine), are difficult and tedious, and the chemical methods for the determination of amino acids require much skill and practice. On the other hand, microbiological methods are relatively quick and inexpensive and do not require any special or elaborate apparatus other than that to be found in a well-equipped bacteriological laboratory.

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The Determination of Fat in Mixtures containing Fatty Acids, and the Determination of Unsaponifiable Matter in Oils and Fats

BY N. D. SYLVESTER, A. N. AINSWORTH AND E. B. HUGHES

ALTHOUGH the determination of fat in admixture with fatty acids is not a procedure frequently required in fat analysis, it is of importance, for example, in the analysis of soap stock fatty acids, and it is possible that such determinations would be more widely made if a simpler method were available.

The method now described is to pass a solution containing fat and fatty acids through a column of aluminium oxide, when the fat (and any unsaponifiable matter) is readily washed through by a suitable solvent such as chloroform or ether, the fatty acids remaining strongly adsorbed on the column. The position of the fatty acid band is clearly defined if the column is tinted with bromothymol blue. It is apparent that this adsorption method for the removal of fatty acids can be applied to the determination of unsaponifiable matter in oils and fats after acidification of the saponified product, but in view of the relatively large quantity of fatty acids to be removed the following procedure is preferred. After the soap solution has been extracted with ether to remove the unsaponifiable matter, the extract is washed with dilute acid to decompose the small amount of soap present, and the resulting fatty acids are removed by passing the ether soln. through an adsorption column. The method is obviously applicable also to the determination of unsaponified matter in soaps.

No efficient method for the determination of unsaponifiable matter has previously been reported that avoids the laborious washings necessary to obtain accurate results, less elaborate procedures having been proved not to be of general application. Four methods were compared in a report to the Association of Official Agricultural Chemists by G. Kirsten,[1] who concluded that the method specified in 1933 by the Society of Public Analysts and Other Analytical Chemists[2] was the only one giving practically complete extraction of the unsaponifiable matter. The simpler procedure now proposed has been found to yield results agreeing closely with those obtained by the Society's method, and no difficulty has been experienced in the analysis of any of the products examined.*

This adsorption process for the removal of acidic bodies has also been applied to the determination of fully saturated glycerides in fats, after their oxidation by permanganate in acetone according to the method of Christian and Hilditch,[3] and this work will form the subject of a subsequent communication.

EXPERIMENTAL

PREPARATION OF THE ADSORPTION COLUMN—The tinting of the columns of aluminium oxide† with bromothymol blue is effected by mixing with the alumina an amount of strongly coloured aluminium oxide sufficient to give a slight but definite bluish tint. This strongly coloured alumina is prepared by adding a 3% soln. of the indicator in alcohol; any excess of liquid is poured away and the wet product is then dried by first stirring on a hot plate and finally heating in an oven at about 100° C.

* Since these investigations were commenced, the use of an indicator for locating the position of fatty acids on an adsorption column has been described by Graff and Skau,[4] who used phenol red on a magnesium oxide column for the chromatographic separation of fatty acids. For the type of work described in the present communication bromothymol blue is preferred, inasmuch as the colour-change from blue to yellow is the more distinct.

† Aluminium oxide (standardised for chromatographic adsorption analysis) purchased from Savory & Moore, Ltd., has been used for this work. It is not suggested that other brands would not prove satisfactory or that other alkaline adsorption agents could not be used.

In the preparation of the columns, it has been found convenient to use tubes of 2 cm diameter into which a No. 2 sintered glass plate is fused near the bottom. The tube is widened for about 10 cm at the top, providing a volume of about 50 ml for reception of the liquid to be passed through the column. Below the sintered glass plate the tube is drawn out to a smaller diameter to facilitate collection of the percolate.

A plug of cotton wool is placed over the sintered glass plate and a thin slurry of tinted aluminium oxide and solvent is poured into the tube. As air entrapped in the column reduces the rate of percolation, it is advisable to remove adsorbed air by heating the alumina and the solvent together for a few minutes with stirring to minimise bumping. The prepared columns should be used without allowing them to run dry.

The size of the column required is obviously dependent on the activity of the aluminium oxide used as well as on the amount of fatty acids to be removed, but, as a rough guide, a 10 cm column in a tube of 2 cm diameter is more than sufficient for the removal of 0·5 g of fatty acids. Care should be taken that conditions are such as to ensure that a clear margin of about 5 cm of unaffected alumina remains after the washing of the column is completed.

The alumina can be reactivated satisfactorily by heating the used residues, after allowing the solvent to evaporate, in a muffle furnace at approximately 500° C. for about 3 hr. After continued reactivation part of the alumina is reduced to a very fine powder, which tends to be washed out of the column; it can be removed by sieving (325-mesh), and the remaining alumina is then suitable for use.

DETERMINATION OF FAT IN PRESENCE OF FATTY ACIDS—The solution containing the fat and fatty acids is passed through the column, the percolate is collected in a weighed flask provided with a standard joint, and the column is then washed with chloroform or ether until the percolate is free from fat. The solvent is finally distilled from the flask in all-glass apparatus, and the residue is dried in a steam-oven to constant weight. A convenient method for testing the washings is that suggested by Edeler,[5] in which a few drops are collected on a ground-glass plate, the presence or absence of a residue being clearly visible when the solvent has evaporated; it is necessary to wash the outside of the lower end of the adsorption tube, to remove any adherent fat, before the percolate is tested.

TABLE I

RECOVERY OF FAT FROM MIXTURES OF FAT AND FATTY ACIDS

Chloroform			Ether		
Fatty acids	Fat	Fat recovered	Fatty acids	Fat	Fat recovered
mg	mg	mg	mg	mg	mg
500	500	501	300	700	697·5
540	460	460·5	500	500	498·5
700	300	301	600	400	401·5
700	300	300	200	250	250·5
780	220	219	780	220	220·5
800	200	200	800	200	200
800	200	199·5	900	100	98
900	100	101·5	400	100	99·5
900	100	99·5	400	100	99

Of the various solvents tested, chloroform has been found to be the best. Satisfactory results can be obtained with ether, but with carbon tetrachloride or benzene complete removal of fat from the columns was found to be very difficult.

A series of results obtained for solutions of known amounts of fat and fatty acids in ether and in chloroform is given in Table I. The fat used was a cooking compound of negligible acidity and the fatty acids were prepared in the laboratory from beef fat. The volume of solvent used for washing the columns was about 200 ml of chloroform or 250 ml of ether, and the results have been corrected for the unsaponifiable matter present in the fatty acids used.

DETERMINATION OF UNSAPONIFIABLE MATTER—The determination, which is carried out according to the following instructions, follows the Society of Public Analysts' method to the stage where the extraction of the unsaponifiable matter is completed.

From 2 to 2·5 g of fat are saponified in all-glass apparatus by heating under reflux for 2 hr. with 25 ml of $N/2$ alcoholic potash, recently prepared. While still slightly warm, the soap soln. is poured into a separating funnel, and the flask is washed successively with 50 ml of water and 50 ml of ether, which are poured into the separating funnel. The contents of the funnel are shaken vigorously and, after separation, the lower layer is run out into the

original saponification flask. The upper layer is poured from the mouth of the funnel into a second separating funnel containing about 20 ml of $N/10$ (approx.) hydrochloric acid and a drop of methyl orange indicator solution. The soap soln. is extracted again in the same way with two further 50-ml portions of ether, and the combined ether extracts in the second separating funnel are shaken vigorously with the dilute acid. After separating, the aqueous layer, which should still show an acid reaction, is removed and the ether solution is washed with 20 ml of water. As much as possible of the water is removed, including that in the stop-cock, and the stem of the funnel (which should preferably be short) is dried with a piece of filter-paper. About 15 g of anhydrous sodium sulphate are added to the ether extract and shaken vigorously.

An aluminium oxide column (tinted with bromothymol blue) is prepared in a tube of 2 cm diameter, the column being about 10 cm long. A plug of cotton wool is placed on the top of the alumina and the extract of unsaponifiable matter is passed through. The separating funnel and the column are washed with five successive 50-ml portions of dried ether, special care being taken to wash the end of the stem of the separating funnel, and the lower end of the adsorption tube. The ether is distilled from the percolate and washings in all-glass apparatus, and the unsaponifiable matter is finally heated to constant weight at 80° C. For accuracy in weighing, a small flask (150 to 200 ml) should be used for this purpose.

TABLE II

DETERMINATIONS OF UNSAPONIFIABLE MATTER

	Weight taken	Unsaponifiable matter, %	
	g	"S.P.A." method	Adsorption method
Premier jus, 1	2·5	0·37; 0·37	0·33; 0·35
,, ,, 2	5	0·25	0·31
Lard, 1	2·5	0·20; 0·20	0·26; 0·26
,, 2	5	0·18	0·24
Lard oil	2·5	0·71; 0·72	0·75; 0·76
Olive oil	2·5	0·81; 0·82	0·83; 0·84
Cotton seed oil	2·5	0·81; 0·89; 0·79	0·87; 0·78; 0·93
	5	0·86	0·87
Ground nut oil	2·5	0·45; 0·46	0·51; 0·55
Cocoa butter	3·5	0·48	0·43
	2·5	—	0·45; 0·44
Hard whale oil, 1	2·5	1·19; 1·23	1·24; 1·28
,, ,, ,, 2	2·5	1·34; 1·29	1·22; 1·21
,, ,, ,, 3	5	1·27	1·34
Cod liver oil	2·5	1·03	1·02
Crude cod liver oil	2·5	1·04; 1·03	1·07
,, shark liver oil, 1	2·5	3·46; 3·52	3·54; 3·64
,, ,, ,, 2	2·5	6·75; 6·80 (0·22)	6·93; 7·15
,, ,, ,, 3	2·5	7·97 (0·25); 8·03 (0·25)	7·79; 7·83 (0·29)
	2·5	7·73 (0·47); 7·85 (0·39)	7·80 (0·48); 7·90 (0·42)
Shea butter, 1	2·5	7·03; 7·21	*7·18; 7·28
	2·5	—	*7·23; 7·32
,, ,, 2	2·5	6·65; 6·73	*6·67; 6·72
	2·5	—	*6·58; 6·62
,, ,, 1 plus 0·3% of ferric stearate	2·5	7·00; 7·03	7·21
Rosin	2·5	4·82 (0·40); 4·83 (0·43)	4·84 (0·24); 4·98 (0·27)
	2·5	4·81 (0·21)	4·88 (0·37)

Results obtained by this method are given in Table II, where they are compared with the corresponding results given by the "S.P.A." method. The latter determinations were made by the full method[2] (the only modification being the use of all-glass apparatus), and the special procedures recommended for shea butter, rosin and fats containing metallic soaps were followed. No special procedures were required for the adsorption method. When the percentage of unsaponifiable matter is small, it is preferable in both methods to make the determinations on more than 2·5 g of material, a corresponding increase being made in the amounts of the reagents used.

In all cases the unsaponifiable matter was examined for the presence of fatty acids by titration with $N/10$ alcoholic sodium hydroxide soln. In general, the titration was less than 0·05 ml for the adsorption method and within the specified limit of 0·10 ml for the "S.P.A." method, the only exceptions for both methods being rosin and the shark liver oils. In these

instances (but especially for shark liver oil, No. 3), the titrations were probably inaccurate because of the dark colour of the unsaponifiable matter, and there was no indication that, within the limits of experimental error, the higher titrations were associated with high results in the determinations. Where the titration of the unsap. matter exceeded 0·20 ml of $N/10$ alcoholic sodium hydroxide, the actual titrations are given in brackets after the respective results in Table II. Apart from these occasional high titrations, no difficulty was experienced in making any of the determinations, and the agreement between the results obtained by the two methods is satisfactory.

The adsorption method is considered easier to carry out and the time taken for the determination is reduced; the passage of the solution through the column and the subsequent washing operations take not more than 1 hr. The method appears to be of general application.

Where the results in Table II are marked by an asterisk, the duplicate pairs of determinations were made by different analysts, one of whom had had no previous experience of the method.

We wish to thank J. Lyons & Co., Ltd., for their permission to make this communication and also Mr. J. P. Van den Bergh, Ministry of Food, who kindly supplied some of the samples analysed.

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The Colorimetric Determination of Phosphorus, with Special Reference to Blood Phosphorus

BY J. I. M. JONES

SMALL quantities of phosphorus are usually determined colorimetrically by means of the blue colour produced by reduction of phosphomolybdate. Since its original use by Osmond,[1] this reaction has been extensively investigated, with conflicting results by different workers. Phenylhydrazine, 1-2-4-aminonaphtholsulphonic acid, quinol and stannous chloride have been used as reducing agents. Some of the main findings in the literature are outlined below.

Bell and Doisy[2] developed the method, using hydroquinone in alkaline solution as reducing agent. Briggs[3,4] improved the stability of the colour by using acid solns. Fiske and Subbarow[5] obtained better stability, using 1.2.3-aminonaphtholsulphonic acid. Stanford and Wheatly[6] applied the method to blood, using quinol, and found that acidity affected colour development and that colour was not proportional to phosphorus content. Martland and Robison[7] did not confirm this. Kuttner and Cohen[8] made a more detailed investigation and found stannous chloride to be the best reducing agent; they also established optimum concns. of acid and reagents to effect rapid development of a relatively stable colour. Addition of trichloroacetic acid up to 4%, used as a protein precipitant, was found not to interfere. Reference may also be made to later studies by Kuttner and Lichtenstein,[9] by Youngburg and Youngburg,[10] and by Bodansky,[11,12] who pointed out the failure of the colour to obey Beer's Law and developed a correction formula.

More recently Dickman and Bray[13] found that presence of an anion other than that of the acid used in the reagent affected the colour and showed Beer's Law to be obeyed up to 5 p.p.m. Sherman[14] confirmed the effect of acidity and temperature and gave the optimum range as 0·015 to 0·1 mg of phosphorus. Stoloff,[15] by a spectrophotometric method, using hydroquinone as reducing agent with a sodium succinate buffer, found Beer's Law obeyed up to 0·14 mg P in 10 ml. Sideris[16] extracted the yellow phosphomolybdate with *iso*butyl alcohol and then effected reduction with stannous chloride, and found that Beer's Law held good up to 0·09 mg per ml.

In a recent investigation of the effect of vitamin D on serum phosphorus and phosphatase in chicks it became necessary to ascertain the range of phosphorus concentrations over which

the colorimetric method could be safely applied and also the effect of other factors on colour production. The outcome of this work is described below.

METHOD—The method and reagents used were those of Kuttner and Cohen[8] and Bodansky,[11,12] with solutions of potassium dihydrogen phosphate of known concn.

Reagents—(1) *Sulphuric acid*: 10 Normal. (2) *Sodium molybdate*: 90 g of molybdenum trioxide dissolved in 250 ml of 5 *N* caustic soda AR and diluted to 2 litres. For use, 1 vol. was mixed with 1 vol. of 10 *N* sulphuric acid. The reagent was mixed fresh daily. (3) *Stannous chloride*: 10 g of AnalaR reagent dissolved in 25 ml of conc. hydrochloric acid. For use, a soln. of 0·75 ml in 100 ml of water was prepared fresh daily. (4) *Phosphate standard*: 0·439 g of pure KH_2PO_4 was dissolved in distilled water and made up to 1 litre (1 ml ≡ 0·1 mg of P); 10 ml of this soln. were diluted to 100 ml with water (1 ml ≡ 0·01 mg of P). (5) *Trichloroacetic acid*, 4·5%: In the determination of inorganic phosphate in blood serum, 1 ml of serum is treated with 9 ml of 5% trichloroacetic acid and the resulting filtrate (usually 1 ml) is taken for the determination of phosphate. 1 ml of 4·5% trichloroacetic acid was therefore added to aliquots of standard phosphate to determine its effect on the colour developed. (6) *Phosphatase substrate*: In determining phosphatase in serum, 1 ml of serum is incubated with 10 ml of a soln. containing 5 g of sodium glycerophosphate and 4·24 g of sodium diethylbarbiturate per litre. After incubation, protein is pptd. with 9 ml of 10% trichloroacetic acid, and the filtrate is used for determination of phosphate. Accordingly 1 ml of a solution containing the following was added to aliquots of standard phosphate to determine the effect on the colour—glycerophosphate-barbiturate substrate, 10 ml; trichloracetic acid (10%), 9 ml; water, 1 ml.

Procedure—The necessary amount of phosphate soln. was introduced into test-tubes and sufficient water was added to make with the reagents a total volume of 10 ml. Additions such as trichloroacetic acid, phosphatase substrate or sulphuric acid were made next, followed by the molybdate reagent (2 ml in each test) and the stannous chloride (2 ml unless otherwise stated below). The colour which developed was estimated in a Hellige Colorimeter of the plunger type. Blanks without phosphate were made to check the reagents; generally a faint green tinge not measurable on the colorimeter was obtained.

In general, a series of tubes containing different amounts of phosphate was prepared and that containing 0·02 mg was used as the basis for comparison, the colorimeter setting for this being 20. Since, on the plunger type of colorimeter the reading varies inversely as the content of material, the quantity 20 ÷ reading was calculated in investigating proportionality.

FACTORS INVESTIGATED—The factors investigated and the findings are given below.

(1) *Stability of the phosphomolybdate blue*—The freshly prepared blue colour was matched with the following mixture—$CuSO_4.5H_2O$ (A.R.) sat. soln. 18° C., 25·6 ml; cobalt chloride (A.R.) 20% soln., 1·9 ml; $K_2Cr_2O_7$, 0·01% soln., 19·5 ml.

At a colorimeter setting of 40, this solution was matched by a soln. containing 0·02 mg of phosphorus at a reading of approx. 16. Colour development was instantaneous, although there was appreciable fading in the first 10 min. but only slow change from 10 min. to half-an-hour. Behaviour was the same in presence of trichloroacetic acid and phosphatase substrate. On standing, the colour becomes greener.

Series of standards containing 0·01 to 0·05 mg of phosphorus were prepared and read at intervals against the 0·02 member. The readings were the same after ½, 1 and 2 hr., showing that the colour-change with time is proportional to concn. Thus there need be no trouble with fading of colour if the standards are prepared at the same time as the unknown samples.

(2) *Departure from Beer's Law*—There is a linear relationship between phosphorus content and colour intensity up to 0·05 mg. There is some uncertainty in matching the 0·01 mg of phosphorus, since the colour is greener than that of stronger solutions. For reliable results, it is necessary to take an aliquot of unknown solution containing between 0·02 and 0·05 mg of phosphorus.

(3) *Effect of stannous chloride*—Increasing the amount of stannous chloride reagent increased the depth of colour developed, but the colour became progressively greener as the amount of stannous chloride increased. There is no reason to depart from the amount, *viz.*, 2 ml, recommended by Kuttner and Cohen,[8] but it must be accurately measured.

(4) *Reproducibility of colour with the same fixed amount of phosphorus*—Table I shows the colorimeter readings for a series of 5 solutions each done in sextuplicate.

Tube No. 1 of expt. 1 was used as the basis of colour comparison for expts. 1, 2 and 3; for expts. 4 and 5 tube No. 1 in each expt. was the basis of comparison for that expt.

TABLE I

Additions	1 None	2 4·5% CCl_3·COOH	3 Phosphatase substrate	4 As Col. 2	5 As Col. 3
Tube No. 1 ..	*20*	19·6	20·4	*20*	*20*
2 ..	20·6	19·5	21·3	20·0	20·8
3 ..	20·2	20·4	19·9	20·7	19·7
4 ..	20·0	20·4	19·8	21·0	19·6
5 ..	19·8	19·7	20·0	20·0	19·6
6 ..	21·1	20·7	19·9	20·9	19·7
Means ..	20·28	20·05	20·22	20·43	19·90
Standard deviation	0·484	0·509	0·571	0·484	0·465
Coeff. of variation	2·39%	2·54%	2·82%	2·37%	2·34%

There is no significant difference between the means of expts. 1, 2 and 3, showing that addition of trichloroacetic acid and phosphatase substrate does not affect the colour. This confirms the findings of Kuttner and Cohen.[8]

(5) *Stability of slope of graph connecting colour intensity and amount of phosphorus*—It has already been shown that over the range 0·01 to 0·05 mg of phosphorus there is a linear relationship between colour intensity and amount of phosphorus. Table II gives the results of a series of observations made in sextuplicate.

TABLE II

Phosphorus, mg		20÷Colorimeter reading 0·01	0·02	0·03	0·04	0·05
No additions ..	Mean	0·6846	1	1·3374	1·6754	1·9880
	Standard deviation ..	0·0361	—	0·0943	0·0592	0·1034
1 ml of 4·5% trichloro-acetic acid.	Mean	0·6192	1	1·3556	1·6908	2·0182
	Standard deviation ..	0·0245	—	0·0316	0·0575	0·0714
1 ml. of phosphatase substrate soln.	Mean	0·6243	1	1·3192	1·6330	1·9457
	Standard deviation ..	0·0500	—	0·0283	0·0283	0·0469

The equations to the three lines deduced by the statistical method of least squares are—

(i) No additions $x = 0{\cdot}0303y - 0{\cdot}0104$
(ii) Trichloroacetic addition $x = 0{\cdot}0295y - 0{\cdot}0097$
(iii) Phosphatase substrate addition $x = 0{\cdot}0317y - 0{\cdot}0117$

where x = phosphorus present in mg, and y = 20 ÷ colorimeter reading.

Statistical analysis shows that there is no significant difference between these equations, *i.e.*, that addition of trichloroacetic acid or of phosphatase substrate is without effect on the colour under the conditions described. A common equation can therefore be used. That deduced from the combined data, with due regard to the number of digits which are significant, is $x = 0{\cdot}03y - 0{\cdot}01$.

These findings do not confirm those of Bodansky,[11,12] whose formula for the same conditions, without additions of trichloroacetic acid, etc., is $\text{P in mg} = \dfrac{0{\cdot}48}{\text{colorimeter reading}} - 0{\cdot}0040$.

Additional corrections are necessary when additions of trichloroacetic acid or phosphatase substrate are made.

Table III compares the percentage errors, using Bodansky's formulae and the one now put forward.

It is worth noting that the departure from Beer's Law is due to interference with the reaction by which the colour is produced. Any of the coloured solns. when diluted obeys Beer's Law accurately over a wide range of dilutions.

(5) *Effect of sulphuric acid*—Determination of total acid soluble phosphorus in blood serum involves digestion with 10 *N* sulphuric acid. Increasing additions of 10 *N* sulphuric acid to standard phosphate solutions diminished the colour. Further there was a gradual increase in depth of colour on standing, and the colour was greener than that produced in absence of sulphuric acid. The linear relationship between colour and amount of phosphorus persists over the same range, *viz.*, 0·01 to 0·05 mg, but the slope of the line is different, and there are changes with the time of standing, as shown in Table IV.

TABLE III

Additions	True P content mg	Mean colorimeter reading	Calculated P content: Bodansky	Error	% Error	Jones	Error	% Error
None	0·01	29·28	0·0124	+0·0024	24	0·0105	+0·0005	5·0
	0·03	15·02	0·0280	−0·0020	−6·7	0·0300	0·0000	0
	0·04	11·95	0·0362	−0·0038	−9·5	0·0402	+0·0002	0·5
	0·05	10·08	0·0436	−0·0064	−12·8	0·0495	−0·0005	−1·0
Trichloroacetic acid.	0·01	32·34	0·0110	+0·0010	10·0	0·0086	−0·0014	−16·0
	0·03	14·76	0·0288	−0·0012	−4·0	0·0305	+0·0005	1·7
	0·04	11·84	0·0369	−0·0031	−7·8	0·0407	+0·0007	1·7
	0·05	9·92	0·0448	−0·0052	−10·4	0·0505	+0·0005	1·0
Phosphatase substrate.	0·01	32·13	0·0112	+0·0011	12·0	0·0087	−0·0013	−13·0
	0·03	15·17	0·0285	−0·0015	−3·0	0·0296	−0·0004	−1·3
	0·04	12·25	0·0363	−0·0037	−9·3	0·0390	−0·0010	−2·5
	0·05	10·28	0·0440	−0·0060	−12·0	0·0484	−0·0016	−3·2

TABLE IV

Time of standing	Slope of line
10 min.	0·0320
75 ,,	0·0264
19¼ hr.	0·0204

SUMMARY—An investigation of factors affecting the development of the blue colour by reduction of phosphomolybdate with stannous chloride shows that reliable and reproducible results are obtainable if conditions are rigidly controlled. The method is applicable over a range of 0·01 to 0·05 mg of phosphorus in 10 ml of solution. Addition of trichloroacetic acid or veronal-buffered solns. of sodium glycerophosphate, as used in serum analysis, do not affect the colour, and need not be made to the standards. Standards must be prepared at the same time as the samples being tested.

Addition of relative large quantities of sulphuric acid has a marked effect and similar additions must be made to the standards.

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THE CROOKES LABORATORIES
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Magnetic Stirring in the Analytical Electrodeposition of Metals

BY H. W. WEBB

(*Read at the Meeting, April* 4, 1945)

IN recent years considerably increased interest has been shown in assay laboratories in the rapid deposition of metals by electrolysis of stirred solutions and many very attractive designs have been produced by the laboratory furnishing firms. In the main, agitation has been effected by rotating either cathode or anode, or by a mechanical stirrer with Fischer type

stationary electrodes, but there has also been a revival, more particularly in the U.S.A., of the magnetically stirred solution. Apparatus for this purpose has been described by Frary[1] and by Heath,[2] but very little information seems to be available in the literature regarding the practical use of this method of agitation.

In order to find out how magnetic rotation compared with the mechanical method, an electromagnet of the Heath type was made up. The following instructions (with diagram) are as given by Scott[3]: "500 turns of No. 13 B. and S. gauge magnet wire are wound upon a copper cylinder, $2\frac{3}{4}$ in. diameter, $3\frac{1}{4}$ in. high, 3/32 in. thick, brazed watertight at the bottom to a $5\frac{1}{2}$ in. disc of 3/32 in. soft steel. Another disc, with a hole to fit the cylinder, is brazed to the top. The bottom disc has a 1 in. hole drilled in the centre for the insertion of a rubber plug through which glass tubes may be inserted for inlet and outlet of air or water to cool the electrolytic beaker. The solenoid may be in series in the electrolytic line, or excited separately."

Scott gives the dimensions of the cathode as constructed of gauze 40 mesh to the inch with a depositing surface of 100 sq.cm., slit to permit quick removal from the electrolyte without removing the anode, but does not give any details of the anode employed. A current of 4·5 amp. is mentioned for both electrolysis and solenoid.

Slight differences between the magnet described and that actually used were that the centre cylinder was continued above the top cheek for $\frac{1}{2}$ in., with an overflow tube brazed into this section (this dispensed with the glass outlet tube), an inlet tube brazed into the bottom cheek, and a small platform provided for the beaker $\frac{1}{4}$ in. above this cheek, in place of the knob in the Heath design.

Experiments showed that the stirring effect is controlled by: (1) Strength of the magnetic field. (2) Value of the electrolysing current. (3) Size of the anode. (4) Position of the electrodes in respect to the field and electrolyte. (5) Quantity of solution (electrolyte).

For maximum stirring the highest possible field strength must be attained, and as high a current density on the cathode and anode used as is practicable. When either the field strength or the current density drops, stirring becomes less efficient.

Rotation is also very largely controlled by the size of the anode. Other conditions being equal, the smaller the anode the greater the stirring. The small anode becomes heated when large currents are used and this causes the solution to become hot, but water cooling is advocated in any event, and the Heath design makes provision for this.

A small anode precludes the reliable determination of lead, manganese or other anodic deposits, but if these elements are required, for instance in a bronze, after removal of copper on the cathode, a fresh electrode may be immersed in the electrolyte in place of the cathode and the current direction reversed. The revolution effect is the same whether the larger electrode be cathode or anode.

The electrolysis beaker should be small, a 150-ml size being recommended, and the cathode, which should be of gauze, should fit fairly tightly into it, to prevent the formation of stagnant areas on the outside of the electrode. The anode should be carefully adjusted to be in the centre of the solution, and equidistant from all points of the cathode. The reason for this is that a vortex tends to be created and maintained more easily, and therefore the stirring action is greater, when these conditions are observed. The greater the volume of electrolyte used, the more inertia there is to be overcome; hence the electrolyte should be kept at low volume.

EXPERIMENTAL TESTS—Experimental depositions were carried out, using in each test 1 g of copper as nitrate, 5 ml of free nitric acid and 2·5 ml of sulphuric acid; total bulk with water, 100 ml in a 150 ml beaker.

An attempt to count the rate of rotation of the solution was made with the aid of a small splint of wood which circled around the anode. Whilst it is realised that this method does not give very accurate figures for the r.p.m., nevertheless it does give a fairly reliable estimate of the comparative stirring efficiency of the electrodes and current densities used.

For the results shown in Table I three types of platinum anode were used. First, the cylindrical type of gauze anode weighing about 30 g, 40 mm high and 25 mm across; secondly, the simple helical wound wire with 6 turns, 10 mm across, 7 mm spacing; thirdly, a straight wire 2 mm thick. The current for the electrodes was in series with the magnet. Chaston and Webb[4] report that the speed of deposition and nature of the deposit are in no way influenced by the use of a small anode in efficiently stirred solns., and this has been verified in the present work.

A slow stream of water circulated through the apparatus at approximately the same rate during each expt.

The deposits with the gauze anode were not appreciably better than would be expected from depositions at the same current density from solns. completely unstirred except for the agitation effect of gas evolution and heat. Drop tests were taken out at frequent intervals into a dip tile containing fresh hydrogen sulphide water until negative results were obtained. No attempt was made to weigh the deposits, the facts of complete deposition being so well-known, and the results were judged solely by appearance. The very marked improvement shown as the anode size was reduced and the rotation increased is sufficient proof of the necessity for effective stirring at high current densities.

TABLE I

		Volts	Amp.	Volts at electrodes	Temperature ° C. start	Temperature ° C. finish	Time (min.)	Revs.	Colour and adherence of deposit
Gauze anode	1	12	5	3	22	27·5	60	50	Fair
,, ,,	2	12	10	3	26	30	35	80	,,
Helical anode	3	12	5	3·25	24	25	50	80	Good
,, ,,	4	12	10	5	28	32	25	150	Excellent
Straight wire	5	12	5	4	26	24	50	240	,,
,, ,,	6	12	10	5	26	43	20	too fast to count	,,

Observations (Table II) were then made on the comparative rotation of the solution, using varying current conditions, with the helical and straight wire anodes, and with separate excitation and electrolysing current.

TABLE II

	Electrolysing current amp.	Magnet current amp.	Rotation r.p.m.
Helical wire	2·5	10	52
	2·5	5	25
	2·5	2·5	10–15
Straight ,,	2·5	10	200
	2·5	5	120
	2·5	2·5	72
Helical ,,	5	10	90
	5	5	60
	5	2·5	26
Straight ,,	5	10	too fast to count
	5	5	200
	5	2·5	100
Helical ,,	10	10	120
	10	5	80
	10	2·5	50
Straight ,,	10	10	too fast to count
	10	5	250
	10	2·5	170

The straight wire is bound to be more efficient as a stirrer than the helical wire, as vortex formation is hindered in the latter type.

CONCLUSIONS—Good results can be obtained from magnetic stirring if the current density and magnet current are kept high enough, and small anodes are used. The system is cheaper than mechanical stirring devices, and there cannot be any mechanical breakdown. At the same time it is realised that there is not the elasticity as to conditions which can be used, such as volume of electrolyte, shape and size of electrodes, etc., as can be obtained by means of mechanical stirring. My personal preference is for mechanical stirring if available, but I would rather use magnetic stirring than still electrolysis, or agitation by a stream of air bubbles.

PERMANENT MAGNETIC FIELD—It seemed reasonable to assume that equivalent results might be obtained by the use of a permanent magnetic field, thus cutting down complexity of manufacture, and rendering magnet conditions fixed and foolproof. Experiments with a view to obtaining equal stirring effect to the Heath magnet were conducted, using the only magnets obtainable, in various positions.

If this system could be employed successfully, it would allow the electrolysis to be watched far more conveniently than can be done with the Heath magnet. Water cooling could be arranged by using a glass cell, and the assay observed continuously throughout the operation, which is not possible with the electro-magnet.

A constant current of 5 amp. was used at the electrodes, with a straight wire anode, other conditions being the same as before, *viz.*, 1 g. of copper as nitrate, 5 ml of free nitric acid, 2·5 ml of sulphuric acid; total 100 ml in a 150 ml beaker. In each test the electrolysis beaker was immersed in a water-cooled cell (copper).

The results show that under optimum conditions a permanent magnet might well be substituted for the Frary or Heath magnet, and the resulting arrangement would be cheap, foolproof, and if intelligently used, efficient.

Four "Alni" magnets supplied by Messrs. Darwins Ltd and one open cylindrical magnet supplied by Messrs. William Jessop & Sons, Ltd., were used. The "Alni" magnets were cylinders 3 in. long by $1\frac{9}{10}$ in. in diam., with a central hole used for bolting on the soft iron pole pieces in various positions as shown below. The open cylinder magnet was $3\frac{1}{8}$ in. high by $3\frac{3}{8}$ in. external diam. with a 5/16 in. wall thickness. In this instance the magnet completely surrounded the electrolysis beaker, but the rotation speed was not so good as the single magnet position.

It is not yet certain that the best possible results have been obtained with this system. A properly cast conductor and a magnet made specially for the purpose would, it is believed, give much improved results. Up to the moment, owing to war conditions, such apparatus has not been obtained.

SUMMARY—Experiments using magnetically stirred electrolytes in the electro-deposition of metals are described, and the suggestion is made that permanent magnets might be substituted for electro-magnets.

In conclusion, I would like to express my thanks to Mr. A. R. Powell and Dr. J. C. Chaston for their interest and criticism, and also to the Directors of Messrs. Johnson, Matthey & Co., Limited, for permission to publish this paper.

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RESEARCH LABORATORIES
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December, 1944

Notes

ESTIMATION OF VITAMIN A BY THE ANTIMONY TRICHLORIDE METHOD USING THE SPEKKER PHOTOELECTRIC ABSORPTIOMETER

THE transient nature of the blue colour developed by the interaction of antimony trichloride and vitamin A in chloroform solution renders impracticable the null method for the colorimetric estimation of that vitamin by the Spekker absorptiometer. A modification of the null method has, however, been developed and has been found practicable for the estimation of the vitamin A content of the livers of sharks.

METHOD—*Reagents*—(*a*) A saturated solution of antimony trichloride in fresh C.P. chloroform. Fresh reagent should be used or, alternatively, it may be stored in 5-ml aliquots in ampoules until required; (*b*) Acetic anhydride.

Setting the Spekker Absorptiometer—The galvanometer supplied with the instrument was replaced by a reflecting galvanometer with a sensitivity of 450 mm per micro-ampere. A 50-cm scale was used in conjunction with the galvanometer and placed about $5\frac{1}{2}$ feet from it.

To set the absorptiometer, a 1-cm cell containing distilled water was placed in front of the iris diaphragm, and a 1-cm cell containing 2 ml of chloroform, one drop of acetic anhydride and 5 ml of antimony trichloride reagent was placed behind the calibrated variable aperture. The 5 ml of reagent and the 2 ml of chloroform were sufficient just to fill the cell. The acetic anhydride was added to prevent cloudiness due to the pptn.

of antimony oxychloride and, as an additional precaution, the cell was closed with a cover glass. The red filters supplied with the Spekker instrument were used to increase the sensitivity of the instrument.[1]

With the cells in place the drum was set to 0·70 and the galvanometer was set to give a scale reading of 40. The iris diaphragm was then adjusted so that the galvanometer showed zero deflection from the scale reading of 40 when the instrument was turned on. The drum was then set to 0·80 and the sensitivity control adjusted to give a galvanometer deflection from 40 to 25. Next the drum was reset at 0·70 and small adjustments were repeated at 3-minute intervals until there were no irregularities. About six such repetitions were found to be sufficient to eliminate the slight fluctuations that followed the initial setting. The usual precautions in the use of red filter were observed.[1]

Careful adjustment of the zero deflection point (drum reading 0·70) and the sensitivity control (drum reading 0·80) ensures that readings for the standard graph and subsequent readings with samples will be made under the most comparable conditions possible.

The Standard Graph—A standard soln. of vitamin A was prepared by dissolving 0·5 g of distilled vitamin A concentrate (100,000 units) in chloroform, and diluting to 1 litre. Aliquots of 25·0, 22·5, 20·0, 17·5, 15·0, 12·5, 10·0, 7·5, 5·0 and 2·5 ml were withdrawn into 50-ml graduated flasks and made to the mark with chloroform. One-ml aliquots from these flasks represented a range of from 50·0 to 5·0 units of vitamin A.

A 1-ml aliquot equiv. to 5·0 units was placed in a 1-cm cell. One ml of chloroform and 1 drop of acetic anhydride were then added and the cell was placed on the slide. The drum was set at 0·7 and 5 ml of antimony trichloride reagent were quickly added to the cell and the maximum deflection of the galvanometer was noted. Speed is essential in sliding the cell into place and switching on the absorptiometer after addition of the reagent. The same procedure with the 1-ml aliquots, equiv. to 10, 15 . . . 50 units, gave a series of galvanometer deflections (Table I) from which a graph could be prepared. When the log. of the concentration was plotted against the log. of the deflection a straight line graph resulted.

TABLE I

Vitamin A units per 7 ml (*i.e.*, per 1-cm cell full)	0	5	10	15	20	25	30	35	40	45	50
Galvanometer deflection, cm	0	4·7	11·0	15·0	19·0	23·4	26·5	29·4	34·2	36·4	39·3

For the estimation of the vitamin A content of sharks' liver oil 5 drops of oil were weighed and dissolved in chloroform, and the soln. was made up to 50 ml. A 1-ml aliquot of this soln. was then treated exactly as described for the standards and the vitamin A content was found from the graph.

SUMMARY—A method is presented for the colorimetric estimation of vitamin A by a modified technique in the manipulation of the Spekker absorptiometer. The method is rapid and sufficiently accurate for classifying the livers of various species and varieties of sharks according to their vitamin A content. The method should be capable of wider application.

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DEPARTMENT OF SCIENCE AND AGRICULTURE
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March, 1945

It is obvious that the results given in Table I apply only to the technique described. In this country alcohol-free chloroform is used as the solvent, so that the colours obtained may differ from those found by the authors with C.P. chloroform (which contains alcohol) as solvent.—EDITOR.

THE COLORIMETRIC ESTIMATION OF MORPHINE

THE official method[1] for the estimation of morphine in small quantities is based on the reaction with nitrous acid described by Radulescu.[2] A nitroso compound is formed by addition of sodium nitrite to an acid soln. of morphine, and subsequent addition of excess alkali produces an orange-brown colour, the intensity of which is a function of the amount of morphine present. Various applications of the reaction to the rapid and approximate estimation of morphine have been described,[3,4,5,6] but only one attempt,[7] employing the Zeiss Step-photometer, appears to have been made to use it for more precise determinations.

In investigating the optimum conditions for the production of the colour with pure morphine hydrochloride solutions, we used the Spekker absorptiometer, with 4-cm cells and an Ilford 601 screen. Addition of ammonia immediately after the nitrite, as in the B.P. procedure, gave erratic results, and a study of the time during which the nitrite is allowed to react in acid solution showed that it is necessary to allow 15 minutes before making ammoniacal. The curves obtained for various quantities of morphine showed the rapid increase in final colour with increasing time of reaction in acid solution. When the reaction is allowed to proceed for 15 min. the colour produced conforms with Beer's law.

Optimum quantities and concns. of acid and nitrite were also established. These were 20 ml of 0·1 *N* hydrochloric acid and 8 ml of 1% sodium nitrite soln. Temperature variation from 15° to 25° C. produced a difference in the extinction of the final solution of less than ±1%. The apparent morphine content differed from the true value by −4% at 28° C. and −8% at 30° C. Identical results were obtained with a freshly-prepared 1% soln. of sodium nitrite and a soln. 14 days old, which had been kept in a glass-stoppered amber bottle; but all recorded results were obtained with freshly-prepared nitrite. Adequate stability of the colour in the final soln. was found, a series of readings taken at 5-minute intervals showing a gradual steady decrease in the extinction value, amounting to less than 1% in 1 hr.

Under the optimum conditions described, no measurable colour was given by quantities up to 5 mg of codeine, hyoscine, narcotine, papaverine, atropine and pethidine. The method would naturally be useless in presence of large quantities of any bases insoluble in ammonia, without preliminary separation of the morphine by, *e.g.*, lime-water extraction.

For the construction of a standard curve, two series of measurements were made with 0·1% morphine solns. prepared independently from pure morphine base which had been recrystallised from aqueous alcohol. This pure morphine was assayed by solution in standard acid and back-titration with sodium hydroxide to methyl red. In practice the difference method for measuring extinction was employed, *i.e.*, setting the instrument with the blank in position at a drum reading of 1·0. The difference between 1·0 and the observed drum reading is then the extinction value plotted.

Duplication of results and checks against standard macro assays indicate that the error in morphine estimation by this method does not exceed ±2%. Using the "compensated blank" technique[8] it has been found possible to apply the method accurately, without preliminary extraction, to "Omnopon" (Roche Brand of Papaveretum) and to "Omnopon"-Scopolamine solns. for injection. Extension of the method to various galenicals is being investigated.

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ANALYTICAL LABORATORIES
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D. C. M. ADAMSON
F. P. HANDISYDE

March, 1945

Ministry of Food

STATUTORY RULES AND ORDERS*

1945—No. 725. Order, dated June 15, 1945, amending the Feeding Stuffs (Regulation of Manufacture) Order, 1944. Price 2d.

This Order, which *came* into force on July 1, 1945, increases the proportion of compounds, concentrates and livestock mixtures which may be manufactured; alters the composition of National Compounds by reducing the animal-protein-rich substances, reducing the total albuminoid content, and increasing the cereal contents; and prohibits the inclusion, except under licence, of iodine or iodine derivatives (other than potassium iodide) in any compound or mixture.

— **No. 798. Order dated June 28, 1945, amending the Meat Products, Canned Soup and Canned Meat (Control and Maximum Prices) Order, 1944.** Price 1d.

This Order, *which came into force on July 5*, 1945, provides for the use of a reduced quantity of pork in pork sausage, pork sausage meat, and pork slicing sausage. The quantity of pork used must be at least 80% instead of at least 90% of the meat content of the sausages or sausage meat. There is no alteration in the total meat content (*viz.*, 50%).

The following definition of "Pork sausages" is substituted for that given in the former Order:—

" 'Pork sausages' means sausages which are ordinarily known and sold as pork sausages and the casings of which are hog or sheep casings."

PROPOSED STANDARD FOR SALAD CREAM AND MAYONNAISE†

THE Ministry of Food has under consideration the issue of an Order under Regulation 2 of the Defence (Sale of Food) Regulations, 1943, prescribing a standard for Salad Cream and Mayonnaise.

The Inter-Departmental Committee on Food Standards has made the following recommendations as to the standard:—

1. Salad Cream and Mayonnaise should be required to contain not less than 25% by weight of edible vegetable oil, and not less than 1·35% by weight of egg yolk solids.
2. The standard should not apply to a product sold under the description "Salad Dressing" or "Salad Sauce," and, if necessary, products sold under these descriptions should be specifically excluded from the ambit of the Order creating a standard for salad cream and mayonnaise.

In a précis of the Committee's report recently issued by the Ministry it is stated that products described as mayonnaise are similar to, although usually more viscous than, salad cream, the latter term having been coined in the 1920's to describe a smooth, non-separable, vegetable oil emulsion then being introduced on the market.

Although the proportion of oil contained in salad cream and mayonnaise ranged from 25 to 48% before the war, the manufacturers' suggestion that 25% by weight should be the minimum proportion of oil has been adopted in view of the restricted supply of vegetable oil, and as a means of maintaining the output of a reasonable product. The standard will require that the oil used in salad cream be of vegetable origin: it was considered improbable that any manufacturer would wish to use animal or other fats instead.

The Committee accepted the view that egg is an essential ingredient, and that a content of 2% of whole egg solids, corresponding to 7·5% of liquid whole egg, would be a reasonable minimum. Although whole

* Obtainable from H.M. Stationery Office. Italics signify changed wording.
† Press Notice 3696a, July 2, 1945.

egg is of necessity the ingredient at present in use, manufacturers may wish, in the future, to utilise yolk only, either liquid or dried, as soon as it becomes available Since it is the yolk fraction which confers on the emulsion both stability and palatability, the white being relatively unimportant in this respect, the Committee decided that the minimum of 2% of whole egg solids should be expressed as yolk solids, the equivalent figure of 1·35% of egg yolk solids being recommended.

Although a reference to the presence of sugar was included in the standard submitted to the Committee, no proportion was specified. Further enquiry showed that unanimity did not prevail among manufacturers as to the desirability of the inclusion of sugar, and, therefore, the Committee cannot regard sugar as an essential ingredient to which reference is necessary in the standard.

If an Order prescribing a standard for salad cream and mayonnaise is made under the Defence (Sale of Food) Regulations, 1943, the effect of the Food Standards (General Provisions) Order, 1944, as amended, would be to apply the standard also to products sold under such descriptions as might lead an intending purchaser to believe he was purchasing salad cream and mayonnaise. Should it be considered that terms such as "Salad Dressing" or "Salad Sauce" might lead a purchaser to believe he was purchasing a preparation conforming to the standard, then it is recommended that the Order creating the standard should make it quite clear that the standard does not apply to a product sold under these descriptions.

In this connection, the Ministry of Food announces that when a standard for salad cream and mayonnaise is made, steps will be taken by amendment of the Labelling of Food (No. 2) Order, 1944, to make it clear that the requirement that the ingredients shall be specified on the label will apply to any other salad sauce or other salad dressing, though it will not apply to products complying with the standard to be prescribed.

Manufacturers or others were asked to send any comments on these proposals to the Ministry not later than July 21, 1945.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

Food and Drugs

Vapour Pressure Measurements as an Index to Moisture in Dehydrated Vegetables. H. Fischbach (*J. Assoc. Off. Agr. Chem.*, 1945, **28**, 186–191)—A critical examination of oven-drying methods for determination of moisture in dried vegetables suggests that the results are data related to the moisture content rather than the true moisture-content. Vapour pressure measurements are suggested as a more accurate criterion for the state of "wetness" of a food material, an important property in relation to quality and stability. The apparatus is a modification of that used by Makower and Myers (*Proc. Inst. Food Tech.*, 1943, 156). It consists of two 500-ml bulbs connected at their bases by means of a manometer tube containing Octoil, a low vapour-pressure oil (Distillation Products, Rochester, New York). The bulbs are connected also by a horizontal tube fused into their sides and carrying a stop cock. The neck of one bulb is connected with a vacuum pump and that of the other with a 50-ml sample flask by means of a wide glass tube, each bulb being provided with a stop cock.

Place the sample of dried vegetable in the 50-ml flask and gently open the stop cock to bring the pressure inside the flask down to the low pressure (0·1 mm) to which the remainder of the system has previously been evacuated. Immediately submerge the 50-ml flask in a freezing-mixture of acetone and "dry ice" and evacuate the system to a pressure of less than 0·1 mm (*e.g.*, 25 to 50 μ). By means of the stop-cock on the horizontal tube shut off the two bulbs from each other. Replace the freezing-bath by a constant temp. bath at 35° C. and allow the system to attain equilibrium, *i.e.*, the point at which the manometer reading remains constant for 30 min. Obtain a second reading by shutting off the sample flask, re-connecting the two bulbs, evacuating the system so that the manometer reads zero, disconnecting the two bulbs by closing the tap on the horizontal tube and gently re-connecting the sample flask with the system. The second reading is considered the more accurate because removal of the vapour collected for the first reading reduces to a minimum the effect of atmospheric humidity. It was found that the difference between the first and second readings is equal to the result of a blank determination made with the sample flask empty. For routine purposes therefore the first reading may be accepted after correction for the blank result. Curves are given showing the relation between the apparent moisture content as determined by the A.O.A.C. method for moisture in dried fruits and the vapour pressure. With dried potatoes and beets of unknown variety and mode of manufacture the apparent moisture content predicted from vapour pressure measurements was within 0·1% of the result of drying in the vacuum oven for 22 hr. at 70° C. Within the range of the expts. the particle size of the material affected only the time of attaining equilibrium in the apparatus. A. O. J.

Munson-Walker reducing Values of some Less Common Sugars and of Sodium Glycuronate. L. E. Wise and D. C. McCammon (*J. Assoc. Off. Agr. Chem.*, 1945, **28**, 167–174)—In the investigation of mucilages used in paper-making and of the composition of hemicelluloses of pulpwoods, the action of various yeasts on certain sugars and uronic acids was studied, the fermentation being followed by using the Munson-Walker method (*J. Amer. Chem. Soc.*, 1906, **28**, 663; ANALYST, 1906, **31**, 337). Data could not be found in the literature for the Munson-Walker reducing values of some of the sugars under investigation. Consequently the reducing values, over a limited range, of *d*-mannose, *d*-galactose, *d*-xylose, *l*-arabinose, *l*-rhamnose and *l*-fucose were determined, no attempt being made to emulate the precision or refinements attained by Munson and Walker or by Hammond (*J. Research Nat. Bur. Standards*, 1940, **24**, 597). The procedure used was that of the A.O.A.C. ("*Methods of Analysis*," 1940, 500), the cuprous oxide being weighed directly. Since the fermentation usually involved 20 to 75 mg of sugar the cuprous oxide values of most interest were those within this range, but in some instances the

range was considerably wider. Ordinarily from four to six different wts. of the sugar were used in a series and the precision was usually within 1%. The data for a particular sugar were correlated graphically on a large scale, and the classical method of least squares was not used for computation. In general that section of each curve that was of greatest interest in the fermentation studies fell on a nearly straight line. Tables were constructed correlating the wt. of cuprous oxide (20 to 275 mg in 1-mg increments) with the corresponding wt. of the sugar.

A specific example of the use of the data is quoted. To determine small amounts of galactose in presence of mannose, fructose, glucose, xylose, arabinose and glycuronic acid a method depending upon differential fermentation with two yeasts was used (Wise and Appling, *Ind. Eng. Chem., Anal. Ed.*, 1944, **16**, 28; ANALYST, 1944, **69**, 194). The yeast N.R.R.L. No. 379 ferments galactose and the other common hexoses, and N.R.R.L. No. 966 leaves galactose unfermented. Neither organism appreciably ferments the pentoses or glycuronic acid. The wt. of cuprous oxide found after fermentation with No. 379 subtracted from that obtained after the use of No. 966 gives a direct measure of the galactose present, the wt. of which may be read from the table. A somewhat similar fermentation method has been devised for the specific detn. of xylose in presence of other sugars and glycuronic acid by means of the micro-organism *Hansenula suaveolens*. Browne (*J. Amer. Chem. Soc.*, 1906, **28**, 439; ANALYST, 1906, **31**, 265), using Allihn's method determined the wts. of copper corresponding with definite amounts of arabinose, xylose, fructose and galactose over a range of 50–250 mg of sugar. From Allihn's tables he then took the glucose value corresponding with each copper value and computed the ratio of glucose to each of these reducing sugars. The average ratios found by Browne were 1·032 for arabinose, 0·983 for xylose and 0·898 for galactose. Analogous ratios were computed from the data now tabulated and Munson and Walker's tables. The average ratios were 1·031 for arabinose (range, 17–75 mg), 0·992 for xylose (range, 25–125 mg) and 0·901 for galactose (range, 25–125 mg). These ratios are very similar to those of Browne. Over a range of 25–142 mg of sodium glycuronate, the glucose-glycuronate ratio was quite constant, the extremes being 0·725 and 0·740 with an average for all experimentally determined values of 0·733. A. O. J.

Determination of the Peroxide Value of Edible Fats and Oils. Influence of Atmospheric Oxygen in the Chapman and McFarlane Method. C. H. Lea (*J. Soc. Chem. Ind.*, 1945, 106–109)—Small amounts of atmospheric or dissolved oxygen seriously affect the peroxide values determined by the Lips, Chapman and McFarlane photometric ferric thiocyanate method (*Oil and Soap*, 1932, **9**, 89) or the volumetric ferrous salt method (*Ind. Eng. Chem.*, 1931, **23**, 1254; *id.*, ANALYST, 1936, **8**, 198). Peroxide values by a modified ferric thiocyanate method were approximately twice as high as those obtained by the iodimetric method. The modifications were: (1) heating for 11 min. in a thermostat at 50° C. instead of as in the less reproducible original method, and (2) replacement of the Coleman spectrophotometer set at 485 $m\mu$ by the Zeiss Pulfrich photometer with filter No. 2 (470 $m\mu$). At peroxide values below 5 mg-equiv./kg the weight of fat taken was increased; at values above 80 it was reduced. When oxygen is rigorously excluded from the tests, by methods described in detail, peroxide values are reduced to about one quarter of the original values. While it is possible that free oxygen may be necessary for completion of the reaction between preformed peroxide and reducing agent, the necessity for assuming an equivalent of 8 for peroxide oxygen in order to correlate oxygen absorption with peroxide value suggests that the latter values are at least twice too high. The degree of further oxidation occurring during the determination is roughly proportional to the quantity of peroxide already present.

Oxidation in the ferrous salt method seems to occur when the fat and reagent are brought together in presence of dissolved oxygen, *i.e.*, during or immediately preceding reduction of the peroxide. Before or after this stage the system is much less sensitive. E. B. D.

Polyethenoid Acids of the C_{18} Series in Milk and Grass Fats. T. P. Hilditch and H. Jasperson (*J. Soc. Chem. Ind.*, 1945, 109–111)—Results of spectrographic analyses of glycol solutions of an unsaturated C_{18} ester-fraction from cow milk fat, of methyl esters of a concentrate of the more unsaturated C_{18} acids of goat milk fat, and of mixed fatty acids of pasture grass are given; also results of spectrographic analyses of the same samples, in glycol, after isomerisation by alkali at 170° C. for 15 min. and at 180° C. for 60 min. These indicate the presence in the milk fats of octadecadienoic acids, which yield conjugated diene and triene acids on isomerisation. In grass fats, large amounts of octadecatrienoic acid also occur. These observations, with earlier ones, indicate that the diethenoid C_{18} acids of the milk fats consist of cis-Δ^9-trans-Δ^{12}- and/or trans-Δ^9-cis-Δ^{12}-octadecadienoic acids. Approximate compositions of the component acids are given. Cow milk fatty acids contain both conjugated and non-conjugated forms of the octadecadienoic acid, with traces of octadecatrienoic acids. It is calculated that the original goat milk fatty acids contained *ca.* 1·1% of non-conjugated and 0·7% of conjugated octadecadienoic acids, with *ca.* 0·9% of non-conjugated and a trace of conjugated octadecatrienoic acids. The fatty acids of both milks consist mainly of oleic acid. The non-conjugated triethenoid C_{18} acids (probably linolenic) are the chief component acids in grass fatty acids, and, while oleic acid forms a considerable proportion of the acids (*ca.* 32%, from iodine vals.), the proportion of non-conjugated diethenoid C_{18} acids (including linolic) is the lowest (*ca.* 11%) of the three main categories of unsaturated acids. E. B. D.

Black Pepper from Sierra Leone. G. T. Bray and F. Major (*Bull. Imp. Inst.*, 1945, **43**, 6–7)—The following results were obtained for black pepper grown in Sierra Leone:—moisture 12·1; total ash 3·7; ash insol. in HCl 0·06; fixed oil 5·5; volatile oil 1·1; starch, by acid hydrolysis, 44·7; crude fibre 9·6; crude proteins 9·7; piperine 2·95%. (Proteins were calculated as 6·25 times the difference between total N and N in fixed oil; piperine, as 20·36 times the N in fixed oil.) Results previously recorded for (1) fixed oil, (2) piperine and (3) starch were (1) 6·10 to 9·64; (2) 4·89 to 9·78 and (3) 28·0 to 43·47. U.S.A. and Australian standards for (1) are not under 6·75 and 6·0% respectively; for total ash, not over 7·0%. As the authenticity of the material is beyond doubt, the Ministry of Food consider that

the analytical figures obtained would not hinder the free sale and utilisation of the pepper in the United Kingdom. E. B. D.

Biochemical

Estimation of the Albumin and Globulin Contents of Human Serum by Methanol Precipitation. L. Pillemer and M. C. Hutchinson (*J. Biol. Chem.*, 1945, **158**, 299–301)—The method is based on the observation that normal human serum can be satisfactorily separated into its globulin and albumin components by treatment with methanol. At 0° C. all the serum albumin is soluble in 42·5% methanol at a *p*H of 6·7 to 6·9 and at ionic strength of about 0·03, whilst the globulins are almost quantitatively pptd. The results agree within 5% of the values obtained by electrophoresis. All manipulations should be carried out at 0 to 10° C. Pipette 2·0 ml of fresh serum into a 15-ml conical centrifuge tube, and add 1 ml of acetate buffer (72 ml of *M* acetic acid and 12 ml of *M* sodium hydroxide in 1 litre of water), with stirring, followed by 7·0 ml of cold methanol (mix 607 ml of methanol with 393 ml of water, cool to 0° C, and then make up to 1 litre with cold methanol). Mix thoroughly, leave at 0° C. for ½-hr. and, if a refrigerated centrifuge is available, centrifuge at 0 to 2° C. at 3000 r.p.m. for 15 min., and decant the clear supernatant liquid. If such a centrifuge is not available, filter the suspension in a cold room through a fluted No. 42 Whatman filter-paper. Analyse the clear supernatant or filtrate for nitrogen, and calculate the albumin percentage from the total protein nitrogen. F. A. R.

Routine Estimation of Haemoglobin as Oxyhaemoglobin. G. H. Bell, J. W. Chambers and M. B. R. Waddell (*Biochem. J.*, 1945, **39**, 60–63)—Visual colorimetric estimations of haemoglobin are probably not sufficiently accurate even for clinical purposes and a description is given of a method involving the use of a photoelectric colorimeter. A number of procedures have already been described in which such instruments are used, but the methods are rather complicated. A simpler procedure, involving the use of oxyhaemoglobin, was suggested by Bell and Guthmann (*J. Sci. Instrum.*, 1943, **20**, 145); this has been further developed.

To 0·5 ml of blood in a 50-ml flask add gradually 2 ml of conc. sulphuric acid and leave overnight. Add 2 ml of sat. potassium persulphate soln., leave for 1 hr. and dilute to *ca.* 25 ml with water. Add 2 ml of 10% sodium tungstate soln., leave for 1 hr., make up to 50 ml and filter. To 20 ml of the filtrate add 1 ml of sat. potassium persulphate soln. and 4 ml of 3 *N* potassium thiocyanate soln. Evaluate the colour of the ferric thiocyanate exactly 3 min. later, using an Ilford Micro 2 light-filter.

One disadvantage of the method compared, *e.g.*, with the alkaline haematin or the cyanmethaemoglobin method is that, although reduced haemoglobin is converted into oxyhaemoglobin, methaemoglobin and other abnormal pigments are unaffected. Errors due to the presence in the blood of several forms of haem-pigment would obviously be reduced if a wave-band could be found at which all the pigments present in blood have the same optical density. With the green filter recommended there is very little difference in the density of these pigments, and even if 10% of the haemoglobin in a blood sample were in the form of one of these pigments there would be less than 1% alteration in the density reading.

The results agreed well with those obtained by the titanous chloride method of King, Gilchrist and Delory (*Lancet*, 1944, **1**, 239). F. A. R.

Simple Test for the Detection of Bile Pigments in Urine. J. Yudkin (*Brit. Med. J.*, 1945, i, 445)—A qualitative test for the detection of bile pigments in urine using the principle of "adsorption colorimetry" (Yudkin, *J. Trop. Med. Hyg.*, 1945, 48; *Nature*, 1945, **155**, 50) is described. *Method*—To *ca.* 10 ml of urine contained in a test tube add silica gel powder (60–120 mesh) from a scoop graduated to contain *ca.* 0·25 ml and mix by swirling every 1 to 2 min. during 10 min. Allow to settle, decant the urine, and wash once with a few ml of water by decantation. The presence of bile pigments is indicated by a brown coloration of the silica gel. Under these conditions mepacrine is also adsorbed (*loc. cit.*) to give a yellow colour, but the presence of the drug could be ascertained from the history of the patient. It is claimed that the method is more sensitive than current "bedside tests" and compares favourably with more elaborate laboratory methods. Using a solution of pure bilirubin in urine, the minimum concns. detectable by the various tests were found to be—Iodine, 3 mg/100 ml; Gmelin, 1·5 mg/100 ml; silica gel, 0·2 mg/100 ml; Fouchet, 0·1 mg/100 ml. Of 100 samples of urine examined, 58 were negative by all four tests, 42 positive by the silica gel and Fouchet tests, 8 positive by the Gmelin and only 1 by the iodine test. All but one of the positive samples were from patients with known hepatic damage. J. A.

Use of Specific Decarboxylase Preparations in the Estimation of Amino Acids and in Protein Analysis. E. F. Gale (*Biochem. J.*, 1945, **39**, 46–52)—In previous papers specific enzyme preparations for the decarboxylation of *l*(+)-glutamic acid and *l*(+)-ornithine (Gale, *Biochem. J.*, 1941, **35**, 66), of *l*(+)-lysine (Gale and Epps, *Biochem. J.*, 1944, **38**, 232), of *l*(−)-tyrosine (Epps, *Biochem. J.*, 1944, **38**, 242) and of *l*(−)-histidine (Epps, *Biochem. J.*, 1945, **39**, 42) have been described. These preparations have now been used for the rapid analysis of amino acids in protein hydrolysates. The method depends on the manometric measurement of the carbon dioxide liberated from the amino acids by the specific decarboxylase at the optimal *p*H and at 30° C. Use Warburg manometers containing measured amounts of amino acid solution or protein hydrolysate, together with 1·5 to 2·0 ml of buffer in the main cup and 0·5 ml of enzyme preparation made up in the same buffer in the side bulb. For lysine and ornithine use 0·2 *M* phosphate buffer, *p*H 6·0, for tyrosine 0·2 *M* citrate buffer, *p*H 5·5, and for glutamic acid and histidine 0·2 *M* acetate buffer, *p*H 4·5. At *p*H 5·5 or less retention of carbon dioxide is very slight, but at *p*H 6·0 as much as 8 to 10% of the liberated carbon dioxide may be retained in solution. For lysine and ornithine therefore it is advisable to use the "acid-tip" manometric method of Woods and Clifton (*Biochem. J.*, 1937, **31**, 1774) to ensure the complete liberation of retained carbon dioxide, 0·25 ml of 8 *N* sulphuric acid being added from the second side bulb at the end of the experiment. It is important that the "acid-tip" should be carried out in control manometers on all reagents.

Prepare protein hydrolysates by dissolving 1 g

in conc. hydrochloric acid, adding water to make the final concn. of acid 20% and then hydrolysing for 24 hr. under a reflux condenser. Evaporate to dryness *in vacuo*, add water, again evaporate and repeat 3 or 4 times to remove the free hydrochloric acid. Dissolve the dried material in *ca.* 15 ml of water, adjust to *p*H 5 and make up to a convenient volume, *e.g.*, 25 ml, so that a sample of 0·3 to 1·0 ml liberates 0·1 to 0·4 ml of carbon dioxide.

The lysine, histidine, tyrosine, glutamic acid and ornithine contents of a variety of proteins obtained by this method were compared with those reported in the literature and with analyses carried out in parallel by other methods. On the whole, agreement was very good. F. A. R.

Enzymatic Micro-analysis of Purine Compounds. H. M. Kalckar (*J. Biol. Chem.*, 1945, **158**, 313–314)—Hypoxanthine, xanthine and allantoin do not absorb, or absorb only slightly, at 290 *mμ*, whereas uric acid absorbs very strongly at this wavelength. Hypoxanthine can therefore be estimated by incubating with purified xanthine oxidase and following the increase in absorption at 290 *mμ*. A lag phase is encountered in the reaction owing to the formation of xanthine as an intermediate. With xanthine as substrate, the same rise in absorption at 290 *mμ* occurs, but without the lag phase. One μg of hypoxanthine, or 0·9 μg of xanthine, per ml on oxidation causes an increase of 0·075 in the extinction at 290 *mμ*, and it is possible to determine 0·1 μg of hypoxanthine in 0·1 ml with an accuracy of about 5%. If inosine is the substrate, no change occurs with xanthine oxidase, but with a nucleosidase prepared from rat liver a rapid increase in absorption at 290 *mμ* takes place. With inosinic triphosphate as substrate, phosphatase, as well as xanthine oxidase and nucleosidase, must be added before a rise in absorption occurs. In the same way guanine can be estimated by means of guanase with xanthine oxidase. Uric acid can be estimated by measuring the decrease in absorption at 290 *mμ*, after oxidation to allantoin by means of purified uricase. One μg of uric acid per ml on oxidation causes a decrease in the extinction at 290 *mμ* of 0·065. F. A. R

Estimation of Formaldehyde in Biological Mixtures. D. A. McFadyen (*J. Biol. Chem.*, 1945, **158**, 107–133)—The reaction of chromotropic acid with formaldehyde was discovered by Eegriwe (*Z. anal. Chem.*, 1937, **110**, 22), who showed it to be highly specific. It was made the basis of a quantitative method of estimating formaldehyde by Boyd and Logan (*J. Biol. Chem.*, 1942, **146**, 279); this has now been improved. A solution containing formaldehyde is mixed with chromotropic acid in sulphuric acid, and the mixture is heated in a boiling water-bath for 30 min. or longer; the reagent is standardised by means of hexamethylene-tetramine. The recommended concns. of sulphuric acid, chromotropic acid and formaldehyde are 9 to 10 *M*, 2 mg per ml and 0·2 to 1·7 μg per ml respectively. Under these conditions formaldehyde in combination with proteins in such preparations as vaccines and toxoids is estimated together with free formaldehyde, the complex being broken down. Where an estimate of the amount of free formaldehyde only is required, the reaction must be repeated in presence of Vorländer's reagent (*Z. anal. Chem.*, 1929, **77**, 241), dimethyldihydroresorcinol, which reacts with free formaldehyde to give methylene-*bis*-(dimethyldihydroresorcinol). This compound is not hydrolysed in 9 to 10 *M* sulphuric acid, so that when Vorländer's reagent is present only combined formaldehyde reacts with chromotropic acid.

Chromotropic acid reaction—Put a known volume of the soln., containing not more than 1·7 μg per ml of formaldehyde, into a reaction tube, and add enough chromotropic acid—sulphuric acid reagent to bring the soln. to a definite volume (10 to 50 ml). Prepare the reagent as follows. Dissolve 1 g of chromotropic acid in 100 ml of water, filter to remove insol. sulphones, and add 12·5 *M* sulphuric acid to bring the volume to 500 ml. The concn. of sulphuric acid should be 9·5 to 10 *M*, so that, on mixing with the soln. to be tested, the concn. of sulphuric acid is not less than 9 *M*. The reagent should not be used if the % transmission at 570 *mμ* falls below 75, as it generally does after a week or longer. Prepare a blank soln. containing all the reagents except formaldehyde, and immerse the tubes in a boiling water-bath, taking care to prevent condensed steam from running into the solns.; heat for 30 min. or longer. Cool the tubes to room temp. and, after bringing back to the original volume if necessary, evaluate the colour at a wavelength of 570 *mμ*. If the solns. are kept in the dark, no change in the colour occurs for at least 3 days. If the solns. are turbid, filter through sintered glass. If the amount of formaldehyde present exceeds 1·7 μg per ml, dilute with 9–10 *M* sulphuric acid.

Vorländer's reaction—Mix 2 ml of the soln., containing not more than 150 μg of formaldehyde, with 2 ml of dimethyl-dihydroresorcinol soln. (0·2 g in 100 ml of McIlvaine's 0·1 *M* citric acid—0·2 *M* Na_2HPO_4 buffer soln. of *p*H 7 to 8, prepared at weekly intervals) and warm the mixture at 37° C. for 30 min. Remove 2-ml aliquots for the chromotropic acid reaction and, without waiting for the soln. to cool, mix with the chromotropic-sulphuric acid reagent and measure the colour as before. Calculate the amount of formaldehyde from the extinction coefficient of formaldehyde at 570 *mμ*, which has been shown experimentally to be 0·57. The amount of free formaldehyde is calculated by subtracting the result obtained in the Vorländer reaction from that obtained in the chromotropic acid reaction, and dividing by 0·985 to compensate for the fact that, under the conditions described, Vorländer's reagent reacts with only 98·5% of formaldehyde. F. A. R.

Estimation of Deuterium Oxide by means of the Hill-Baldes Vapour Tension Apparatus. N. Lifson, V. Lorber and E. L. Hill (*J. Biol. Chem.*, 1945, **158**, 219–229)—At ordinary room temperature, the vapour pressure of D_2O is less than 90% of that of H_2O, so that when H_2O is placed on one junction of a thermocouple in Baldes' modification (*J. Scientific Instruments*, 1934, **11**, 223; *Biodynamica*, 1939, No. **47**, 1) of A. V. Hill's vapour tension apparatus (*Proc. Roy. Soc.*, A, 1930, **127**, 9), and a mixture of D_2O and H_2O on the other junction, the galvanometer indicates that the D_2O–H_2O drop possesses a higher temperature, corresponding to a lower vapour pressure, than the H_2O drop. As the D_2O leaves the D_2O–H_2O drop by diffusion, the process can be followed continuously by observation of the galvanometer deflection. A micrometer burette, to which was attached a hypodermic needle, gauge 22–24, was used to deposit drops of uniform size. The bevel of the needle was removed, and paraffin was applied to the outer portion of the tube to prevent creeping of the drop. The time was measured immediately before beginning exposure of the drop at the tip of the needle and, 10 sec later the thermocouple

was placed in the moist chamber of the apparatus. This was immersed continuously in a water-bath maintained at 33° C. and the walls were moistened with water. Galvanometer readings were begun at a uniform time (2 to 4 min.) after deposition of the drop, and readings were taken at intervals. These were extrapolated to zero time and the D_2O concn. was calculated from curves obtained by plotting the deflection after 0, 140 and 250 sec. against the D_2O concn. of solns. of known strength. The D_2O concn. is obtained with less accuracy by this method than by the falling-drop method, but it affords a rapid means of carrying out an approximate D_2O estimation on a minute amount of material. The accuracy could be improved by reducing the bath temperature and increasing the drop size.

F. A. R.

Effect of Light on the Stability of the Carr-Price Colour in the Estimation of Vitamin A. M. J. Caldwell and D. B. Parrish (*J. Biol. Chem.*, 1945, **158**, 181–186)—The intensity of the incident light has a marked effect on the rate of fading of the blue colour formed in the Carr-Price reaction. As the intensity of the light in a Coleman photometer was reduced from 100 to 13% of full normal brilliance, the rate of loss of colour during the first minute decreased from 39 to 18·2%, and exposure of the soln. to light at 13% of normal for just long enough to read the galvanometer reduced this loss further to 11·7%. A still smaller loss of 7·8% was observed with light of low intensity, such as that used in the Evelyn photometer. Extrapolation of all the curves to zero time tended to bring these to a common point. Reducing the light intensity by means of a perforated diaphragm gave the same result as when a rheostat was used. Light affected the blue colour much more rapidly than it affected vitamin A itself. The fading of the blue colour was shown to be due to radiations in the red region of the spectrum. It is recommended that vitamin A estimations be made with light of low intensity and, where a Coleman photometer is used, the light should be decreased to less than 10% of full brilliance. Calibration curves should of course be prepared with the same light intensity as that used in the estimations. F. A. R.

Chemical Estimation of N¹-Methylnicotinamide. M. Hackberg, D. Melnick and B. L. Oser (*J. Biol. Chem.*, 1945, **158**, 265–278)—It was shown by Huff and Perlzweig (*Science*, 1945, **97**, 538; *J. Biol. Chem.*, 1945, **150**, 395, 483) that the urinary metabolite of nicotinic acid is N¹-methylnicotinamide, and that the methods in use for detecting nicotinic acid deficiency depend on the estimation of this compound either colorimetrically or fluorimetrically. A modification of the fluorimetric procedure is now described, using N¹-methylnicotinamide chloride as standard. Collect a 24-hr. sample of urine in a bottle containing 20 ml of 3·5 *N* sulphuric acid and measure the total volume. Dilute a 6-minute aliquot to 50 ml with an acetate buffer of *p*H 4·5 (dissolve 15·0 g of sodium acetate in 2·5 litres of water and add 2·0 ml of conc. sulphuric acid). Run the soln. at the rate of approx. 2 drops per sec. through a column of zeolite, approx. 50 mesh, at the top of which a reflux condenser is attached by means of a ground-glass joint. The zeolite should previously have been activated by stirring in bulk with four 10-vol. portions of 3% acetic acid for 10 min. each, and with 5 vols. of neutral 25% potassium chloride soln. for 15 min. between the second and third acid washes and then washed successively with water, alcohol and ether, and air-dried. Wash the zeolite column with 30 ml of water while steam is passed through the jacket of the condenser, allowing the water to be heated for ½-min. and then drawing it through the zeolite with full suction. Elute the N¹-methylnicotinamide by immediately running 15 ml of 25% potassium chloride soln. through the hot condenser, and collect the eluate at the rate of 1 drop per 2 secs. Finally, wash the zeolite column with 300 ml of water with steam passing through the jacket and then with 75 ml of water after turning off the steam. The apparatus is then ready for the next sample. In the same way run a standard soln., containing 50 μg of N¹-methylnicotinamide chloride in 50 ml of the acetate buffer, through the column. Pipette 5·0 ml of the eluate into each of two 30-ml separating funnels or glass-stoppered centrifuge tubes and to one (the blank) add 1 ml of water and 16·5 ml of *n*-butanol, and to the other the same volume of *n*-butanol and 1 ml of 15% sodium hydroxide soln. Shake the two funnels vigorously, separate the two layers by centrifuging, and discard the aqueous layers. Shake each butanol layer with 1 g of anhydrous sodium sulphate and, after 15 min., evaluate its fluorescence in a fluorophotometer.

The amount of N¹-methylnicotinamide chloride in the 24-hr. urine sample, expressed in terms of nicotinamide (μg per day), is calculated as follows: $50 \times \frac{Gu}{Gs} \times \frac{60 \text{ min.}}{M \text{ min.}} \times 24 \text{ hr.} \times 0{\cdot}707$, where Gu and Gs are the galvanometer deflections of the unknown and the standard, both corrected for their respective blanks, M is the size of the urinary aliquot in terms of minutes, and 0·707 is the factor for converting the N¹-methylnicotinamide chloride into nicotinamide. The reproducibility of the results in replicate assays was within ± 5% of the average figure and, provided the standard was carried through the zeolite adsorption in the same way as the test soln., and that 3 to 12-min. aliquots were used for analysis, the recovery of added N¹-methylnicotinamide chloride was quantitative. F. A. R.

Effect of Unsaturated Fatty Acids on *Lactobacillus Helveticus*. E. Kodicek and A. N. Worden (*Biochem. J.*, 1945, **39**, 78–85)—Previous workers have reported that certain fatty acids stimulate the growth of *L. helveticus* in presence of suboptimal amounts of riboflavin, whilst other fatty acids may have an inhibitory effect. These results have been confirmed and extended. The riboflavin-free medium of Snell and Strong (*Ind. Eng. Chem. Anal. Ed.*, 1939, **11**, 346; *Univ. Texas Publication* No. 4137) was modified by the incorporation of xylose, pantothenic acid and nicotinic acid, as suggested by Barton-Wright and Booth (*Biochem. J.*, 1943, **37**, 25). Addition of 160 μg of stearic or palmitic acid to 10 ml of the medium caused an increase in acid production during the first 24 hr. of incubation. Caprylic and caproic acids showed little or no effect. Similar results were obtained with each of these four acids on the same medium freed from lipids by extracting twice with chloroform. Oleic acid was without effect in the unextracted medium, but in the extracted medium 160 μg per 10 ml completely inhibited growth and acid production during 24 hr. and partially inhibited them during 48 hr.; after 72 hr., the acid production was almost the same as in control samples without oleic acid. A much more pronounced effect was obtained with linolic acid, 160 μg of which per 10 ml completely inhibited growth for 72 hr. in both unextracted and

extracted media. Linolenic acid behaved in much the same way. The inhibitory action of these acids was reversed by addition of surface active compounds such as lecithin or cholesterol. In view of these observations it is recommended that in the micro-biological assay of riboflavin, lipids should be removed from the medium and from the material under test by extraction with chloroform, and that the tubes should be incubated for 72 hr. rather than for shorter periods. It is also suggested that a standard inoculum of known concn. is advantageous, as it avoids the high blanks which result from the use of a heavy inoculum. F. A. R.

Calculation of the Results of Microbiological Assays. E. C. Wood (*Nature*, 1945, **155**, 632)—The customary method of calculating the results of microbiological assays, in which a standard curve is constructed from the responses obtained by applying the assay to a series of graduated dilutions of the pure nutrient factor concerned (together with a blank) and correlating the responses obtained from the several assay levels of the test preparation with the amount of nutrient factor present from this curve, is criticised as being theoretically unsound and likely in practice to give results materially in error. The relation between the dose, D, of the standard preparation and the response, Y, is, over a certain range of dosage levels, practically linear for many microbiological assays; hence the curve relating these values has the equation $Y = A + B.D$, where A is the intercept on the Y axis and B is the slope of the curve. Further, if the hypothesis upon which all microbiological assays are based, *viz.*, that the response in the test is due exclusively to the nutrient factor concerned without modification, is true, the curve relating the dosage, d, with the response, y, for the test preparation must have the equation $y = a + b.d$ where a is the intercept on the y axis and b is the slope of the curve. Thus: (1) Marked lack of linearity of the test prep. curve renders the assay suspect. (2) The only sound measure of the relative potencies of the test and standard preps. is given by b/B, the "slope ratio" of the two lines and this is a valid statement of the mean potency of the test prep. over the range of linearity of the standard prep. irrespective of the slope of the curve outside these limits, provided the fundamental hypothesis referred to above is true: (3) Theoretically, a and A should be equal, each being independent of the dosage: *i.e.*, if the two curves are plotted on the same graph with the same origin, they should intersect on the axis of response. A difference between the intercepts greater than could be accounted for by experimental error casts suspicion on the validity of the assay, *i.e.*, on the fundamental hypothesis. The magnitude of the allowable difference between a and A should be determined statistically for any particular test. (4) It can be shown that unless $(A-a)$ is *ca.* zero, the usual method of calculating the results of microbiological assays may give results agreeing *inter se* to within 10%, but which differ from the "slope ratio" by much more than this figure. Although the whole assay is suspect, it is very likely that the "slope ratio" result is nearer the correct one than any other. An actual example from an assay of riboflavin is quoted to illustrate the above argument. J. A.

Agricultural

Rapid Method for Determining "Crude Fibre" in Distillers' Dried Grain. K. Whitehouse, A. Zarow and H. Shay (*J. Assoc. Off. Agr. Chem.*, 1945, **28**, 147–152)—Scharrer and Kurschner (*Tierernährung*, 1931, **3**, 307) devised a rapid method for the estimation of crude fibre, but it has been found unsuccessful in its original form. Of a number of modifications investigated, the most suitable one is described and is recommended for its rapidity and reliability. The reagent used for digesting the sample contains 500 ml of glacial acetic acid, 450 ml of water, 50 ml of nitric acid (sp.gr. 1·42) and 20 g of trichloroacetic acid.

Method—Treat 1 g of grain from which the fat has been removed with 100 ml of the reagent, carefully rinsing down the sides of the flask, and immediately boil the mixture gently under reflux for 40 min. from the addition of the acid. Immediately transfer the residue to a linen cloth, wash it thoroughly with water and transfer it into a previously prepared Gooch crucible packed loosely with *ca.* 0·5 g of ignited asbestos. Dry the crucible at 105° C. to constant wt., ignite until all carbonaceous matter has been destroyed, cool and reweigh. The loss of wt. is crude fibre. The % fibre on the dry basis is

$$\frac{\text{wt. of fibre} \times 100}{1 + \frac{\%\ \text{fat}}{100}}.$$

The determination can be completed in *ca.* one half the time required by the official method, and the probability of error is greatly lessened by the reduction of the number of transferrings of the residue from five to three. Filtration is rapid, never exceeding 3 min. and errors due to variations in time of digestion and amount of heat are minimised. Statistical analysis of the results obtained with a variety of samples of brewers' grains showed that the proposed method gives highly reproducible results. A. O. J.

Determination of Undecomposed DDT Spray Deposits on Apples from Total Organic Chlorine Content. J. E. Fahey (*J. Assoc. Off. Agr. Chem.*, 1945, **28**, 152–158)—The high chlorine content of DDT and the fact that organic chlorides are not commonly found in other sprays applied for control of the codling moth suggest a method for determination of DDT in the deposits on sprayed fruit. Weigh the sample (10 to 25 fruits) in a tared container, add 100 to 500 ml of acetone (benzene is equally effective and other solvents may prove satisfactory) and shake the sealed container for 5 min. Transfer the solvent into a volumetric flask, rinse the fruit and container twice with *ca.* one-third of the vol. of solvent used for stripping and adjust the combined extract and rinsings to a definite vol. If a less volatile solvent is used (*e.g.*, benzene or toluene) a carefully measured vol. may be added for stripping and the vol. assumed to remain unchanged. Concentrate an aliquot of the strip soln. to contain chlorine ≡ 0·5–5·0 mg of DDT per ml. The apparatus for recovery of the chlorine consists of a combustion tube of 12 mm diam. bent twice at right angles at the lower end to form one short limb with an orifice of 1 mm diam. This serves as the burner and the other end of the tube is connected with the gas supply. The tube is heated by means of a coil of No. 26 nichrome wire 3·5 ft. long wound round the short limb and the base of the tube and connected with the current supply through a variable transformer. Over the orifice is fitted a chimney made from a 25 mm adapter, which is bent and inserted into an absorption tube similar to that of Cassil (*J. Assoc.*

Off. Agr. Chem., 1941, **24**, 196) permitting the use of much smaller amounts of absorbing liquid than the conventional gas scrubbing bottle. Such an absorber can be made by fusing a short length of 12 mm tubing closed at its lower end into the base of a 25-mm test tube and fusing an appropriate length of 8 mm tubing to the adapter to serve as a bubbler. Place a 1-ml aliquot of the conc. extract in the bent portion of the combustion tube and remove the solvent by evaporation in a water-bath. If larger aliquots are required add successive 1-ml portions in the same manner. In the absorber place 15 ml of a soln. made by dissolving 12 g of sodium hydroxide in 50 ml of water, dissolving 5 g of arsenious oxide in this soln. and finally diluting to 1 litre ($\equiv 0{\cdot}3\ N$ sodium hydroxide, $0{\cdot}1\ N$ arsenious oxide) and 4 ml of nitrobenzene. Connect the outlet of the absorber to a vacuum pump and adjust the suction to supply enough air to support the burner flame and draw the products of combustion through the absorber. Light the gas, place the chimney over the burner and when the suction has been finally adjusted switch on the current at 20 volts and after 5 min. increase it to 35 volts. Avoid rapid volatilisation of the sample and consequent formation of soot. When the sample has been completely volatilised, as indicated by the colour of the flame, transfer the absorbent soln. into a stoppered 125-ml flask, rinse the absorber, keeping the vol. of absorbent and rinsings below 50 ml. Add excess of $0{\cdot}007\ N$ silver nitrate (1 ml $\equiv$ *ca.* 0·5 mg of DDT), 1 ml of conc. nitric acid, 1 ml of ferric sulphate indicator (10 g per 250 ml containing 2 ml of conc. nitric acid) and shake the flask violently for 1 min., taking precautions to allow escape of carbon dioxide. Titrate the residual silver nitrate with $0{\cdot}007\ N$ potassium thiocyanate until a pink tinge persists after agitation of the stoppered flask for 10 min. Considerable experience is necessary to recognise the true end-point, and the silver chloride has to be completely protected by adsorbed nitrobenzene from the aqueous phase to prevent fading of the end-point. The silver nitrate soln. should be standardised by submitting a measured amount of DDT to the whole procedure. Since different grades of DDT vary in chlorine content, the primary standard should be of the same grade as the DDT used in the spray. Unsprayed fruit may give a blank result corresponding to as much as 0·08 mg of DDT per fruit.

A. O. J.

Adaptation of the Wagner Procedure to the Chemical Evaluation of Fused Tricalcium Phosphate. W. H. MacIntire and G. Palmer (*J. Assoc. Off. Agr. Chem.*, 1945, **28**, 158–167)—The chief component of fused tricalcium phosphate prepared by defluorination of rock phosphate is the α-form of the tertiary phosphate set free from apatite combination during fusion. Its effectiveness as a fertiliser and its compatibility with other fertilisers have been demonstrated, and the present investigation was undertaken to ascertain if a modification of the Wagner procedure (*"Methods of Analysis of the A.O.A.C.,"* pp. 22, 39) would indicate for the fused tricalcium phosphate a fertiliser value agreeing with that shown by plant response in pot-culture expts. Earlier work showed that solubility of the phosphoric anhydride content of fused rock phosphate in neutral ammonium citrate and in carbonated water is dependent upon its degree of defluorination. Four samples were used containing respectively 0·06, 0·4, 0·7 and 1·25% of fluorine, the last two being included as controls and not as representative products. At the fusion temp. (1600–1650° C.) the intermediate compound $Ca_{10}FOH(PO_4)_6$ is not stable and the fluorine content is therefore to be attributed to residues of apatite in matrices of tricalcium phosphate. The extracting reagents used were neutral ammonium citrate soln. and 2% citric acid soln.

The main conclusions reached in the investigation were as follows. Agitation of the sample with ammonium citrate for 1 hr. at 65° C., with or without the inclusion of filter-paper, gives values for soluble phosphoric anhydride lower than those indicated by pot-culture expts. When 1 g of the tertiary phosphate is digested with 100 ml of the ammonium citrate reagent the *p*H of the reagent rises and its solvent power is lowered. Doubling the vol. of reagent used brings into solution some of the fused apatite. Phosphoric anhydride dissolved by 2% citric acid can be determined accurately when pptd. directly from suitable aliquots by ammonium molybdate. With a given ratio of reagent to sample the soluble phosphoric anhydride found decreased with increasing residual fluorine. Removal of the citrate ion by treatment of the extract with milk of lime and potassium permanganate, evaporating, igniting and dissolving the residue in nitric acid gave higher values for phosphoric anhydride than the results obtained when both citrate ion and silica were removed by fuming with perchloric acid. The lower values may be caused by loss of phosphorus as phosphine or as a phosphofluor compound before the perchloric acid is sufficiently concentrated to effect oxidation and dehydration. Mechanical end-over-end agitation of 1-g samples in 100 ml of solvent in 250-ml flasks was found to effect continuous migration of the sample through the solvent. The value for phosphoric anhydride content obtained by continuous agitation for 30 min. at room temp. in 100 ml of 2% citric acid of 1 g of material passing an 80-mesh sieve proved very nearly equal to the calculated amount of phosphoric anhydride present in the readily available form of α-tricalcium phosphate and agreed with the results of comparisons with superphosphate in pot-culture expts. with substantially defluorinated products. The amount of tricalcium phosphate present was calculated by deducting from the total phosphoric anhydride (determined in solns. of the material in *aqua regia*) the phosphoric anhydride accounted for by the apatite equiv. to the residual fluorine. Material of the degree of fineness passing an 80-mesh sieve was found suitable for analytical work.

The following analytical procedure is therefore recommended. To 1 g of the sample, ground to pass an 80-mesh sieve, in a 250-ml fertiliser flask add 100 ml of 2% citric acid reagent. Agitate the stoppered flask for 30 min. in an end-over-end agitator at 25 r.p.m. Filter and collect 50 ml of the clear filtrate. To 10 ml add 15 g of phosphorus-free ammonium nitrate, dilute to 60 to 75 ml, add 5 ml of ammonium molybdate reagent slowly, agitate for 5 min., add 50 ml more of the reagent and agitate continuously for 30 min. Collect the ppt. by light suction on a Shimer filter and wash the flask and filter with 6 successive portions of water free from carbon dioxide. Place the pad and ppt. in the pptn. flask, disintegrate the pad by means of a stream of water and dilute to 75–100 ml. Add 1 ml of 1% alc. phenolphthalein soln. and dissolve the ppt. in sodium hydroxide soln. standardised against Bureau of Standards rock, adding an excess of 2 ml. Back titrate the soln. as described in *"Methods of Analysis of the A.O.A.C.,"* p. 22, par. 12.

A. O. J.

Occurrence of Auxins in Organic Manures. J. H. Hamence (*J. Soc. Chem. Ind.*, 1945, 147–148)—The auxins have been determined in organic manures of the following types. (1) Excreta: farmyard manure, etc.; (2) animal and fish by-products: meals (various) and dried blood; (3) other materials: peat, hop manure, old sewage sludge, leaf mould. The total auxins (as β-indolylacetic acid, mg/100 g) (A), and β-indolylacetic acid (mg/100 g) (B) were determined by the Went pea method and modified perchloric method respectively (ANALYST, 1944, **69**, 229). Manures richest in auxins occur in (1), being highest in a two-weeks old poultry manure: (A) 7·66 and (B) 3·83. In dried poultry manure part of the auxin activity is lost. For fresh farmyard manures (including urine) results were: (A) 1·41 and (B) 0·36. After 4 months and two years respectively, the auxin contents of samples were: (A) 0·15, 0·12; (B) 0·07, 0·08. Peruvian guano contained (A) 0·16, (B) 0·16. In group (2) blood, which is the most active member, increases in auxin content as the blood becomes stale; the auxin content of dried blood thus depends on its freshness when dried. Results on three samples were: (A) 0·90, 0·36, 0·28; (B) 0·33, 0·14, 0·19. A hoof meal sample contained (A) 0·64 and (B) only 0·03. Activity of peat is slight: (A) 0·08; (B) ? (pigment interferes with the test). Leaf mould contained 0·007 only of (A). All the above results were calculated on a dry basis. Auxins were present in all the manures examined, but in old sewage sludge the amount was inappreciable, and in some other manures very small. These auxins are not always present as β-indolyl-acetic acid (or heteroauxin), although in some manures this forms almost the entire auxin content.

E. B. D.

Pyrethrum Flowers from Nigeria. F. Major (*Bull. Imp. Inst.*, 1945, **43**, 7–8)—Pyrethrum flowers grown in the Cameroons Province and submitted for examination by the Director of Agriculture, Nigeria, contained 9·8% of moisture, 0·54% of pyrethrin I and 0·50% of pyrethrin II (1 ml of *M*/100 potassium iodate soln. ≡ 0·0057 g of pyrethrin). Flowers with the total pyrethrin content of 1·04% would be readily saleable, and if the sample of flowers examined had been gathered earlier the total pyrethrin content would probably have been higher. Kenya pyrethrum flowers (the richest in pyrethrin) are usually sold with guaranteed total pyrethrin content of 1·3%, but pre-war Japanese-grown flowers were usually sold on the basis of a minimum of 0·9% of pyrethrin. E. B. D.

Organic

Alumina Catalysts for Organic Reactions. F. J. L. Bentley and C. G. Feachem (*J. Soc. Chem. Ind.*, 1945, 148–149)—The activities of alumina catalysts can be increased or restored by alternate hydration and dehydration, the hydration being effected by means of hot water or steam below *ca.* 150° C. The lower temp. limit was not found. A useful catalyst can thus be made from initial material of very low activity. If water is used, there is a washing action also (*cf. J. Amer. Chem. Soc.*, 1925, **47**, 2749) and the effect is greater. During washing, some of the active γ-alumina is hydrated to boehmite and the increase in activity is attributed, probably, to the breaking down of the surfaces of the crystals by the treatment. For each initial material there is an optimum combination of washing (or steaming) time and number of washes. Complete dehydration, as determined by X-ray examination, may be essential finally. Probably, for any given partial pressure of water vapour, there is a "transition temp." below which boehmite and above which γ-alumina are the stable solids; this is stated to lie between 154° and 256° C. at one atmosphere pressure of water vapour. The phases were identified by X-ray examinations. E. B. D.

Inorganic

Determination of Aluminium in Spelter. F. F. Pollak and E. F. Pellowe (*J. Chem. Soc.*, 1945, 300–301)—Aluminium in quantities of 0·01 to 0·1% added to the "hot-dip" galvanising bath improves the coating. The procedure given ensures the accurate control of the bath. Gently heat 5 g of fine "magneted" drillings with 25 ml of diluted sulphuric (1 + 4) and 25 of diluted hydrochloric acid (1 + 9). When the attack is over, add 1 g of pure zinc and warm for 5 min., filter into a 400-ml conical beaker and wash 5 times with 10 ml of water. Cool the filtrate, add 4 ml of ammonium acetate soln. (500 g per litre) and 5 *N* ammonia 1 ml at a time during agitation until the liquid remains turbid. Add 3 or 4 drops of glacial acetic acid and slowly, with constant stirring, 20 ml of ammonium benzoate reagent (100 g per litre, with 1 mg of thymol); gently boil for 5 min., set aside for a short time, and filter while hot (No. 41 Whatman). Re-filter the first portion if turbid. Wash the ppt. (containing the iron and aluminium, with a little zinc) 5 times with hot ammonium benzoate soln. (10 g per litre, to 100 ml of which 2 ml of acetic acid are added), and once with hot water, then dissolve it on the filter with 50 ml of a hot soln. of 30 g of tartaric acid and 150 ml of strong ammonia in 1 litre, receiving the filtrate in the pptn. beaker and washing the filter with hot tartrate wash (5 ml of the above soln. diluted to 50 ml). Treat the hot filtrate with a rapid stream of hydrogen sulphide, add filter pulp, and set aside in a warm place. Filter, wash 5 times with ammonium sulphide and tartrate wash, and ppt. the filtrate at 90° C. with excess of oxine reagent (10 g of 8-hydroxyquinoline and 50 ml of diluted acetic acid [1+1] in 500 ml). Set aside hot until the ppt. coagulates, collect, after another 15 min., on a No. 540 Whatman paper, and wash with hot water until the washings are colourless. Dissolve the ppt. on the filter in 50 ml of hot diluted hydrochloric acid (1+1) into the pptn. beaker and wash with 50 ml of hot water. Dilute to 150 ml, cool, and add 3 drops of indicator (0·5 g of indigo-carmine in 100 ml), slowly run in from a burette 0·1 *N* bromate-bromide soln. until the colour is pure yellow then 3–5 ml in excess (2·784 g $KBrO_3$ and 13 g KBr per litre; 1 ml ≡ 0·2248 mg of Al). Add 1 g of potassium iodide and titrate back with 0·1 *N* thiosulphate (starch indicator) standardised against the bromate soln. Carry out a blank titration on the same amount of indicator soln. Time, 2½ hr. W. R. S.

Effect of Silica on the Quantitative Reduction of Nitrates with Devarda's Alloy. J. A. Brabson and J. H. Karchmer (*J. Assoc. Off. Agr. Chem.*, 1945, **28**, 142–147)—When the analysis of ammonium nitrate was attempted by first distilling off the ammonium nitrogen with sodium hydroxide and then adding Devarda's alloy to reduce the nitrate, low results were obtained for nitrate nitrogen. Investigation disclosed that silica dissolved from the Kjeldahl flask during the determination of ammonium nitrogen subsequently inhibited the reduction of nitrate by Devarda's

alloy. The official procedure ("*Methods of Analysis of the A.O.A.C.*," 1940, **27**, 30) was followed with the regular Kjeldahl apparatus fitted with Davisson scrubbers (*J. Ind. Eng. Chem.*, 1919, **11**, 465; ANALYST, 1919, **44**, 251), and with condenser ends of the Miller design (*Ind. Eng. Chem.*, 1936, **8**, 50) immersed in the standard sulphuric acid. Modified methyl red ("*Handbook of Chemistry and Physics*," Chemical Rubber Publishing Co., 25th Ed., 1264) was used as indicator.* The sample was treated with 5 ml of 42% w/w sodium hydroxide soln. (3·04 g of sodium hydroxide) and, after dilution to 325 ml, with 3 g of Devarda's alloy. Distillation was at the rate of 250 ml per hr. and the results were corrected by a blank determination. Solns. containing known amounts of potassium nitrate were boiled for 1 hr. in Pyrex Kjeldahl flasks with the amount of sodium hydroxide specified in the A.O.A.C. method for nitrate nitrogen. When the nitrate nitrogen was subsequently determined the results were invariably low, but when the Pyrex flasks were replaced by Monel beakers the recovery of nitrate nitrogen was complete. Since silicon and boron are the elements most likely to be absorbed from Pyrex flasks by hot sodium hydroxide soln., small amounts of silicates and borates were added to the sample solns. Presence of borate had no effect upon the recovery of nitrate nitrogen, but in presence of silicate the recovery was low. Gravimetric determination of the silica dissolved by boiling 3·04 g of sodium hydroxide in 325 ml of water in Kjeldahl flasks for various periods showed that the glass was attacked by the alkali, the action increasing with increasing time and concn. of the alkali. When the vol. of liquid had been reduced to 270 ml (25 min.) the silica dissolved was 26·1 mg, and when the vol. had diminished to 40 ml (76 min.) the silica content was 65·9 mg. Further confirmation of the interference of silica was afforded by parallel determinations of the ammonium and nitrate nitrogen content of ammonium nitrate solns. in glass and copper flasks. With glass flasks recovery of nitrate nitrogen was low, whereas with copper flasks recovery was complete. To determine the quantitative effect of silica on nitrate reduction, 25-mg increments of silica (as sodium silicate) were added to a series of aliquots of potassium nitrate soln. containing 45·05 mg of nitrate nitrogen. To each soln. 3 g of Devarda's alloy, 3·04 g of sodium hydroxide and enough water to bring the vol. to 325 ml were added and the nitrate nitrogen was determined in copper flasks. The results showed that addition of silica reduced the recovery of nitrogen from 99·7% (no silica) to 63·4% (150 mg of silica), intermediate figures being 99·4% (25 mg), 97·3% (50 mg), 92·2% (75 mg). When compared with the amounts of silica dissolved from glass by boiling sodium hydroxide soln., these figures show that the error in nitrogen determination in glass flasks may be considerable. Expts. showed that when sodium hydroxide is replaced by calcium oxide or magnesium oxide the amount of silica dissolved is insufficient to cause appreciable interference with the subsequent reduction of nitrate by Devarda's alloy. Magnesium oxide removed less silica from glass than did calcium oxide and apparently little of what was removed remained in the soluble form. Addition of magnesium oxide to a nitrate soln. containing 100 mg of silica as sodium silicate apparently pptd. the silica, for the recovery of nitrogen was complete. Interference by silica is lessened by reducing the particle size of the alloy, increasing the amount of alkali or increasing the rate of distillation. Excessive frothing encountered under these conditions may be reduced by addition of alcohol, but blank determinations are higher than is desirable.

The results of the investigation indicate that soln. of silica from the flask does not interfere with determination of nitrate nitrogen by the current A.O.A.C. methods. Ammonium nitrogen and nitrate nitrogen can be determined accurately in the same soln. by determining the ammonium nitrogen by the official A.O.A.C. procedure (*loc. cit.*) and by subsequently determining nitrate nitrogen according to the A.O.A.C. modification of the Devarda method. The recovery of both forms of nitrogen is complete also when the amount of sodium hydroxide used is *ca.* 5 times that of the official amount as in Devarda's original method (*Chem. Ztg.*, 1892, **16**, 1952). The explanation is advanced that when total nitrogen is determined by Devarda's method, reduction is complete before the concn. of alkali is sufficient to attack the glass, whereas when the ammonium nitrogen is determined before addition of the alloy the subsequent concn. of alkali causes appreciable action on the glass. Silica may interfere by forming a film on the alloy, the film being broken by high alkali concn. and rapid rates of heating. Reduction of the particle size of the alloy increases the surface and thus diminishes the effect of the film. Devarda's alloy contains 45% of aluminium, and the interference of silica with reduction of nitrates may be related to the phenomenon of the inhibition of the corrosion of aluminium by silicates (Baker, *Ind. Eng. Chem.*, 1935, **27**, 1358).

A. O. J.

* 0·50 g of methyl red and 1·25 g of xylene cyanole FF, or 1·25 g of methyl red and 0·825 g of methylene blue, dissolved in 1 litre of 90% alcohol. —EDITOR.

Microchemical

Micro-determination of Water. G. B. Levy, J. J. Murtaugh and M. Rosenblatt (*Ind. Eng. Chem. Anal. Ed.*, 1945, **17**, 193–195)—By rigid exclusion of extraneous moisture, the Karl Fischer reagent may be used on the micro scale. The method was developed for the examination of penicillin sodium salt, but is applicable to a variety of substances. Back-titration technique is used, the end-point being determined electrometrically. The titration vessel is a 16 mm diameter Pyrex test-tube cut down to 50 mm long. Two 26-gauge platinum wire electrodes are fused through the bottom, while the upper end is closed by a tight sleeved rubber stopper. The micro-burettes are fitted with 22-gauge hypodermic needles, which may be thrust through the rubber stopper. A 27-gauge needle is also inserted as a vent to equalise the pressure. The air supply to the burettes must be thoroughly dry. *Procedure*—Dry the titration vessel and cap at 105° C. and store over phosphorus pentoxide until required. Introduce the sample rapidly and deliver the Karl Fischer reagent (Smith, Bryant and Mitchell, *J. Amer. Chem. Soc.*, 1939, **61**, 2407; ANALYST, 1939, **64**, 911) from a micro-burette until an excess of 5 to 10% has been added. Withdraw the needles and shake until solids are dissolved. Connect the electrodes to the electrometric apparatus (McKinney and Hall, *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 460) and back-titrate with methanol containing water (2 to 5 g of water added to 1 litre of anhydrous methanol). Agitate gently until incipient decolorisation and then approach the end-point by dropwise addition.

Determine the water-eqvt. of the Karl Fischer reagent by titrating against standard water solution (92 to 95% ethanol; determine the water content by accurate density measurement), and standardise the back-titrating soln. against the Karl Fischer reagent. J. T. S.

Micro-detection of Sulphur in Insoluble Sulphates and in Organic Compounds. F. L. Hahn (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 199)—The test is based on the liberation of nitrogen from a sodium azide—iodine soln., the reaction being catalysed by sulphides (and certain other sulphur-containing groups). *Non-volatile substances*—Clean the end of a piece of copper wire (0·1 to 0·2 mm), dip into satd. potassium hydroxide soln. and pass through an alcohol flame so as to leave a small bead of fused alkali at or near the end of the wire. Bring the bead in contact with the substance to be tested and re-introduce into the flame, moving slowly into the reducing cone. After a few sec., allow to cool near the wick, cut off the portion of wire covered by the bead and transfer to a microscope slide. Cover with 1 drop of reagent (1 g of sodium azide dissolved in 50 ml of 0·5 *N* iodine soln.) and examine under the microscope. The presence of sulphur is indicated by active evolution of nitrogen. Alternatively, drop the bead into a short m.p. tube closed at one end, introduce a droplet of reagent and bring it in contact with the bead by rapidly swinging the tube. Invert the tube and observe any collection of gas. A strongly positive result is obtained with less than 1 μg of barium sulphate. *Volatile Substances*—Silver internally a length of glass tubing (0·5 to 2 mm bore) by filling with a reagent which contains silver nitrate (about 0·1 *N*), ammonium hydroxide, a sol. tartrate and sodium hydroxide. After leaving overnight, rinse and dry by warming while passing a current of air through the tube. Pull out the end into a capillary about 2 to 3 mm long and 0·1 to 0·2 mm in diam. Touch the liquid substance to be tested (if a solid, melt or dissolve in a sulphur-free solvent) with the capillary tip so as to draw in a small quantity, then close the tip by touching with a hot flame. Heat the tube 2 to 3 cm from the sealed end and move the flame slowly so as to volatilise the sample. Allow to cool, break off the tip, allow a droplet of reagent to enter, and observe under the microscope. With 0·02 μg of sulphur the test is positive; with 0·01 μg it is doubtful. J. T. S.

Determination of Sulphate by the Benzidine Method. D. S. McKittrick and C. L. A. Schmidt (*Arch. Biochem.*, 1945, **6**, 411–417)—The estimation of sulphate by the benzidine method is convenient, but is subject to error from occlusion and incomplete pptn. A modification has now been devised in which the errors from these sources are reduced. Neutralise the material to be tested (which should contain not more than 2 mg of sulphate) with sodium hydroxide, using bromophenol blue as indicator, add 0·1 *N* hydrochloric acid until the blue colour disappears and then 2 ml of *M* acetate buffer of *p*H 2·8; benzidine sulphate has minimum solubility at this *p*H. Add 20 ml of a 0·044 *M* soln. of benzidine dihydrochloride in 0·4 *N* hydrochloric acid in 5-ml increments at 5-min. intervals, followed, 3 to 10 min. later, by a vol. of acetone equal to one-quarter of the final vol. Leave the mixture at 0° C. for 20 hr., filter off the benzidine sulphate and wash the flask and ppt. with 2 ml of 50% aqueous acetone followed by four 2-ml portions of pure acetone. Wash the ppt. from the filter with water, and titrate hot with 0·02 *N* sodium hydroxide, using phenol red as indicator. Under these conditions the method gives recoveries accurate within 0·5%. Although the method of Fiske (*J. Biol. Chem.*, 1921, **47**, 59) gives moderately accurate results with urine free from added salts, the error may be as much as 5% when these are present. The new procedure applied to the same samples gives results accurate within 0·5%. F. A. R.

Physical Methods, Apparatus, etc.

Micro-calomel Electrode for Polarographic Measurements. P. W. West and E. S. Amis (*Science*, 1945, **101**, 71–72)—The electrode assembly is of pencil form for insertion through a hole in the stopper of a Heyrovsky-type cell. It can be removed and stored in satd. potassium chloride soln. The electrode proper is of the satd. type and is made from 4-mm glass tubing. It has a small hole blown in the side to permit junction with the solution in the salt bridge container. The electrode slips into the salt bridge container, which is a length of 6-mm outside diam. Pyrex tubing drawn down at the lower end to retain an agar plug, which is saturated with potassium chloride or other suitable electrolyte. Alternatively, a soft glass ground-in plug may be used. The internal resistance of the assembly is less than 1500 ohm, and half-wave potentials determined with it agree with those in the literature. J. T. S.

Light Absorption Spectrometry. M. G. Mellon (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 81–88)—Current spectrophotometric practice as applied to analytical problems is reviewed generally. In connection with instrumental details, the importance of wavelength calibration and of a knowledge of the spectral band width used is emphasised. Of the various alternative methods of presenting the results, transmission/wavelength curves are most easily plotted from the experimental data and, in this form, are of value in calculating numerical colour specifications. Optical density ("extinction") /wavelength curves magnify absorption maxima and thus make them definite and easily read; such curves also facilitate computation from one thickness to another. Log. extinction/wavelength curves have the advantage that their shape is independent of thickness or concentration (if they obey Beer's law) and such curves are thus useful for identification. In theoretical studies on the relation of absorption and constitution, frequency is of more fundamental importance than wavelength, and curves should be plotted on this basis. Examples of qualitative and quantitative analysis are given. B. S. C.

Analytical Applications of Emission Spectrometry. J. R. Churchill (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 66–74)—In the general analytical laboratory the d.c. arc is recommended as the most generally applicable excitation source for non-routine applications. The a.c. spark may be of use in such a laboratory, (*a*) when the results obtained using the d.c. arc are not sufficiently quantitative, (*b*) when a metal specimen is to be analysed with a minimum of damage to the sample, and (*c*) when the test is to be restricted to the surface or to a particular localised region of a metal sample. Emission spectroscopy is of particular value to the analytical chemist in the following applications—general qualitative analysis; alloy

identification; comparison between materials showing different properties; corrosion investigations; identification of platings and coatings; identification of minute samples; assessment of suitability of reagents for particular chemical analyses.

B. S. C.

Mass Spectrometry. H. W. Washburn, H. F. Wiley, S. M. Rock and C. E. Berry (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 74–81)—In the mass spectrometer, as applied to the analysis of gas or liquid samples, the gas or liquid vapour is allowed to flow at constant rate through an ionisation chamber where it is subjected to electron bombardment. This converts some of the neutral molecules into positive ions which, under the influence of electric and magnetic fields, form a fan of ion beams, each beam containing ions of one mass only. Gradual variation of the electric or magnetic fields causes this fan of ion beams to move past an exit slit and fall successively on a target. The current thus imparted to the target is amplified and fed to a recording galvanometer system. The resulting record, which gives the relative abundance of ions of different masses, is the "mass spectrum." Minor variations in the arrangement of atoms in a molecule, such as occur, *e.g.*, between 2,2,3-, 2,2,4- and 2,3,4-trimethyl pentane, produce very marked differences in the relative proportions of the positive ions produced on ionisation. It is this characteristic which enables the technique to be used for the analysis of isomeric paraffin mixtures containing as many as ten components. For the determination of small amounts of impurity in a relatively pure substance, mass spectrometry is often the most accurate method, *e.g.*, for the determination of small amounts of diethylbenzene in ethyl benzene, of pentenes in isoprene and of pentenes or butenes in butadiene. In such instances the minimum amount that can be determined is approx. 0·01% for automatic recording, and less than 0·001% if manual recording is employed. B. S. C.

Quantitative Spectrographic Method for Analysis of Small Samples of Powders. E. J. Fitz and W. M. Murray (*Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 145–147)—For very small samples of inorganic powders, such as corrosion products, non-metallic inclusions and ash from organic materials, a rapid spectrographic technique has been devised. One mg of the sample is mixed with 20 mg of an internal standard/buffer mixture consisting of equal parts of barium nitrate and ammonium sulphate. The combination is well mixed by grinding in an agate mortar and made into a pellet, 2 mm. in diam., using a micro-press. The pellet is inserted in a small crater in the lower positive electrode (0·47 cm diam. specially pure graphite) of a d.c. arc run at 7 amps. The method is then conventional except that working curves are prepared by plotting galvanometer deflection ratio, instead of the more usual relative intensity, against log. concn. This method involves some loss of accuracy but gives a marked gain in speed of working. An analysis of up to 10 constituents can be completed in 2 hr. For constituents present to the extent of less than 50% of the whole sample, the method gives results within 10% of the actual amount present. The elements investigated, and lines used, are—Tin (2429·5A), silicon (2435·1A), aluminium (2652·5A), iron (2723·6A), magnesium (2783·0A), copper (2824·4A), manganese (2933·1A), nickel (2943·9A), calcium (3158·9A), titanium (3168·5A). Barium (2771·4A) is used as the internal standard. B. S. C.

Abrasion Resistance of Paper Base Plastics and Associated Materials. E. R. Hoffman (*Paper Trade J.*, 1945, **120**, 25th Jan., *TAPPI Sect.*, 31–34)—The Taber abrasion tester is described. It consists of 2 abrading wheels (diam. 2 in.), made of a rubbery composition containing an abrasive material, which rest under a load (which can be adjusted from 125 to 1000 g) on the square horizontal specimen; this is carried on an air-cooled, circular turntable rotating at 70 r.p.m. The wheels are 2·5 in apart, and are equidistant radially from the centre of the turntable. As the table turns, the specimen passes diagonally under the faces of the wheels, causing a rubbing action as the wheels are forced to rotate. The wheels are made in 5 grades of coarseness, and their surfaces are "standardised" by using a standard grade of emery cloth in place of the specimen. The abrasion resistance is the loss in wt. of the specimen after a given no. of revs. of the turntable for a given load and grade of wheel. The effects on it of the resin content, the R.H., the no. of sheets in the panel, and the materials in the paper used for the base of the laminate, are discussed. J. G.

Review

SEMI-MICRO QUANTITATIVE ORGANIC ANALYSIS. By R. BELCHER, F.R.I.C., and A. L. GODBERT, M.Sc., Ph.D. Pp. viii + 168. London: Longmans, Green & Co. 1945. Price 10s. 6d.

This publication appears to be the first of a series of "Textbooks on Modern Analytical Chemistry," edited by Dr. C. L. Wilson and Mr. Belcher, and it augurs well for those to follow. The authors have attempted, successfully, the difficult task of satisfying the needs of both the experienced analyst and the student; to the former the book may serve for reference, while the clear and detailed descriptions of the various techniques should be sufficient guidance to the latter.

As far as the reviewer is aware no other book covers the same field, but much of the technique is similar to that familiar to readers of the established texts on quantitative organic semi-micro- and micro-analysis. The methods, however, are not always those which the more conservative microchemical analysts will regard as standard. After a general introduction, an account is given of the balance and its use, including setting it up, cleaning it and testing its behaviour; the ordinary analytical balance is satisfactory. Notes are given concerning

the purification of materials by the usual procedures of filtration, crystallisation, solvent extraction, sublimation, distillation and adsorption. The remainder of the book is devoted to detailed descriptions of the determinations outlined below, where attention is drawn to the unusual features. All the procedures have been fully tested in the authors' laboratory, both by experts and by students.

Carbon and hydrogen are determined by combustion in a stream of oxygen controlled by means of a flowmeter and a Mariotte bottle. Ingram's tube filling is used, *viz.*, copper oxide—lead chromate and cerium oxide enclosed in copper gauze; the lead peroxide is placed in a boat, so that none of the tube filling comes into contact with the combustion tube.

The Dumas method for nitrogen is described, together with a modification for substances which are difficult to burn; potassium chlorate is then used. A warning is given that in the digestion for the Kjeldahl process, decomposition is not complete immediately the acid mixture becomes colourless. For the distillation (with caustic soda and sodium sulphide) a modified Parnas and Wagner apparatus is recommended; the ammonia is absorbed in boric acid, methyl red plus methylene blue being the indicator for the final titration.

Sulphur is determined by combustion in oxygen, using platinum contacts, or in air, the gases passing through heated sintered silica discs which serve as catalyst. In both methods the sulphur oxides are absorbed in hydrogen peroxide. Excess of barium chloride is added and, after removal of the barium sulphate, the excess of barium is precipitated as chromate; the barium chromate is dissolved in hydrochloric acid and titrated with ferrous ammonium sulphate (barium diphenylamine sulphonate as indicator).

In the determination of halogens the hydrochloric acid or hydrobromic acid plus bromine mixture produced by combustion in oxygen (platinum contacts) is absorbed in heated barium carbonate and finally titrated with silver nitrate. Iodine formed by a similar combustion is absorbed in caustic potash solution and is oxidised to iodate; iodine liberated on addition of potassium iodide is titrated in the usual manner.

For phosphorus determination the organic matter is oxidised by sulphuric and nitric acids, the resulting phosphoric acid being precipitated by acid sodium molybdate and nitrato-pentammine cobaltinitrate and weighed as $[Co(NH_3)_5NO_3]H_3PMo_{12}O_{41}$. Arsenic is oxidised by the acid mixture to arsenic acid and is determined iodimetrically.

In the Zeisel method for alkoxyl groups the authors prefer to use as absorbent a solution of sodium acetate and acetic acid containing bromine. Iodine monobromide is produced and is estimated volumetrically after oxidation to iodate. For acetyl group determination the acetic acid produced on hydrolysis is distilled and titrated with caustic soda solution.

The following physical determinations are also described, the methods being adapted to the semi-micro scale; specific gravity of liquids, melting and boiling points in capillaries, and molecular weight by ebullioscopic, cryoscopic and Victor Meyer procedures.

This book should prove both interesting and helpful, not only to the experienced micro-chemical analyst, but also to those who are less familiar with the subject. G. H. WYATT

MICROCHEMISTRY GROUP

A JOINT MEETING of the Group, the Newcastle Section of the Royal Institute of Chemistry and the Newcastle Section of the Society of Chemical Industry, will be held in Newcastle on Friday, September 14th, 1945.

Programme—During the afternoon it is hoped to visit a local works. The arrangements are not yet complete and final details will be given in the circular notice to Group members later.

At 6.30 p.m., in the Lecture Room, Chemistry Department, King's College, the following papers will be read:

(1) "Inorganic Quantitative Microanalysis for University Students," by Dr. Christina C. Miller.
(2) "A Review of Methods for Micro-filtration," by Dr. G. H. Wyatt.
(3) "Some Aspects of the Microchemical Analysis of Ferrous Alloys," by Mr. C. Whalley.

An invitation to attend the Meeting is extended to all Northern Section members.

The next meeting of the Group will be held in London in January, 1946.

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