THE ANALYST

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

PHYSICAL METHODS GROUP

THE Ninth Ordinary Meeting of the Physical Methods Group was held on Tuesday, February 11th, 1947, in the rooms of the Chemical Society, Burlington House, London, W.1., with the Chairman of the Group, Mr. R. C. Chirnside, F.R.I.C., presiding. The subject of the meeting was Fluorimetric Analysis, and the following papers were presented and discussed: "Apparatus Design for Fluorescence Measurements," by E. J. Bowen, M.A., F.R.S.; "Notes on Fluorescence Quenching," by the same author; "Some Applications of Fluorimetry in Vitamin Analysis," by E. Kodicek, Ph.D., M.D.; "The Use of Fluorimetric Analysis in the Study of Pterins," by Delia M. Simpson, M.A., Ph.D.

The Tenth Ordinary Meeting of the Group was held on Friday, May 2nd, 1947, at King's College, Newcastle-on-Tyne. Mr. Chirnside was again in the chair and about eighty members and visitors were present. The following papers on Physical Methods of Gas Analysis were presented and discussed: "Gas Analysis at Low Pressures," by C. E. Ransley, M.Sc., Ph.D.; "The Analysis of Hydrocarbon Gases by Low-Temperature Distillation," by J. H. D. Hooper, B.Sc., A.R.I.C.; "A New Apparatus for Gas Analysis by the Soap Film Method," by W. J. Gooderham, B.Sc., A.R.C.S., F.R.I.C., M.Inst.Gas E., who also demonstrated his apparatus.

POLAROGRAPHIC DISCUSSION PANEL

The first meeting of the panel was held, under the chairmanship of Dr. W. Cule Davies, in the Physical Chemical Lecture Theatre, Imperial College of Science and Technology, London, S.W.7, on Friday afternoon, April 25th, 1947. The attendance was 39 members and visitors and discussions followed contributions by Dr. G. Jessop on "Notes on Polarographic Technique," by Dr. W. Cule Davies on "Remarks on Polarographs" and by Dr. E. R. Roberts on "The Analysis of Brass Plating."

Dr. Jessop dealt first with the instrumental aspects, in particular the measurement of current and potential and the various ways of determining wave height. He discussed such effects as arise from the ohmic resistance of the circuit and from variations in the anode potential, instancing the peculiar wave forms sometimes encountered when sodium sulphite is used in conjunction with a mercury pool anode.

Dr. Cule Davies, discussing desirable features to be incorporated in a polarograph, considered that means should be provided for the accurate measurement of drop-rate, current and voltage, and that damping, if used, should alter neither the magnitude of the current nor the observed half-wave potential. Published accounts of polarographic work should give the characteristics of the dropping electrode, wave heights being expressed in units of current.

Dr. Roberts pointed out that in the bonding of rubber to cast iron the latter must be brass-plated and described polarographic methods of control of the plating, by means of which six bath samples could be analysed within half an hour, while plating could be stripped and examined similarly. De-oxygenation by sodium sulphite gave peculiar results for zinc, but sulphur dioxide was both rapid and effective. A simple cell incorporating an external reference electrode was developed for routine use and enabled results to be obtained by measuring the current at three predetermined potentials.

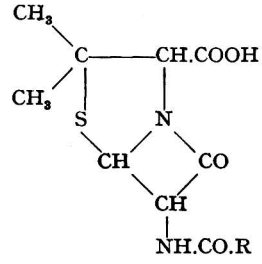
Chemistry of Penicillin

By F. A. ROBINSON

(*Read at the meeting of the Society on May 7th, 1947*)

THE constitution of penicillin was elucidated as the result of collaborative research carried out in many laboratories in Great Britain and the U.S.A. during 1943–45.[1] In the course of this work, it was discovered that several different penicillins exist. These vary according to the strain of *Penicillium notatum* or *P. chrysogenum* used and with the cultural conditions. Indeed, the proportion of certain penicillins in a mixture can be increased by addition to the medium of certain "precursors" which the mould is apparently able to assimilate and incorporate into the penicillin molecule.

All the penicillins have the common basic structure



in which the group R varies from one penicillin to another. In the original penicillin produced by Sir Howard Florey and Dr. E. Chain and their colleagues at Oxford, R for the major constituent is a Δ^2-pentenyl group. This species of penicillin was originally termed penicillin F, in order to identify it with the penicillin discovered by Fleming and to distinguish it from another antibiotic produced by *P. notatum*, which was originally called penicillin A and later notatin; it has a structure entirely different from that of the penicillins. The term penicillin F is still used in the U.S.A., but in this country this form is now generally known as penicillin I. Mild methods of reduction convert penicillin I into dihydro penicillin I, in which R is an amyl group, and the activity is apparently undiminished or even slightly increased by the hydrogenation. In America the penicillin originally isolated was not penicillin F, but a different penicillin with approximately the same activity. It was considerably easier to isolate and more stable than penicillin F and was called penicillin G by the Americans; in this country it is known as penicillin II. In this instance the group R is a benzyl group, and the presence of this form in the original American penicillin was associated with the use of a different strain of *P. notatum* and with addition to the medium of corn steep liquor, which contains benzyl derivatives that favour the production of penicillin II in preference to penicillin I. Since the adoption of corn steep liquor for routine production in this country, penicillin II is also the predominant form in British penicillin. Another form of penicillin produced by some strains of *P. notatum* and *P. chrysogenum*, especially in presence of the appropriate precursor, is penicillin III or penicillin X, in which R is a *p*-hydroxy benzyl group. Even under the best conditions, however, it is produced in limited amounts only, but, as it has rather unusual biological properties, interest in this form of penicillin may be expected to increase.

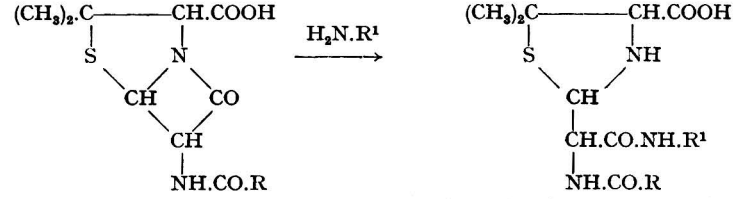
In 1945 a new strain of *P. chrysogenum*, known as Q.176, was generally adopted for submerged culture, because it produced much larger quantities of total penicillin than the strain X.1612 previously employed. It was not immediately realised, however, that, in absence of precursors, the predominant form of penicillin so produced is not the therapeutically valuable penicillin I or penicillin II, but another penicillin termed penicillin K, in which R is a *n*-heptyl group. This form of penicillin is rapidly inactivated in the animal or human organism and is therefore clinically less effective than penicillin I or II. Even in presence of precursors, substantial amounts of penicillin K are produced. The relative effectiveness of the various forms of pencillin is still a matter of controversy, but the balance of evidence is in favour of reducing to a minimum the proportion of penicillin K in material to be used for clinical purposes.

A substance isomeric with penicillin I and with similar biological properties is produced

by *Aspergillus flavus* and has been given the names flavicin and flavicidin.[2] In this substance R is a Δ^3-pentenyl group.

The discovery that different forms of penicillin do not have the same therapeutic value has made it imperative to find methods of estimating the individual penicillins in commercial penicillin, and this is proving to be an extremely difficult task. For a long time the only method of estimating the amount of total penicillins in a mixture was the bacteriological method of assay, and although this has been improved so that the errors of the method now in use are surprisingly small, such a method must always suffer from certain practical disadvantages: for example, that the result can only be known several hours after submission of the sample for test, and that occasional highly erroneous results are obtained. Within recent months several chemical methods of estimating total penicillin have been described. Scudi,[3] for example, published details of a colorimetric method and of a fluorimetric method of assay based on the fact that the β-lactam ring of all the penicillins is opened by organic bases to form substituted amides and, by using an amine containing a chromophoric group (N-(1-naphthyl-4-azobenzene)-ethylene diamine) or an amine containing a fluorophoric group (7-methoxy-2-chloro-5-β-aminoethylamino acridine), products which are either coloured or fluorescent are produced and can be estimated by conventional procedures. These methods have very serious limitations, due mainly to the difficulty of completely removing excess of the reagent from the coloured product; moreover, they are tedious and the reagents are difficult to prepare.

Reactions of this type can be represented as follows:

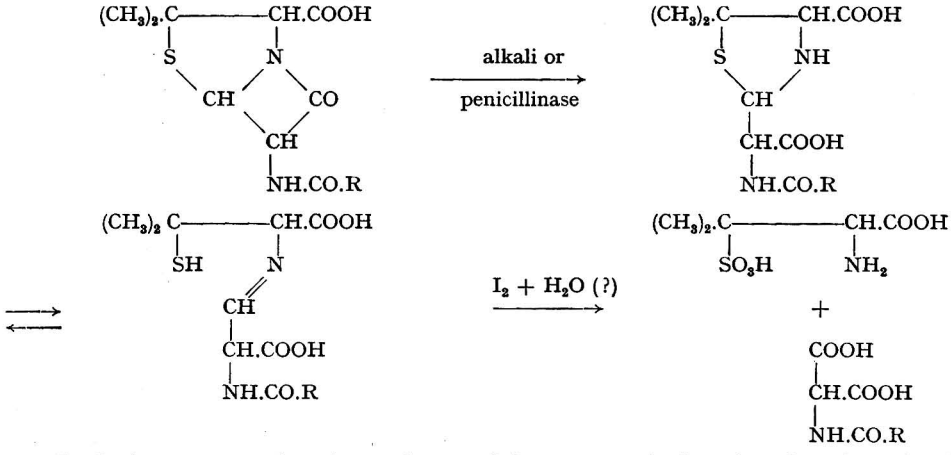


A similar reaction occurs with hydrazines and hydroxylamines and can likewise be utilised for the quantitative estimation of total penicillins. Such methods, however, cannot be used for the differentiation of individual penicillins, as these all behave in the same manner towards these reagents.

A simpler method of estimating total penicillins is based on the fact that, on treatment with alkali or with a specific enzyme known as penicillinase, the β-lactam ring of penicillin is opened, with the formation of an additional carboxyl group, giving penicilloic acid; the amount of alkali required to neutralise the acidity thus produced is proportional to the penicillin content. These two methods are not very accurate, though the penicillinase modification is, as might be expected, highly specific. An improved procedure, which is likely to prove more useful than either of Scudi's methods, is the iodimetric method of Alicino,[4] which is based on the observation that the difference in the amount of iodine consumed under standard conditions before and after inactivation by alkali is proportional to the amount of penicillin present. The alkali converts penicillin into penicilloic acid, which adds on six to nine equivalents of iodine, depending on the conditions used. The mechanism of the reaction is obscure but, provided the conditions are rigidly standardised, the amount of iodine reacting is always constant. The reaction undoubtedly depends on the fact that the thiazolidine ring in penicilloic acid is readily opened to form a free thiol group, whereas ring-cleavage does not occur with compounds, such as penicillin, in which the thiazolidine nitrogen atom is acylated. The free thiol group is then oxidised by the iodine, presumably to a sulphonic acid group, whilst an aldehyde group is probably liberated and then oxidised as in the scheme on p. 276.

The bacteriological method of assay, in addition to its purely practical disadvantages, also suffers from a very serious theoretical objection, for not only do the individual penicillins differ in therapeutic value, but they also differ in the extent to which they inhibit the growth of test-organisms. The degree of inhibition produced by a mixture of penicillins is therefore dependent on the proportions in which the constituents occur, and the biological assay may give an entirely different result from the chemical method of assay and probably neither will give a satisfactory estimate of the therapeutic efficacy. The proportion of two penicillins in a mixture can be determined from the ratio of the response of two different organisms, such as *Staphylococcus aureus* and *Bacillus subtilis*, but the method is not very accurate

and is invalid if a third penicillin is present; with commercial preparations, this is at present the rule rather than the exception.



It is important, therefore, that satisfactory methods of estimating the individual penicillins should be made available as soon as possible. The only chemical method that has so far given satisfactory results is that of Sheehan *et al.*[5] for the estimation of penicillin II. This method depends on the observation that penicillin II forms with N-ethyl-piperidine a sparingly soluble salt which can be filtered off and weighed. The results are said to be quantitative with material containing not less than 50 per cent. of penicillin II and with a potency not less than 800 units per mg., but in our experience the results may not be reliable with material containing less than 90 per cent. of penicillin II. The method has recently been improved in our laboratories by combining the method of Sheehan *et al.* with that of Alicino. A colorimetric method of estimating penicillin II, actually a modification of the Kapeller-Adler method for the estimation of tyrosine, was described by Page and Robinson[6] but is less specific than the method of Sheehan *et al.*, since impurities containing aromatic rings also react, giving rise to coloured products and thus leading to high results. The same disadvantage applies to spectrophotometric methods of estimating penicillin II that depend on the absorption of the phenyl ring, although a method recently elaborated by Philpotts, Thain, and Twigg[7] has given valuable results with material of high potency.

The only chemical method available for the estimation of penicillin K is one recommended by the Food and Drug Administration of the U.S.A., but it does not give reliable results. In this method the penicillin is distributed between a buffer solution of *p*H 6 and chloroform; most of the penicillin K goes into the chloroform phase, whilst most of the penicillin G remains in the aqueous phase. The amount of penicillin in the chloroform solution is estimated by iodimetric titration. Unfortunately the separation is not sharp and other penicillins present may be distributed more equally between the two phases and so increase the error of the method.

The method to be described by Dr. Goodall and Dr. Levi is, like the foregoing, a distribution method, but is of far greater value, since all the penicillins in a mixture can be estimated simultaneously. Morevoer, it is capable of indicating what penicillins are present, so that a qualitative and quantitative analysis of a mixture may be made at the same time. It is without doubt the most useful method yet described for the examination of mixtures of penicillins.

References

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4. Alicino, J. F., *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 619.
5. Sheehan, J. C., Mader, W. J., and Cram, D. J., *J. Amer. Chem. Soc.*, 1946, **68**, 2407; Abst., this vol. p. 313.
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Research and Development Department
Great Burgh, Epsom

A Micro-chromatographic Method for the Detection and Approximate Determination of the Different Penicillins in a Mixture

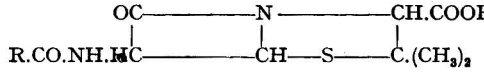
By R. R. GOODALL and A. A. LEVI

(*Read at the meeting of the Society on May 7th, 1947*)

INTRODUCTION—The following Table contains data (as available in February, 1947) on certain characteristics of the penicillins.

TABLE I

PENICILLINS: NOMENCLATURE AND TYPE OF SIDE-CHAINS



Name			Activity per mg.		
British	American	Side chain (R)	*a*, against *B. subtilis*	*b*, against *S. aureus*	Ratio $a \div b$
I	F	Δ^2 pentenyl	970	1550	0·63
II	G	benzyl	1667	1667	1·0
III	X	*p*-hydroxy benzyl	800	900	0·89
K	K	*n*-heptyl	700	2300	0·30
IV	—	unknown	unknown	unknown	—
VI	—	,,	,,	,,	—
VII	—	,,	,,	,,	—

NOTES—

(a) Penicillin IV is not identical with penicillin K as stated by several authors.[7]

(b) Penicillin K is probably identical with a penicillin originally called V (five) by ourselves, but this cannot now be confirmed.

(c) The activities given are those currently available.[8] Differences due to strain variation may be observed in practice. Values for penicillin K are based on unpublished data.

(d) There is some uncertainty about the *B. subtilis* activity of penicillin III (*cf.*[8]).

Commercial samples of penicillin may contain from at least three to seven individual types, the proportions of which may vary widely, depending on the strain of organism, method of culture and method of isolation. Although the penicillins hitherto isolated in a pure state (Table I, I to K) are not widely different in chemical structure, their therapeutic values are not identical, partly because of differences in chemical stability and partly because of possible differences in biological activity against different test organisms.

Methods for determining certain of the penicillins in admixture with others have already been published. Schmidt, Ward and Coghill[8] show how the composition of a two-component mixture can be deduced from the *B. subtilis/S. aureus* assay ratio. The differential assay principle has recently been extended by Higuchi and Peterson[11] to mixtures of three components. Fischbach, Mundell and Elbe[6] describe a macro-chromatographic method for determining penicillin K. Sheehan, Mader and Cram[9] base a method for penicillin II on selective precipitation as the N-ethylpiperidine salt. Penicillin III and K contents can be determined by distribution between appropriate buffer solutions and chloroform.[10]

It is, however, desirable that an analytical method be available for determining directly the proportions of all the different penicillins in a given sample, and this is the aim of the present investigation. We have stated in a preliminary communication[1] that the modified partition chromatogram[2] obtained by using buffer salts in the stationary phase can be adapted to the micro scale, after the manner of Martin and co-workers,[3] who separated a mixture of amino acids and located the position of each component on the paper strip by colour reaction with ninhydrin. In our preliminary attempts to separate penicillin from impurities by the following method, we found that colour reactions were not readily applicable to the treated paper strip, and we therefore tried a biological technique similar in principle to the routine

"cup assay" for penicillin. This technique, which is very simple and highly sensitive, had potential quantitative applications, and it was found possible to establish a method for the approximate determination of the relative proportions of the various penicillins present in a given mixture. This method is described below.

METHOD

SPECIAL REAGENTS REQUIRED—

1. *Stock potassium phosphate buffer solution*—Dilute 500 ml. of phosphoric acid of sp.gr. 1·75 with 1500 ml. of water and add potassium hydroxide solution (containing 906 g. in 3 litres of solution) until 2 ml. of the mixture, diluted to 60 ml. with distilled water at room temperature, has *p*H 6·9 ± 0·04 as measured by glass electrode, using, as standard, potassium hydrogen phthalate solution, *p*H 3·97, at room temperature.

2. *Ether*—Shake vigorously 2 litres of ether (anaesthetic quality) with 250 ml. of water for about 2 minutes. Remove the aqueous layer and store the wet ether in the cold room (0° to 5° C.).

3. *Nutrient agar*—Prepare twenty bottles of 285 ml. of filtered sterile medium containing peptone 10 g., Lemco 3 g., sodium chloride (A.R) 2 g., and agar 25 g. per litre of aqueous solution. Adjust to *p*H 7·5 with 2 *N* sodium hydroxide before filtration.

4. *Buffered filter paper*—Wet Whatman Filter Paper No. 4 with 30 per cent. buffer solution of *p*H 6·9 (Reagent 1). Remove the excess of buffer solution by pressing between sheets of clean blotting paper sandwiched between plate glass. Hang the damp treated paper vertically to dry in the air; it dries in about 2 hours. Cut the dry prepared paper into strips 1·8 by 30 cm. and mark each strip with a pencilled cross on the centre line 6 cm. from one end. The width should be as close to the above figure (1·8 cm.) as possible, and strips with ragged edges should be rejected.

5. *Photographic material*—Extra contrasty gaslight paper (16 by 16 inches), etc.

SPECIAL APPARATUS REQUIRED—

1. *Micro-pipettes*—A. *Automatic pipettes for aqueous solutions*—Draw out thick-walled capillary tubing approximately to the dimensions given in Fig. 1. Cut the glass at point O so

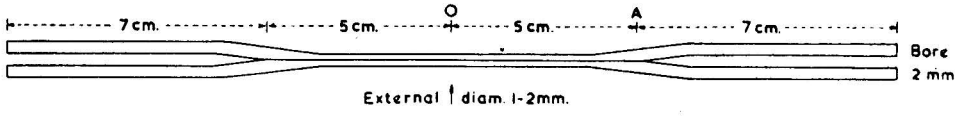


Fig. 1. Dimensions of tubing for automatic micro-pipettes, type A.

as to form two jets. Coat the inside of each jet with paraffin wax. Holding each tube vertically, dip the tip into toluene or xylene. The solvent will rise to a definite height. Remove the toluene by pressing the tip of the pipette on to filter paper. Repeat the toluene treatment four times and then dry the pipette by blowing air through it gently. Dip the tip of the pipette

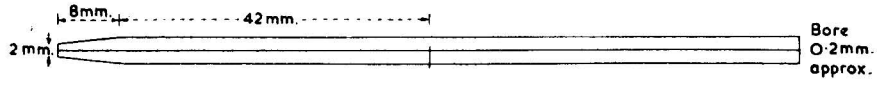


Fig 2. Approximate dimensions of capillary micro-pipette, type B.

into distilled water and note that the liquid rises by capillarity to the boundary of the paraffin coating. Discharge the pipette by pressing gently on to filter paper. Cut down the jet of the pipette until the amount of water delivered in this way is constant at from 1 to 1·5 mg., and mark the position of the liquid meniscus.

B. *Capillary micro-pipettes for solvent extracts*—Prepare a 1-microlitre glass pipette as shown in Fig. 2.

2. *Apparatus for chromatographic development*—The apparatus is illustrated in Figs. 3A, 3B, and 3C.

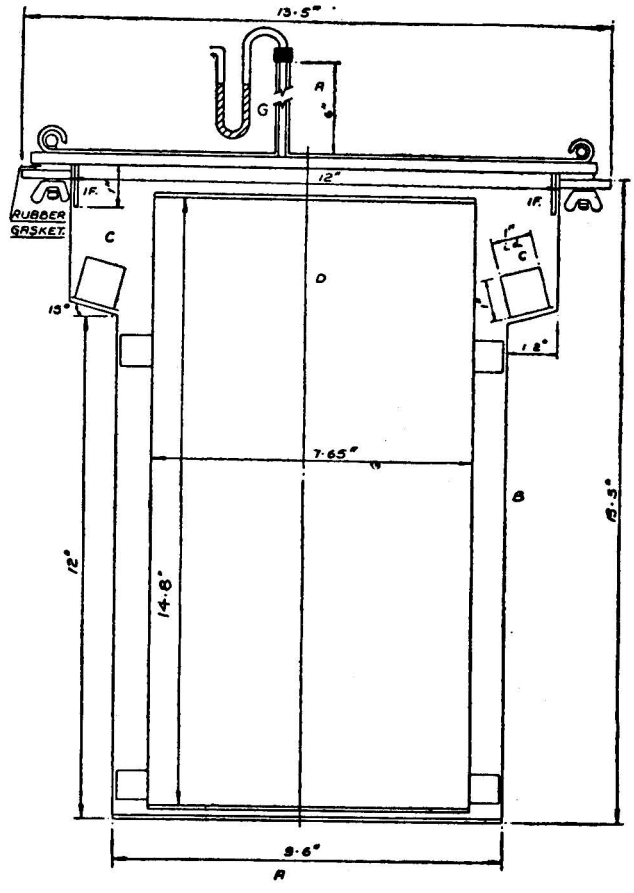


Fig. 3A. Section through 'A' 'A' of apparatus for development of 24 paper chromatograms.

B. Chamber with lid and internal flange, IF., secured by 8 wing nuts on to a soft rubber gasket.

C.C. Ring carrying 24 cups seating on a shoulder 3" from top of chamber.

G. Stainless steel vent tube ($\frac{1}{4}$" O.D.) attached to a short U trap containing mercury.

D. Closed drum with 4 spacing pieces.

Material of construction — body-welded stainless steel sheet 20 gauge, flange and lid $\frac{1}{4}$" stainless steel plate.

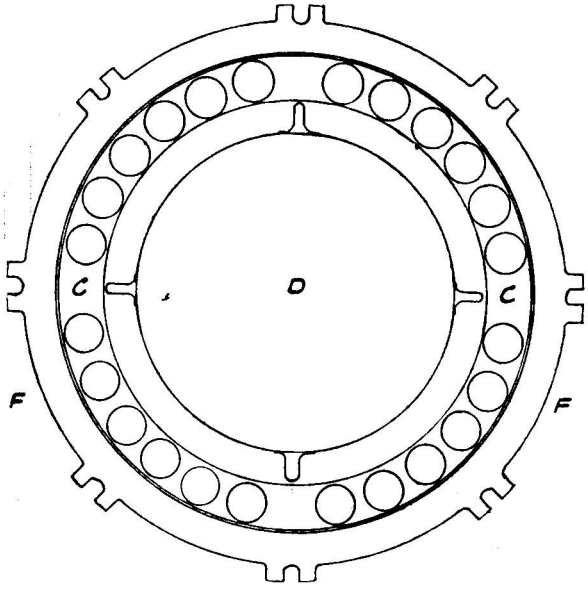


Fig. 3B. Plan of top of chamber with lid removed.

F. Flange with 8 lugs for wing nuts.

C.C. Ring, showing distance between centres of the cups.

For true position of each cup, which is inclined inwards (at an angle of 15°), see Fig. 3A. Each cup holds a glass reservoir (see Fig. 3c).

The vertical surface of the closed drum D (Fig. 3A) is tightly wound with one thickness of cotton bandage, 2 inches wide, and each end is stitched to the adjacent lap to prevent unwinding. Lengths of rustless spring curtain rod (about 23·5 inches long before expansion) are then clipped over the bandage, at 2-inch intervals, to form a set of eight horizontal rings. The bandage is moistened before the apparatus is to be used; the spring rings prevent the paper chromatographic strips from coming in contact with the wet cotton.

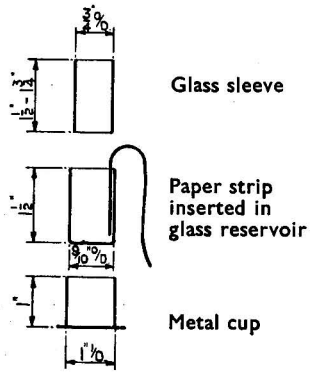


Fig. 3c. Details of glass reservoir, etc., before assembly.

3. *Glass plates for agar medium*—Construct these as detailed in Fig. 4 by cementing plate glass strips, 0·75 inch wide, to a square plate-glass base, 16 by 16 inches, with shellac dissolved in alcohol. Each plate will carry seven chromatographic strips, or six and a strip with standards. Provide a plate-glass lid, 16 by 16 inches, for each of the plates.

A sheet of plate glass, 13·5 by 13·5 inches, just smaller than the space for the agar in the above plates, will also be required.

Clean and sterilise each plate before use (see below).

4. *Dilution pipette*—In general, sufficient sample will be available to permit serial dilution by graduated pipettes. If, however, economy of sample is a prime consideration, the technique of the dilution pipette[5] is readily applicable. These pipettes must not be used for organic solvent solutions.

5. *Burettes for charging ether*—Prepare two rough burettes holding 60 ml. and graduated at intervals of 8 ml. Control delivery of liquid by a spring clip on a rubber tube of 5 mm. bore.

6. *Vessel for humidifying strips*—Obtain a large vessel (*e.g.*, a large drum) with a lid. Provide means of keeping the atmosphere in this vessel damp (*e.g.*, a damp cloth hanging round the inner circumference). Provide a stand on which the strips can be hung, well spaced out from each other, inside the vessel, so as to expose them freely to the atmosphere of the vessel.

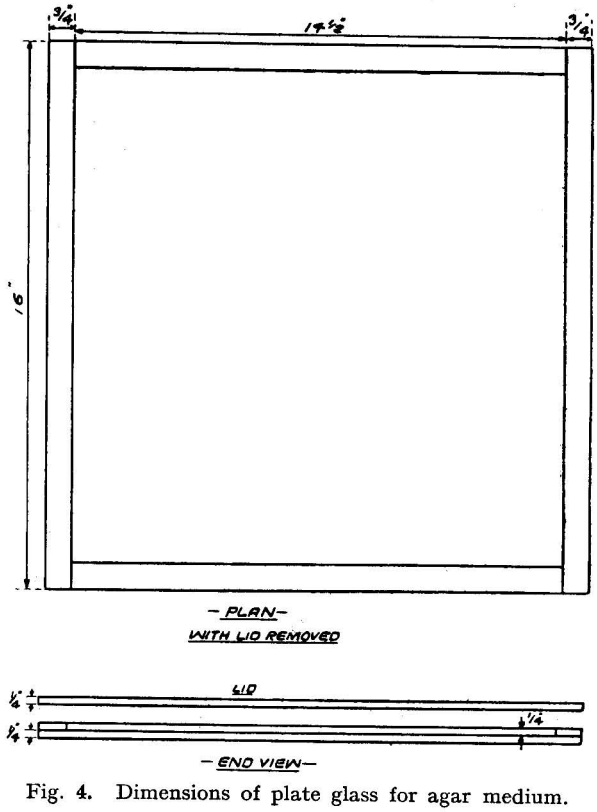


Fig. 4. Dimensions of plate glass for agar medium.

PROCEDURE—

FIRST DAY

Preparation of sample and standards—Prepare a solution of the sample containing from 1 to 40 units per microlitre. Dissolve the sample in half the calculated volume of water and make up to volume with 30 per cent. buffer solution of *p*H 6·9 (Reagent 1). Filter or centrifuge if necessary; 50 to 100 μl. of solution are ample.

For standards make five serial dilutions of a solution of pure sodium penicillin II containing approximately 30 units per ml. in 30 per cent. *p*H 6·9 buffer solution (Reagent 1), each one-third the strength of the previous solution, one volume of solution being diluted with two volumes of buffer at each stage.

All solutions and standards must be kept stoppered in the cold room (0° to 5° C.) except when actually required for use. After 14 days fresh solutions should be made up.

Solvent extracts and samples already in solution—These are used without further treatment. Waxed pipettes must not be used for organic solvent extracts.

"Spotting" solutions of sample, etc.—Fifteen minutes before beginning "spotting," place the required number of strips in the special apparatus 6 to humidify. After this treatment, apply to each strip a 1-μl. spot of the test solution at the marked point. Fix a wire paper-fastener to the bottom end of each strip (*i.e.*, the end remote from the marked point). Remove the central drum from the special apparatus 2. The apparatus should be at room temperature during this operation. Place the top end of each spotted test strip in a glass reservoir supported in the frame of the apparatus and fix it in position by inserting the glass sleeve provided for each reservoir. Ensure that the top end of the strip just touches the bottom of the reservoir and that the "spot" is located about 1 cm. below the edge of the reservoir. Adjust the strips so that the clipped ends hang vertically, *i.e.*, clear of each other and not touching any part of the apparatus.

Put 50 ml. of ether in the bottom of the apparatus to form a pool. Remove the apparatus to the cold room. Wet the cloth apron round the central drum with water and carefully place the drum in the apparatus. Replace the lid.

After 2 hours, and while the apparatus is still in the cold room, remove the lid. Deliver 8 ml. of ice-cold ether into each reservoir. Without delay, replace the lid on the apparatus and make gas-tight. This operation must be carried out as quickly as possible to avoid formation of ice on the strips.

Allow development to proceed overnight.

Cleaning and sterilisation of glass plates—After removing used agar, rinse the plates thoroughly with water. Wipe the surface with dilute hydrochloric acid (0·4 per cent.). Allow the acid to remain on the plates for 2 to 3 minutes, rinse with water, wash with soap and water, and again rinse with water. Careful cleaning of the plates in this way is essential.

Support the plates on top of an asbestos sheet, using cork spacers, and sterilise by heating at 120° to 130° C. for 3 hours. Store the required plates at 37° C.

Spotting out standard solution—Prepare one standard strip carrying undeveloped spots by delivering 1-μl. spots of each solution on to a *p*H 6·9 buffered strip at points 5 cm. apart along the strip. Store in the cold room until required.

SECOND DAY

Melt the agar medium and inoculate it with *B. subtilis* spores at a temperature not below 70° C.; shake and allow the froth to break at 70° C. Pour the medium on to an uncovered glass plate (special apparatus 3) just removed from storage at 37° C. and laid on a level glass surface. If necessary, flame the plate locally to assist covering by medium. Protect the medium with the plate lid, spaced 0·5 inch above the surface: allow to set and evolve moisture during 10 minutes. Then lower the lid on to two felt strips, placed on opposite sides of the plate, invert the plate and allow to cool during 15 minutes in an asbestos-lined box in a cold room (0° to 5° C.).

Plating out the strips—Open the apparatus containing the developed chromatographic strips, remove the central drum carefully and transfer the strips to a clean glass container. If any reservoirs still contain ether, remove the strips from these first, otherwise ice will collect at the top of the strips owing to evaporation of ether.

When the agar plates have been in the cold room for at least 15 minutes, begin plating out the strips in the following way.

Support the 13·5 by 13·5 inch sheet of plate glass (special apparatus 3) on blocks so that it is raised off the bench about 2 inches and the edges are not obstructed in any way. Clean the surface with acetone. Arrange a set of strips evenly on the top of the glass. A card ruled at 1·9-inch intervals from the centre and attached to the under-side of the glass is a convenient guide. Lift the agar plate off the felt supports and lay it gently, face down, on top of the strips. When the strips have wetted out (this is easily observed) lift up the agar plate. The strips will adhere to the agar surface in their correct positions. Replace the agar plate on the felt supports on the lid and transfer with its lid, still with the agar face downwards, to the cold room.

Incubation—After the plates carrying the strips have been in the box in the cold room for four hours, close the plates with the lids by removing the felt strips and place, face downwards, in an incubator at 37° C. Allow incubation to proceed overnight.

THIRD DAY

Reproducing the results—Remove the plates from the incubator and identify the strips with "Chinagraph" markings on the glass. Using the patterns on the plates as a negative, make contact prints on the gas-light paper.

Measurement of zones—Measure the diameters of the circles given by the stationary (undeveloped) standards, estimating to 0·1 mm. Similarly measure the maximum width of each zone on each developed strip.

CALCULATION OF RESULTS—

Relative proportions of B. subtilis *units*—Let the diameters of the circles given by the standard spots be A, B, C, D, E, and F in descending order of magnitude. Then the slope b' (compare conclusions following Table II, p. 286) for the developed zones is given by the expression:

$$b' = 1{\cdot}2\,[5(\text{A–F}) + 3(\text{B–E}) + (\text{C–D})]/35 \log 3$$
$$= 0{\cdot}0719\,[5(\text{A–F}) + 3(\text{B–E}) + (\text{C–D})]$$

Divide the diameter (in mm.) of each developed zone by b'. The resulting value is the logarithm of the number of arbitrary units in the zone. Calculate the mean activity in arbitrary units for each zone, and from these obtain the percentage composition. The arbitrary *B. subtilis* units apply to the particular plate only.

Conversion to relative proportions of S. aureus *units*—

(i) Divide the per cent. activity in each zone (*B. subtilis* units) by the appropriate factor (Table I).

(ii) $$\frac{\text{Units against } B.\ subtilis}{\text{Units against } S.\ aureus} = \frac{100}{\text{Sum of quotients from (i)}} = \text{F}$$

(iii) Proportionate activity (*S. aureus* units) in each zone
$= \text{F} \times$ % activity in *B. subtilis* units.

Composition by weight—

(i) $$\text{Per cent. of total activity in each zone} = \frac{\text{Per cent. activity in } B.\ subtilis \text{ units}}{\text{Activity}^* \text{ (}B.\ subtilis\text{ units) of the appropriate pure component.}}$$
$$= \text{P}$$

(ii) Per cent. of total sample weight in each zone
$= \text{P} \times$ activity of the sample in *B. subtilis* units.

Note—For penicillins of undetermined activity the ratios *B. subtilis/S aureus* have been assumed to be unity, and the activity 1000 units per mg. Where these penicillins occur only as minor components of a mixture, as they have done in many samples we have examined, the error introduced into the over-all analysis by this approximation is probably not great.

RESULTS AND DISCUSSION

As a necessary preliminary, it was established, from the results of a series of experiments designed to detect a loss of 7 per cent. or more of the total activity, that no significant loss took place when the above method was applied to a blend of calcium penicillin (activity 300 units per mg.). Losses, however, occurred if the chromatograms were carried out at room temperature or if serious delay arose before completing the experiment. In these experiments the active zones were stripped from the paper with water and the activity of the resulting solution was compared with that of a freshly prepared solution of the same sample.

QUALITATIVE SECTION

The chromatographic identification of the zones (Fig. 5, A) obtained from an early sample, by reference to the results obtained from known penicillins developed simultaneously on adjacent strips, is illustrated in Fig. 5, A, B and C. The zone A II is attributed to penicillin II not only because it corresponds in situation to the zone C II obtained from pure penicillin II, but also because the increment in zone diameter (B II–A II) due to enrichment of the sample

* See Table I.

with pure penicillin II is confined to the one appropriate zone and is of the expected order of magnitude, according to later deductions. Since the other major constituent of this sample was known to be penicillin I, by the macro-column technique, the identity of the substance producing the other zone in Fig. 5, A is in all probability penicillin I. The identity of penicillin III on the paper strip was established by similar comparative tests using a penicillin III fraction from a macro column. Resolution of penicillins II and III is shown in Fig. 5, D. The above three penicillins all occur in the same order as observed on macro columns.

When the technique came to be applied to products from various sources, further zones of activity were resolved (*e.g.*, Fig. 5, E). Ideally, for the qualitative and quantitative development of the technique, the behaviour of mixtures of the pure individual penicillins should be studied, but only penicillin II was available in the pure state. However, there was little difficulty in identifying the various zones of activity, from the fixed order in which they occur after development. The behaviour is analogous to that of the amino acids reported by Martin and others.[3] Penicillin II was a convenient key zone, for it was usually known to be the major active constituent; if not, it was added as a "tracer," in the manner of Fig. 5, B. The order from the point of application of the solution is: III, VI, II, I, IV, VII and K. The seven zones were detected on one strip (*cf.* reproduction in Fig. 5, E). The existence in Fig. 5, E and Fig. 6, C of the zone attributed to penicillin VII has been repeatedly confirmed. It is also apparent in Fig. 6, C that there is a suggestion of further resolution at the front of the K zone. The existence of penicillin VI was clearly demonstrated in other experiments as shown in Fig. 6, A and B. Penicillins VI and VII have only been found in minor amounts, generally less than 5 per cent. of the activity of unfractionated material.

Work on macro columns in these laboratories (to be published shortly) has confirmed that the zone usually attributed to penicillin I is due to a mixture of two penicillins, called I and IV (penicillin IV is not identical with penicillin K). This is also clearly shown by the present technique (see Fig. 5, E).

It is known that penicillin K is the fastest-moving penicillin.[6]

With further experience it has been found that the method of identification based on ratio of distances traversed by unknown and known "spots"[1] is less satisfactory than was at first thought. Following are comments on certain aspects of the technique.

Control of development

(1) *Humidity*—It cannot be stressed too strongly that the humidification treatment of the strip before and after spotting is of cardinal importance. For example, a prior treatment of the strip for one hour instead of 15 minutes will result in elongation of each zone, the K zone partly overshooting the end of the strip. The syrup obtained by evaporation of buffer solutions of the type used is very sensitive to the state of humidity of the atmosphere. The best method of controlling the moisture content of the strip was found to be to expose the treated paper to a fully humid atmosphere for a specified time. Even this is not entirely satisfactory.

(2) *Concentration of buffer*—This also has very marked effects on distance of development. Strips were impregnated with phosphate buffer solutions ranging from 1 per cent. w/v up to 30 per cent. w/v (Reagent 1) and it was found that the use of the 30 per cent. w/v solution, which is about the maximum convenient concentration, led to zones of the most compact and most regular type; it also produced the farthest separation of the zones, in other words the greatest degree of resolution, obtained.

(3) *pH value*—For the slower-moving zones, variations of ± 0·1 unit in the *p*H of the applied buffer solution have little effect, but a closer control of *p*H is desirable in order to retain the fast-moving zones on the strip. The method outlined above lays down conditions adequate for separating the major constituents, I, II, III and K. For the detection of traces of other penicillins (*e.g.*, VI) some variation in the conditions may be necessary. For example,[1] the existence of the new penicillin VI was revealed between penicillins III and II when a chromatogram on a strip carrying buffer of *p*H 6·78 was repeated on a strip carrying buffer of *p*H 6·25 (*cf.* Fig. 6, A and B).

(4) *Width of strip*—The width specified is a compromise between narrow strips (10 mm. wide) which are more productive of faults such as asymmetric zones (Fig. 5, D II), and wide strips (20 mm. wide), which obscure the appearance of the smaller zones and are an inconvenient size for fitting into the apparatus. Strips must be of uniform width and free from ragged edges. The latter are liable to cause gross irregularities. A mean change in strip width of 1 mm. caused a mean change in zone diameter of the order of 0·4 mm.

(5) *Solvent*—Of the available solvents, ether has been by far the most satisfactory. Adjustments of *p*H will be necessary if other solvents are used.

(6) *Development*—The apparatus (Figs. 3A and 3B) is designed to standardise as far as possible the degree of development, which is related to throughput of solvent and moisture

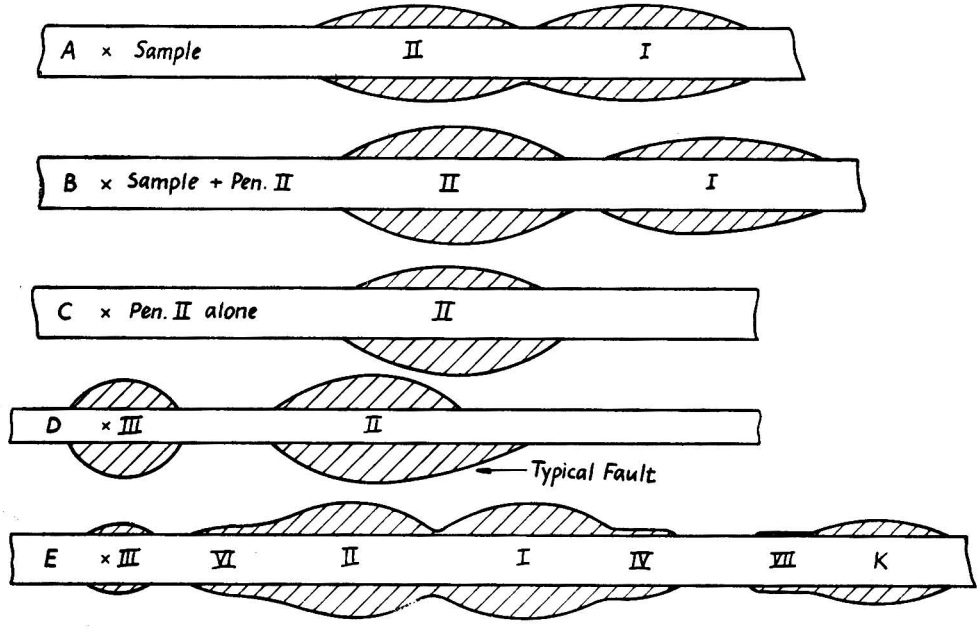


Fig. 5. × = Point at which penicillin solution was applied. The area where growth is inhibited is shaded.

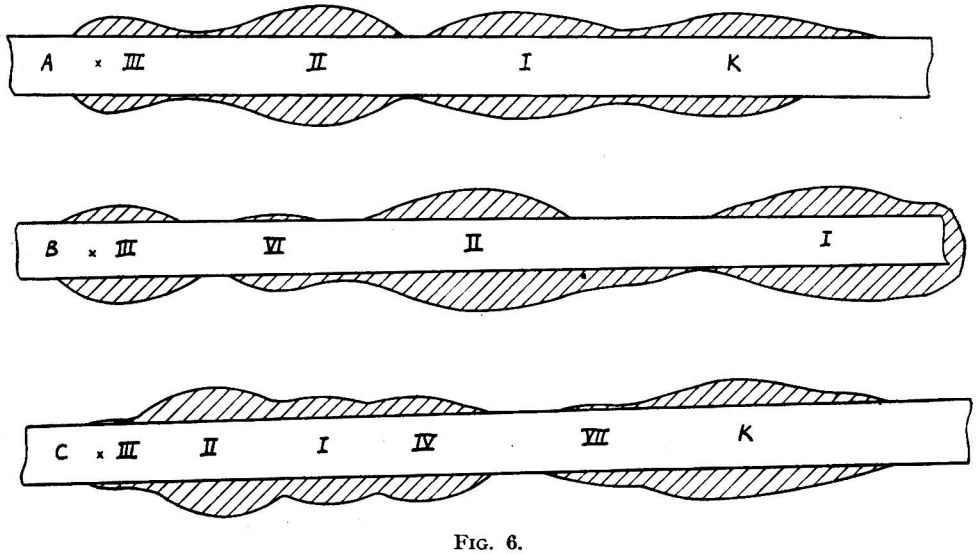


FIG. 6.

content of the treated strip. In order to keep these two factors constant, the apparatus is made gas-tight, with individual reservoirs for each strip and with a large damp surface to keep the atmosphere humid. The junction at the flange and lid is readily made gas-tight by means of a Neoprene sponge rubber gasket.

Biological procedure

(1) *Precautions regarding sterility*—These were confined to the preparation and use of the biological plates, since no practical advantage was gained by sterilising the paper strips before use. Little infection was encountered under the specified conditions.

(2) *Medium*—Inoculated agar sheets of uneven thickness are useless for quantitative work, owing to marked variation of zone diameter with depth of agar. Hence the molten medium must be poured under conditions that permit the agar to form a plane sheet before setting.

(3) *Diffusion*—Larger zones with clearer edges are obtained if a period is allowed for diffusion before incubation. This is not, however, essential. Care must be taken that there is no temperature gradient across the plate. For example, from a plate containing only undeveloped zones kept cold in a domestic-type refrigerator, there was a significant variation in zone diameter across the plate from the side nearest to the cooling coils.

Quantitative

For the development of a quantitative technique, dimensions such as area or length of the inhibition zone were too highly dependent on degree of development and also were not precise when the zones overlapped. However, these objections did not apply to the maximum diameter of the zone, which in preliminary experiments appeared to be proportional to the logarithm of the units in the zone. The precise relationship defining the diameters of the inhibition zones obtainable both from developed and undeveloped spots was investigated in the following way.

A set of solutions of penicillin II crystalline sodium salt (Table II) was prepared by serial dilution. Using 1-μl. spots from each of these solutions a set of strips carrying 1-μl. developed spots were obtained after chromatographic development. These developed spots were arranged on a biological plate together with a set of 1-μl. undeveloped spots from the same set of solutions. The diameters of the resulting zones of inhibition are given in Table II. The experiment was carried out under routine conditions without special supervision, and the figures are probably fairly representative.

Table II

Comparison of inhibition zone diameters (mm.) obtained from "undeveloped" (U) and "developed" (D) spots of known activity

	Sodium penicillin II solution; units/ml. (*B. subtilis*):						Mean (a and a')	Slope (b and b')	b'/b
	10·47	3·49	1·163	0·388	0·129	0·043			
U	44·4	39·6	37·1	32·1	29·9	23·9	34·50	8·18	1·20
D	43·2	41·0	35·8	30·2	26·9	20·0	32·85	9·82	
U	43·2	40·2	36·9	33·2	27·8	22·2	33·92	8·75	1·08
D	43·2	40·6	36·4	30·2	25·1	22·2	32·95	9·41	
U	43·6	39·7	37·2	32·6	29·6	24·2	34·48	7·91	1·24
D	43·2	41·2	34·8	31·1	27·9	19·2	32·90	9·80	
U	42·9	41·4	37·3	33·6	28·5	24·8	34·75	7·96	1·19
D	44·0	40·5	36·8	31·6	27·2	21·5	33·60	9·44	
U	44·9	40·8	38·1	33·0	31·0	24·8	35·43	7·66	1·20
D	45·4	42·0	36·2	32·5	28·2	22·1	34·40	9·77	
U	46·5	43·0	39·8	35·1	31·1	25·0	36·75	8·86	1·10
D	44·0	43·8	37·8	33·1	30·0	20·8	34·93	9·69	
U	43·9	40·7	37·2	32·8	29·3	24·8	34·78	7·66	1·16
D	45·1	41·1	36·5	31·9	26·4	23·7	34·11	8·86	
U	38·3	36·7	33·1	26·8	26·1	21·7	30·45	7·25	1·22
D	40·0	36·9	32·3	28·5	23·8	19·1	30·11	8·87	
U	39·1	36·5	33·0	28·8	25·2	21·2	30·63	7·64	1·16
D	39·5	36·7	32·7	29·1	23·0	18·9	29·99	8·86	

Notes on Table II—

a and a' are ordinates, b and b' are slopes for six results for undeveloped and developed spots respectively. Standard deviation of each zone diameter: U, 0·9; D, 1·24 mm.

The following conclusions were drawn from the data:

(1) For both sets of spots the trend of the results is largely accounted for by an expression of the type

$$\text{diameter of zone} = a + b \log (\text{units in zone})$$

where a and b are constants for a particular biological plate.

Both a and b may differ considerably between plates.

(2) There is, however, a small but significant curvature, which is similar in magnitude for both sets, *i.e.*, the relationship is only approximately rectilinear. A similar result has been observed in the ordinary cup assay when carried out with an extended range of concentrations.

(3) The slopes for the undeveloped zones (b) were significantly lower than those for the developed zones (b').

(4) The ratio of the slopes b'/b was sensibly constant, with a value of approximately 1·2; similar values were obtained on several other occasions.

(5) The over-all standard error, including that due to the curvature, is of the order of ± 1 mm. This corresponds approximately, at 95 per cent. limits of error, to ± 25 to 30 per cent. in the estimation of activity in any one zone. For a sample replicated on 6 strips the 95 per cent. limits of error are thus about ± 15 per cent.

In a further experiment in which solutions containing 30, 3, and 0·1 units were each developed with 1, 3, 5, 7, 9, and 11 ml. of ether, it was shown that zone diameters increased with distance of development for the strongest solution, while the reverse was true for the weakest solution. No effect was detectable at the intermediate strength. This phenomenon accounts for observation (3) above.

OTHER POSSIBLE METHODS OF DETERMINING PLATE SLOPE—

The method given above was adopted after considering three alternatives.

(*a*) Comparison with the results from standard mixtures of the pure penicillins so arranged as to give series of developed inhibition areas appropriate to each penicillin. This is not possible in the absence of the pure components.

(*b*) A planned distribution of the developed test spots and the undeveloped standard spots over several plates. This method is dependent on reasonable conformity of each plate to an average plate slope, but plate to plate variations of slope, even on a single day, were too great.

(*c*) Serial dilution of the sample itself. As the minor zones soon disappeared in the results obtained from a set of serial dilutions, and as there is obviously an upper limit to the load (governed by the area available on the biological plate), this procedure did not permit adequate replication. Nevertheless if samples of suitable composition are available this method should provide useful information about the relative plate slopes of the different penicillins.

In adopting the undeveloped spots as standards, the advantage that the results are, comparatively, of a high order of accuracy will counteract the errors due to the uncertainty of the empirical factor 1·2 used to calculate the slope for the developed spots. Small errors in the value of the slope are important only in connection with very small or very large zones, as, for example, in calculating the proportions of an impurity in a major component.

It is probable that the slopes of the developed zones increase from penicillin III to penicillin K, but the evidence so far obtained is not conclusive. Since penicillin II is developed a moderate distance down the strip, the value of b' for penicillin II is probably a reasonable value for the mean of slopes. Errors due to the use of this figure can be expected; thus differences between proportions of penicillin III in different samples will be minimised; differences between proportions of penicillin K will be exaggerated.

TYPICAL NUMERICAL RESULTS—

In the absence of samples of the pure components for making synthetic mixtures, checks on accuracy have had to be confined to such results as are shown below in Tables III, IV and V.

TABLE III

REPLICATION OF ANALYSES BY ROUTINE OPERATOR

	Penicillins found:					
	III	II	I	IV	VII	K
Sample 1:						
Six-strip assay	0·5	37·5	29·8	19·7	1·4	11·1 per cent.
Repeat	1·1	39·6	28·5	17·7	trace	13·1 „ „
Sample 2:						
Six-strip assay	1·9	15·6	18·8	16·4	—	47·0 per cent.
Repeat	1·8	15·9	22·5	17·8	—	41·7 „ „

TABLE IV

COMPARISON OF CALCULATED AND OBSERVED VALUES FOR DIFFERENTIAL ASSAYS

The figures below represent values of the ratio: units against *B. subtilis* ÷ units against *S. aureus*, for a variety of samples containing from 3 to 7 component penicillins.

Observed:	0·65	0·71	0·98	0·82	0·58	
Calc. (see method):	0·69	0·71	0·98	0·88	0·55	
Observed:	0·83	0·94	0·85	0·80	0·89	0·68
Calc.:	0·89	0·96	0·89	0·86	0·92	0·75

TABLE V

MATERIAL BALANCE (MEGA-UNITS) ON PARTIAL FRACTIONATION OF A PENICILLIN SAMPLE

Fraction No.	Total mega-units (direct assay)	Composition of each fraction, in mega-units calc. from micro-chromatographic analysis and direct assay			
		III	II	I + IV	K
1	0·079	0·079*	—	—	—
2	0·218	0·002	0·175	0·038	0·003
3	0·507	—	0·446	0·060	0·002
4	0·071	—	0·008	0·062	0·001
5	0·532	—	—	0·523	0·009
6	0·212	—	—	0·160	0·052
7	0·477	—	—	0·015	0·462
Total	2·096	0·081	0·629	0·858	0·529
Starting material	2·40	0·068	0·652	1·17	0·503

* Assumed to be penicillin III, not analysed.

Note—The recovery in a fractionation process of this kind is expected to be about 80 per cent.

The data in Tables III to V are fairly representative of the results obtainable under routine conditions. The method described has proved to be very useful, particularly for the examination of complex mixtures of the type shown in Table III, which, for the first time, can now be directly analysed for every component. It is hardly likely that such a complete analytical picture could be obtained by other than chromatographic means. Bearing in mind that the procedure involves both a chromatographic stage and a biological assay, the reproducibility obtained is reasonably good.

The application of the method to samples predominantly penicillin II has recently become of special interest, since clinical-quality salts containing from 90 to 100 per cent. of penicillin II are now being manufactured. In this connection it is pertinent to illustrate how the figure for the relative proportion of penicillin II is not greatly affected by comparatively large errors in the units found in each of the several penicillin zones. For example, let us assume that a sample of relatively pure penicillin II has the following composition in *B. subtilis* units per cent.:

Penicillins: III, 1%; II, 94%; I, 2%; IV, 1% and K, 2%.

Let us further assume that the error in the estimation of the penicillin II zone be ± 15 per cent. (see p. 286), but that the errors in the minor (III, I, IV and K) zones be *as much as* ± 50 per cent. Now although these deviations are large when considered as absolute errors on each individual unitage, it can be calculated that for mixtures of the above type, the *extreme range* for the *relative* proportions of the major constituent (pencillin II) carries a much smaller error. In the case assumed the range is from 90·0 to 97·3 per cent., where the true figure is 94 per cent.; *i.e.*, the *extreme* errors in the relative proportion are − 4·0 and + 3·3 per cent. It is shown below that the reproducibility in the proportions of penicillin II found in replicate analyses (6-strip assays) of this type is well within these limits:

Pencillins present	III	II	I	IV	K	
Sample 1:	trace	98·2	1·0	0·5	0·4	per cent.
Repeat:	0·4	98·0	0·7	0·3	0·6	,, ,,
Sample 2:	0·7	93·0	3·3	0·9	2·0	,, ,,
Repeat:	0·6	94·5	2·5	1·1	1·3	,, ,,

Hence the method serves to provide a reasonably accurate measure of the relative content of the major penicillin in this class of sample.

SUMMARY

A method is described in which the various penicillins in a mixture are separated by a micro partition chromatographic technique, based on differences in distribution of the penicillins between ether (mobile phase) and phosphate buffer (stationary phase) supported on a strip of filter paper. The separated penicillins are invisible, but after incubation in contact with an agar sheet, pre-inoculated with *B. subtilis* spore suspension, elliptical zones free from bacterial growth are clearly visible in the neighbourhood of each active component. The technique is highly sensitive, 1 μl. of solution (1 to 30 international units) being adequate for a qualitative separation. The maximum diameter of each zone is proportional to the logarithm of the number of units in the zone. Quantitative data are given, indicating the order of reproducibility of the method.

It is shown that the activity in crude products is mainly due to penicillins I, II, III, IV, and K. Small amounts of new penicillins (called VI and VII) have also been separated, but in the samples examined these have never represented more than 5 per cent. of the total activity.

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DISCUSSION

Mr. S. G. E. STEVENS congratulated the authors on a method which seemed to have great possibilities in evaluating penicillins and asked whether "halo" formation was observed and whether variations in the concentration of a penicillin introduced any undue vertical as well as horizontal diffusion.

Dr. LEVI in reply said that the edges of the zones were quite sharp at all concentrations and halo effects had not been observed.

Dr. E. LESTER SMITH said the authors were to be congratulated on this extremely elegant application of the chromatographic technique to a difficult and important problem. It seemed probable that chromatography, and in particular partition chromatography, would become of increasing importance to analysts. The speaker had obtained some experience with the procedure on the basis of the authors' earlier brief report, and had rediscovered some of the snags, in particular the vital importance of humidity control. Another difficulty encountered had been a tendency for the zones, even on the standard strips, to merge into one another as though the paper was taking up too much moisture from the agar jelly and so permitting diffusion of penicillin along the strip.

The paper strip chromatograms were remarkably effective in separating individual penicillins and appeared to give sharper separations than buffered silica gel columns. It was suggested that this might be correlated with the observation that penicillin K sodium salt behaves in aqueous solution as a colloidal electrolyte; its solutions froth badly and are capable of emulsifying organic solvents such as chloroform and solubilising certain dyes that are insoluble in water. Penicillin II sodium salt does not behave in this way in dilute aqueous solution but becomes colloidal in presence of strong buffers. The colloidal particles were likely to be adsorbed by the silica and this could account for the bad "tailing" of penicillin zones on these columns.

It was unfortunate that the nomenclature was so confused. The American system using various arbitrary letters was less tidy than the English numeral system, but the latter was rather spoiled because the number "IV" had been allotted to different penicillins. It had to be conceded that I.C.I. workers were the first to claim this number for a new penicillin in a confidential report, whereas others, in ignorance of this report, had assigned the number to penicillin "K"; perhaps we should agree to use "V" for penicillin "K"? A zone on paper strip chromatograms probably corresponding with the I.C.I. penicillin IV was commonly found with submerged-culture penicillin. It seemed likely that this would be found identical with the dihydro-penicillin I recently claimed by American workers as a component of penicillin made by submerged culture.

THE AUTHORS, in reply to Dr. Lester Smith, said they had observed the effects of merging of zones, and diffusion along the edges of the strip, to which he had referred. The main cause was irregularity of the strip edges, but there were undoubtedly other factors still unknown. The effects were certainly less frequent with strong buffer. It was mentioned that it was difficult to cut the strips exactly to size.

Buffered columns on silica gel could give excellent separations, but care must be exercised in the correct choice of initial conditions, such as amount of charge relative to amount of adsorbent, initial concentration, and so forth. Dr. Levi hoped to publish shortly a paper on the theory and practice of this type of chromatogram. Previous publication had been delayed by secrecy regulations.

Mr. F. A. ROBINSON said he had gained the impression that Dr. Lèvi, in studying the effect of various factors, had carried out factorial design experiments only on the chromatographic procedure; he asked whether similar experiments had been carried out to determine whether any of these factors interacted with factors involved in the bacteriological procedure. It was very difficult to obtain an agar film of uniform thickness, and an irregular film might well introduce errors, wrongly attributed to faults in the chromatographic technique.

Dr. LEVI, in reply, said that such experiments had been carried out and that the method of pouring the plates was now very rigidly controlled; he did not believe that the variations in the thickness of the film at different points on the plate were sufficient to introduce serious errors, provided that the specified routine was strictly followed.

Dr. GOODALL said it was important to use a refrigerator so designed as to minimise thermal gradients in the cold atmosphere enclosed. It had been noted that, when a domestic-type refrigerator was used, the diameters of a set of "standard" spots decreased in the proximity of the cooling coil.

Dr. H. DRYERRE, enlarging on a point made by Mr. Stevens, thought that the thickness of the agar film was a factor which must not be neglected. The test was dependent upon the extent of diffusion of the penicillin lateral to the side of the paper strips. It was reasonable to assume that the penicillin diffused also downwards, and any increase in thickness of the agar layer would by diversion leave less penicillin to diffuse laterally.

In reply, Dr. LEVI said there was no doubt that thickness of the agar film had a profound effect on zone diameter. Conditions, however, had been worked out which give a reasonably uniform film. The most important condition was that the agar should be above 70° C. when inoculated and poured. If the temperature were, say, 65° C., then visibly irregular films were liable to be obtained.

Dr. A. J. P. MARTIN asked whether the use of a salt solution instead of water in the inner cylinder would not give a more uniform humidity in the chromatogram strips.

Dr. LEVI said that potassium phosphate solutions of the type used, when evaporated, leave syrups that readily take up or evolve moisture according to the state of the atmosphere. There was, of course, no equilibrium vapour pressure. Salt mixtures had a vapour pressure less than saturation for the particular temperature and tended to dry the buffered paper strips. They were abandoned for this and other reasons. The best method so far found was to expose the strips to a fully humid atmosphere for a specified time. This was still unsatisfactory, as it was difficult to control the state of the strips before humidification was started. Thus strips in the centre of a pile might be more or less damp than those near the top, and so on.

Mr. A. L. BACHARACH asked whether the iodination of penicillin and its hydrolytic product might not be affected by the nature of the side chain. He also enquired whether it was unnecessary, as he gathered, to sterilise the paper strips before attaching them to the agar plates. The problem of producing satisfactory strips of uniform width was surely one that should be referred back to the makers of the filter paper, particularly if the test and its subsequent modifications were, as a result of the excellent work and description by the authors, to be widely used. He also emphasised the importance, even though the error of these tests should be steadily reduced, of so designing them and computing the results that not only the estimate of penicillin activity, but also its error, could be stated.

Mr. ROBINSON said that the iodimetric method gave very satisfactory results, in the sense that these could readily be reproduced by different workers. He agreed with Mr. Bacharach that the individual penicillins might take up different amounts of iodine; this was especially likely to occur with the *p*-hydroxy benzyl group of penicillin III, though he doubted whether the double bond in the side-chain of penicillin I would add on iodine under the conditions employed.

Dr. GOODALL, in reply said that he had modified the iodimetric method of Alicino by increasing the nett titre and controlling the reaction temperature by thermostat. The reproducibility was thereby increased. He confirmed the experience of other speakers that it was necessary for each analyst to evaluate the iodine absorption of pure penicillin II, as it varied according to conditions adopted.

It was not necessary to sterilise the paper strips before the chromatographic analysis. A manufacturer had not yet been found to produce strips, but further enquiries would be made in view of possible increased demand.

Further details available in the paper when published would indicate that the error in estimating the activity in any zone was dependent on the type and proportion of penicillin referred to. Until results on known "synthetic" mixtures were available, it was estimated that for a mixture of several components present in similar proportions, the 95 per cent. limits of error were about ± 15 per cent. for 6 replicates.

Where the sample had a 95 per cent. proportion of penicillin II, the data to be published would show that the errors found were very much less (less than ± 4 per cent.) for the main component.

Mr. STROUD asked if the quality of the ether used in the development of the strips was standard, and was the same quality always used? With respect to the iodimetric method of assay, the interference of unsaturated bonds is eliminated by the control blank experiment.

Dr. LEVI said they always used anaesthetic ether, but he did not think the quality was critical. It was, of course, essential for the ether to be damp.

Dr. E. C. WOOD considered that the necessary paper strips with clean-cut edges and precisely determined width could be easily made by the manufacturer. The makers of the pressure-sensitive adhesive tapes, so widely employed nowadays for sealing purposes, used a machine that should be quite capable of dealing with filter-paper. He would like to ask Mr. Robinson if he would venture a definition of the word "penicillin." The difficulty of defining the word "vitamin" was notorious, and it seemed that a similar difficulty was beginning to arise with the penicillins. If the definition were based on the chemical constitution, it would be difficult to exclude certain substances that have little or no anti-biotic activity. If, on the other hand, a functional definition was attempted, very careful wording would be required if the definition was to be neither too wide nor too narrow.

Mr. ROBINSON referred Dr. Wood to the Therapeutic Substances Amendment Regulations, 1946, in which a statutory definition of penicillin is given.

Mr. S. S. RANDALL asked how many antibiotics the authors had discovered. With similar technique, but different solvents and buffers and with the aid of "differential" organisms, he had found evidence of fourteen.

Dr. LEVI said they had found at least nine. In reply to another question he said that so far they had encountered no differences in results attributable to differences between *B. subtilis* strains.

Dr. HEATLEY asked if penicillin K was not still being called penicillin IV by some workers and if this error had ever been refuted in print.

Dr. LEVI said that they discovered penicillin IV during the war and did work on it which, of course, could not then be published. In the meantime Americans had been working on penicillin K and it had been assumed that the two were identical. But there could now be no doubt that they are in fact quite distinct.

Commenting on Dr. Heatley's question, Mr. ROBINSON said that penicillin K was probably a mixture of several penicillins, with pK values of about 5, and penicillin IV was probably not identical with any of them. Craig, in America, had designed an apparatus capable of partitioning penicillin a large number of times between an aqueous phase and a solvent phase, and the results had indicated the existence of numerous penicillins not yet characterised.

Mr. C. R. BOND, in congratulating the authors on the work they had done, said that the method had enabled a much clearer picture of the complex composition of penicillin as produced by the mould to be formed and was a valuable weapon in work on the purification of penicillin and the separation of individual penicillins. He asked the authors whether they had applied the method to streptomycin, and, if so, whether this was likely to prove as complex as penicillin. He also enquired whether they could provide any information on the nature of the side chain in the newer penicillins such as IV, VI and VII.

With reference to the confusion existing on the nomenclature of the penicillins, particularly penicillins K and IV, he said that the existence of more than one penicillin was first exclusively demonstrated in the I.C.I. laboratories in July, 1943, by the chromatographic separation of what were now known as penicillins I and II. At that time the constitution of penicillin was not known and it was suggested that penicillins as they were discovered should be distinguished by the use of Roman numerals. Penicillin III was discovered in January, 1944, and shown to be a p-hydroxy derivative of penicillin II. Penicillin IV was isolated shortly afterwards in the I.C.I. laboratories but sufficient was not obtained for a complete determination of its constitution. It appeared, however, to be closely related to penicillin I; it was certainly different from penicillin K, which was discovered later.

Dr. LEVI said he would like to see streptomycin put on strips. He knew nothing of the chemical constitution of penicillins IV, VI or VII. There was confusion between penicillins IV and K in America and in this country.

Mr. W. F. WILKINSON asked if differences in quality between different samples of agar had been found to have any effect on the readability and validity of results. In determinations of fungistatic and bacteriostatic values, considerable differences due to this cause had been encountered.

Dr. LEVI thought that might well be, but he had not experienced difficulties in that direction, except with one particular batch of agar, which gave unusually small zones.

Polarography

The following four papers were read at the Meeting of the Physical Methods Group on November 26th, 1946.

Amperometric Titration

By J. T. STOCK

POLAROGRAPHIC technique can be used not only for the direct determination of electro-reducible or electro-oxidisable substances in solution, but also for following the progress of a reaction such as the precipitation of one of the constituents of the solution. It is thus possible to employ polarometry for the location of the end-point in certain titrations. Since the process involves the measurement of current, Kolthoff and Pan[1] proposed the name *amperometric titration* to replace the term *polarometric titration* suggested earlier by Majer.[2] Though subject to some criticism,[3] the newer term appears to have become firmly established.

Titrations with the aid of the dropping mercury electrode were first performed in 1929 by Heyrovský and Berezicky,[4] who studied the precipitation of barium, strontium and calcium as the sulphate, carbonate, etc. These workers added successive measured volumes of the reagent, *e.g.*, potassium sulphate, to the solution to be titrated, *e.g.*, barium chloride, polarographed the latter after each addition and measured the height of the barium wave. The height decreased regularly as the reagent was added, owing to removal of electro-reducible barium ions from solution. After equivalence had been attained, further addition of the reagent caused the wave height decrease to continue at a smaller rate, owing to the suppression of barium ions by the solubility-product effect. The change in the rate of decrease was taken to indicate the end-point, although the latter was actually found by comparing the wave height with that of an artificial equivalence solution.

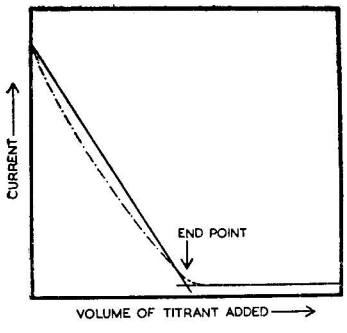

Fig. 1. Typical amperometric titration curve obtained when only the substance titrated is wave-forming.

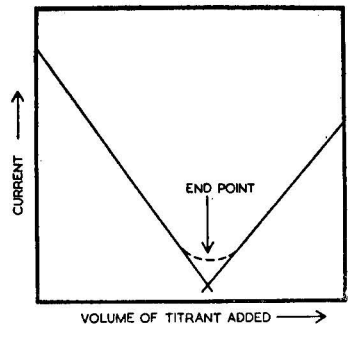

Fig. 2. Curve obtained when both reagent and substance to be determined are wave-forming.

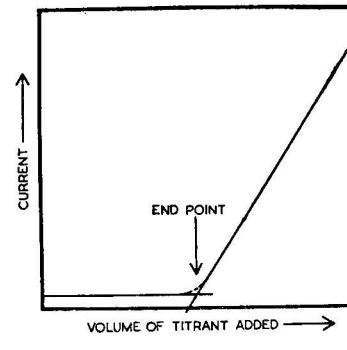

Fig. 3. Curve obtained when the reagent only is wave-forming.

Considerable advances were made by Majer,[2] who discussed the general characteristics of the method and pointed out the simplification, both in manipulation and in the necessary apparatus, which resulted from the use of a fixed applied e.m.f. instead of recording a series of polarograms. Several other Continental workers[5,6,7,8,9,10] have also contributed to the development of the method. The systematic investigations by Kolthoff and his co-workers, the first results of which were published in 1939,[1] have shown that amperometric titration is capable of high accuracy, and have done much to extend the applications of the method.

If a solution of an indifferent electrolyte, such as potassium nitrate, containing lead ions be placed in a polarographic cell and a potential of about −1·2 volt (all potentials are with respect to the saturated calomel electrode) be applied to the dropping mercury electrode, a diffusion current will be obtained, since lead ions are reduced well below this potential. (It is assumed that the usual precautions of oxygen-removal and of maximum-suppression have been observed.) Suppose small successive volumes of potassium oxalate solution are now added, the current being measured after each addition. Usually, a stream of hydrogen or nitrogen is bubbled through the solution for a few minutes to assist mixing; the gas stream

is stopped before the current is read. The current flowing falls progressively as lead oxalate is precipitated, and becomes very small when equivalence is reached. Since oxalate is not reducible under the experimental conditions, its continued addition does not cause further change in the current. If the latter be plotted against the corresponding volume of oxalate solution added, a curve, shown diagrammatically in Fig. 1, consisting of two straight lines intersecting in the form of a rough "L" is obtained. The end-point volume of the titration is indicated by the point of intersection of the two branches of the curve.

In practice, introduction of the titrant unavoidably dilutes the test solution, causing deviation from linearity, as depicted by chain lines in Fig. 1. This effect may be minimised by using a titrant 10 to 20 times more concentrated than the test solution and in precise work may be allowed for by correcting the current readings by the relationship:

$$i_{corrected} = i_{measured} \times (V + v)/V,$$

where V is the initial volume of the test solution and v is the volume of reagent added.[1,6]

Owing to the solubility of the precipitate, a "rounding-off" in the region of the end-point, as shown by broken lines in Fig. 1, is observed. Except when the solubility is appreciable, or when extremely dilute solutions have to be titrated, this curvature does not interfere; the end-point is located by producing the linear portions of the branches to intersect. In most cases, therefore, it is sufficient to plot three or four readings on each side of the end-point and not too close to the latter and to draw through the points a pair of straight lines to intersect at the end-point volume.

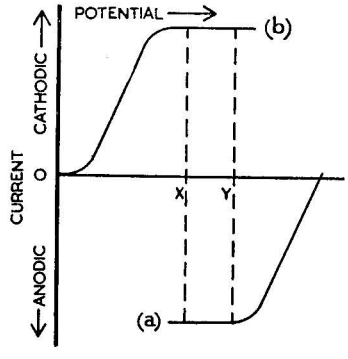

Fig. 4. Anodic and cathodic curves.

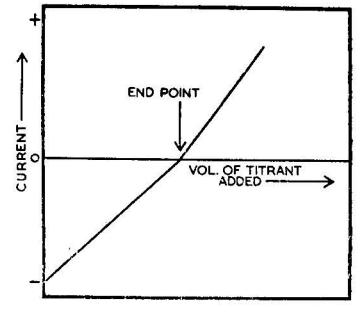

Fig. 5. Titration of an electro-oxidisable substance with a reducible reagent.

Suppose that the titration be repeated with potassium dichromate solution in place of the oxalate. As before, the current falls progressively until the end-point is reached. Since, however, dichromate forms a wave under the experimental conditions, continuance of the titration causes the current to rise progressively. The "V"-shaped titration curve (Fig. 2) obtained is characteristic of titrations in which both reagent and substance to be determined are electro-reducible.

If the titration with dichromate be performed at zero potential and with the lead-containing solution rendered slightly acid, the current, initially small, does not alter appreciably until the end-point has been reached. It then increases progressively as addition of dichromate is continued, giving a titration curve of the "reversed L" type shown in Fig. 3. Such curves are characteristic of titrations in which the reagent, but not the substance to be determined, is reducible.

The titration curves so far discussed resemble those obtained in conductometric titration, although quite different principles are involved. In both conductometric and amperometric techniques the method of finding the end-point does not involve readings near this region. Accordingly, both techniques are applicable to the titration of very dilute solutions, or in other circumstances when methods based on the actual recognition of the end-point fail. However, foreign or indifferent salts, traces of which often have to be guarded against in conductometric work, may be present if the amperometric technique is employed, and are in fact normally added.

The marked increase or cessation of flow of current that occurs in the end-point region of an amperometric titration is reminiscent of the effects observed in the "dead stop" method

of titration.[12] Both methods involve polarisation phenomena, although of different kinds. However, no actual measurement of the current is made when the "dead stop" method is used, hence the term "amperometric" is not strictly applicable to it. Alternative terminology has been proposed by Guzman and Rancano.[13]

Another type of titration curve is obtained when the substance to be determined forms an *anodic* wave (*i.e.*, is *oxidised* at the dropping mercury electrode), whilst, under the same conditions, the titrant forms a cathodic wave. Such titrations were first studied by Strubl.[6] Thus in acidified tartrate solution tervalent titanium forms an *anodic* wave (Fig. 4*a*), the half wave potential of which is −0·44 volt. In the same medium tervalent iron gives a cathodic wave at a less negative potential, as depicted at *b* in Fig. 4. Between the potential limits X and Y, either substance yields its diffusion current. If, therefore, an acid tartrate solution containing titanous ions be electrolysed at a potential lying between X and Y and titrated with ferric chloride solution, the *anodic* current initially obtained decreases to zero as the titanous ions are oxidised by the titrant. Beyond the end-point, however, the ferric iron now present gives rise to a *cathodic* current, the magnitude of which increases as further additions of titrant are made. Thus a *reversal* of the current occurs; the titration curve is shown diagramatically in Fig. 5. Generally, the slopes of the portions of the curve before and after the end-point are not identical. In the reverse titration, *viz.*, when the titrant forms an

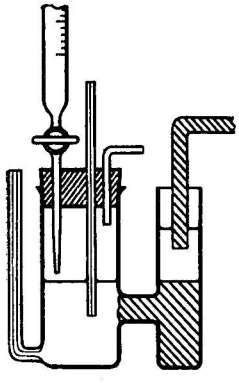

Fig. 6. Titration cell for use with external reference electrode.

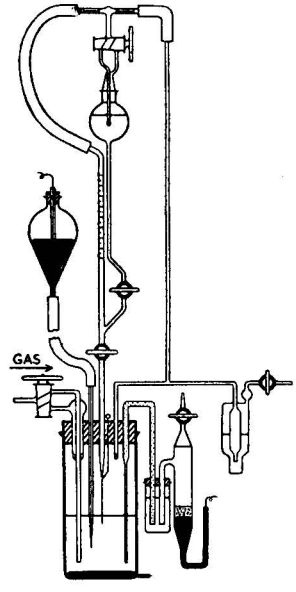

Fig. 7. Kolthoff and Langer titration assembly.

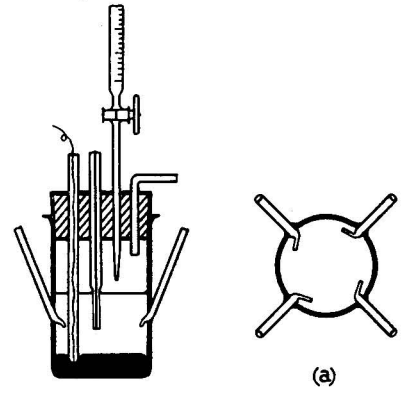

Fig. 8. Cell designed to promote efficient stirring.

anodic wave and the substance to be determined a cathodic wave, the titration curve slopes the opposite way. The work of Strubl was extended by Spalenka,[7] who studied the determination of chromate, ferricyanide, etc., by the titration of titanous chloride solution. Other titrations involving substances that form anodic waves are of halides with soluble salts of silver[14,15] or mercury,[16] of chromate with ferrous ammonium sulphate[17] and of stannous tin with copper sulphate.[18] The last example is interesting in being based upon *compensation* of the anodic current of tin by the cathodic current of copper, no direct reaction between the ions occurring in the bulk of the solution.[19]

Although most examples of amperometric titration hitherto investigated have been performed with the dropping mercury electrode, increasing interest is being shown in the possibilities of the rotating platinum micro-electrode.[17,20,21,22,23,45] This is especially suitable for titrations that can be carried out in an open beaker. In its simplest form the rotating electrode consists merely of a short piece of platinum wire sealed through the wall near the lower closed end of a length of glass tubing, which is rotated at constant speed.

Various forms of titration cell have been described. That shown in Fig. 6 is used in conjunction with an external reference electrode (usually a large saturated calomel electrode) an agar - potassium salt plug allowing connection to be made with the solution to be titrated.[14] Since the titrant may be stored and introduced into the cell in absence of air, the Kolthoff and Langer apparatus (Fig. 7) is suitable for routine work.[24] In a cell designed by Spalenka,[7] the gas stream enters through four tubes bent as shown at (a) in Fig. 8. Intensive stirring of the solution is thus caused. Owing to the danger of breaking fragile electrode capillaries, burette jets, and the like, on attempting removal from rubber stoppers, the writer prefers to use for beaker-type cells the bakelite clamp-on cap shown in Fig. 9. The electrode capillary or burette jet passes through a short glass tube cemented into the cap, a short sleeve of rubber tubing preventing ingress of air.[25] A tall-form 100-ml. lipless pyrex beaker A is used as the cell, and is retained by stirrup B. The thumbscrews CC enable the lip of the beaker to be lightly pressed into rubber joint D, thus forming a gas-tight seal.

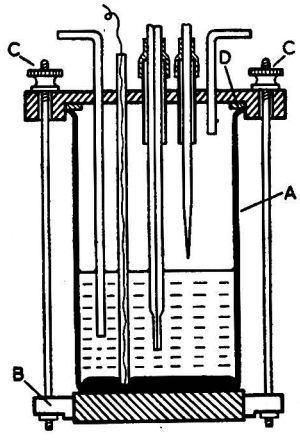

Fig. 9. Beaker-type cell with clamp-on cap.

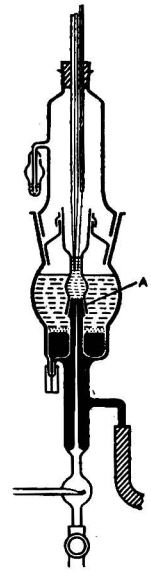

Fig. 10. Micro-cell incorporating calomel electrode.

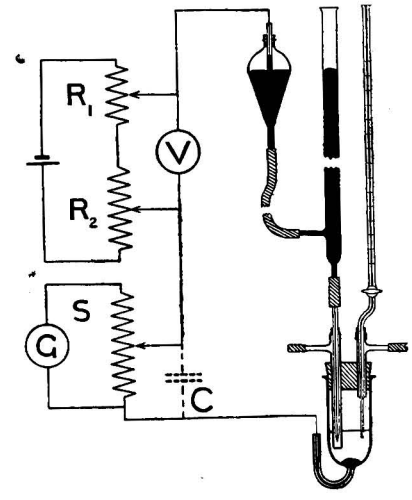

Fig. 11. Circuit arrangement for amperometric titration.

Langer has described both macro- and micro-scale cells.[26] The latter is depicted in Fig. 10. It incorporates a saturated calomel reference electrode which surrounds the titration chamber, junction with the test solution being made through ground joint A. Other cells for the titration of small volumes of solution have also been described.[27,28] Since electrical measurements are made only as a means of end-point location, thermostatic control, which is at least desirable in normal polarography, is unnecessary. For the same reason the dropping mercury electrode capillary need not be calibrated, so replacement is simple if a breakage occurs.

Amperometric titration is normally carried out with an applied e.m.f. that remains constant during the entire operation. Hence the electrical apparatus is comparatively simple. The potentiometer device shown in Fig. 11 may be used to select the desired applied e.m.f., the value of which is indicated by voltmeter V. To measure the current, a mirror galvanometer G, or, if available, a sensitive microammeter, is used. Shunt S permits adjustment of the sensitivity. To damp the galvanometer oscillations, a large-capacity electrolytic condenser C may be incorporated in the circuit.[8,29,30] A compact polarising unit that permits the degree of damping to be adjusted has been described by the author.[28]

Amperometric titrations involving lead have attracted considerable attention. Titration of this metal with potassium dichromate yields very accurate results in solutions as dilute as 0·001 *M*. As indicated above, dichromate in acid solution is reduced at zero potential. The titration may therefore be performed without the aid of a battery or polarising

unit, the dropping electrode being merely short-circuited through the galvanometer to the reference electrode.

Several anions may be titrated with lead nitrate solution. The titration of sulphate, first studied by Majer,[2] has attracted particular attention. A titration curve of the type shown in Fig. 3 is obtained, and, since lead sulphate is appreciably soluble, considerable rounding of the curve occurs. In an attempt to overcome this, Majer evolved a somewhat elaborate graphical method for locating the end-point. Other mathematical studies upon the location of the end-point in titrations involving the formation of appreciably soluble precipitates have also been made.[31,32] To reduce the solubility of lead sulphate, Spalenka[7] added ethanol to the solution before titration; this procedure was extensively studied by Kolthoff and Pan.[11] After oxidation to sulphate, sulphur in coke may be determined in this manner.[47] The determination of sulphate in precipitated alumina presents difficulties owing to the solubility of lead sulphate in aluminium nitrate solutions.[33] However, by adjusting the *p*H, keeping the aluminium content down and adding ethanol, satisfactory results may be obtained.

The amperometric titration of chloride with lead nitrate solution is also rendered possible by addition of ethanol to the solution.[34] The alcoholic content at the end-point should be 55 to 60 per cent. According to Thanheiser and Willems,[10] molybdate may also be determined by titration with lead nitrate solution, and the method may be used for the determination of molybdenum in steels. In the latter instance, iron, and, if present, tungsten must first be removed. Lead nitrate solution may also be used for the titration of ferrocyanide and of oxalate.[11] Another reagent for amperometric titration of ferrocyanide is zinc chloride solution. Langer showed that solutions that are from 0·01 *N* to 0·001 *N* with respect to fluoride can be titrated with thorium or lanthanum nitrate solution, the accuracy being about 0·5 per cent. A potential of −1·7 volt is employed. Fluoride forms no wave, so that a "reversed L" titration curve is obtained.[39] In the titration of phosphate with bismuth oxyperchlorate solution, described by Neuberger,[8] a curve of the same type is obtained. The method was applied to the examination of superphosphate. Even with appreciable amounts of phosphate, results are not very accurate. A much more satisfactory reagent for this anion is uranyl acetate.[36]

In addition to precipitation as sulphate,[2,4] barium in neutral solution may also be determined by titration with potassium chromate solution.[1] If lead is present in addition to barium, the former is precipitated first, since the solubility product of lead chromate is much less than that of barium chromate. If the titration is performed at a potential of −1·0 volt, lead and chromate form waves, but barium does not. Hence as titration proceeds, the current at first falls progressively until precipitation of lead is complete; it then remains at almost zero until sufficient chromate has been added to precipitate the barium. Further addition of reagent causes a progressive rise in current. A titration curve of the type shown in Fig. 12, consisting of *three* linear portions, is thus obtained, permitting the simultaneous determination of lead and barium. Though the second point of intersection corresponds correctly to the complete precipitation of lead and barium, co-precipitation causes the first to occur considerably after the theoretical end-point for lead. The solubility product of copper quinaldinate is much lower than that of the corresponding zinc salt,[37] so that the simultaneous titration of copper and zinc with quinaldinic acid is similarly possible.[38]

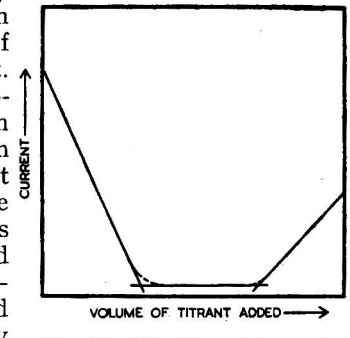

Fig. 12. Titration of two substances in the same solution.

Many of the specific organic reagents for metals are polarographically reducible and have been used successfully in amperometric titration. According to Neuberger,[8] Abresch showed that dimethylglyoxime could be used for the titration of nickel. Little detail is given in Neuberger's paper. The systematic studies of Kolthoff and Langer[24] showed that highly satisfactory results could be obtained, and these workers showed that the method was applicable to the determination of nickel in steels. Copper may be accurately titrated either with α-benzoinoxime[39] or with α-nitroso-β-naphthol solution.[40] The latter reagent is also suitable for the determination of cobalt and palladium. Copper may also be determined by means of salicylaldoxime solution.[9] Excess of the latter is added to the solution to be

examined and the determination is completed by titrating back with standard copper solution. Reversal of titration or employment of back-titration is sometimes useful in reactions in which precipitation is sluggish. This is demonstrated by the curves shown in Fig. 13. Those at (*a*) were obtained by adding 4 ml. of 0·01 *M* copper sulphate to 40 ml. of an acetate buffer of *p*H 4·9 and titrating with 0·02 *M* quinaldinic acid solution. If 4 ml. of the latter are diluted with 40 ml. of the buffer and titrated with 0·01 *M* copper sulphate, titration curves as at (*b*) are obtained. These show that equilibrium is almost obtained within 3 minutes of mixing; when quinaldinic acid is added to the copper-containing solution, attainment of equilibrium takes several times as long.[38]

For the titration of bismuth, 8-hydroxyquinoline has been used.[41] According to Zanko,[42] this reagent may also be used to titrate zinc, copper and aluminium.

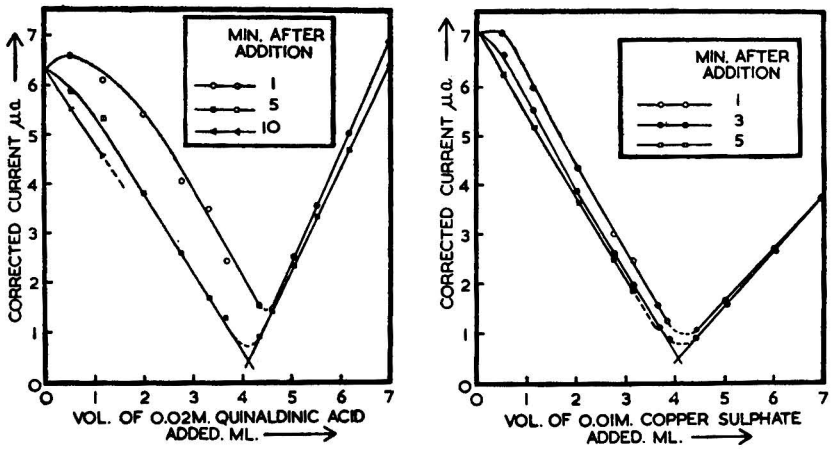

Fig. 13.

(*a*) Titration of copper with quinaldinic acid. (*b*) Reverse titration.

The amperometric titration of α-tocopherol with gold chloride solution is interesting as an example of the determination of an organic substance with an inorganic reagent.[43] In a similar manner, silver nitrate solution may be used for the titration of mercaptans.[21,45] A titration in which both reactants are organic in nature is that of picrolonic acid with methylene blue, which has been used as an indirect method of determining calcium.[44]

Since the principles involved are mainly as in polarography, amperometric titration possesses most of the advantages and disadvantages of the polarographic method. Interference by metals or other substances that form waves at potentials below that at which the particular titration has to be performed is serious. Although use of a counter-current device[29,46] may minimise such interference, the presence of high concentrations of such substances renders titration impossible. By introduction of nitro, azo or other readily reducible groups it might be possible to modify existing specific organic precipitants by rendering them wave-forming and hence usable at low potentials The likelihood of interference would then be diminished.

References

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August, 1946

DISCUSSION

Mr. A. C. MASON asked whether, with precipitation reactions that take some minutes to reach completion, the method would not require undue time for a determination requiring the plotting of several points.

Dr. W. CULE DAVIES asked what were the advantages of amperometric titrations over other well known methods of titration. Possibility of titrating very dilute solutions seemed to be one.

Mr. J. HASLAM asked what order of accuracy should be expected when two points before and two points after the end-point were tested.

Dr. W. STROSS asked how long an amperometric titration took to perform.

Mr. K. GOLDSCHMIDT asked if the author had any information concerning the application of the method to very concentrated solutions. Was it a fact that trouble rather difficult to eliminate occurred?

Mr. F. C. J. POULTON asked if Mr. Stock had experience of amperometric titrations of copper at very low concentrations, of the order of $M/20{,}000$, with α-benzoin oxime. Could he say if in such instances the amperometric method had any advantages over the orthodox polarographic method?

Mr. W. FURNESS asked if Mr. Stock would amplify his statement of the conditions that govern the choice of those points at which tests should be made. If a very insoluble salt is produced it would seem best to choose points close to the point of discontinuity of the curve; but when the salt produced had appreciable solubility points well removed from the equivalence point would seem preferable.

Mr. STOCK, replying to Mr. Mason, said that with the organic precipitants used 2 or 3 minutes for completion of precipitation could be allowed and sometimes less at each point. The total time required for a titration was normally less than half an hour and often twenty minutes were sufficient. In reply to Dr. Cule Davies he said that another advantage of the method was that one did not have to ascertain the end-point directly. The method was comparatively new and other advantages might appear later. In reply to Mr. Haslam he considered that if the selected points were well chosen an accuracy rather better than 1 per cent. should be obtained, especially if a correction was made for dilution by the titrant. He had no information about application of the method to very concentrated solutions. In reply to Mr. Poulton the stock copper concentration he had titrated with α-benzoin oxime was $M/1000$; he had titrated 5 μg. in 500,000, but such titrations were difficult. In reply to Mr. Furness he said that normally he tested three points on either side of the end-point, to make sure that they lay on a straight line. With soluble precipitates the matter was more complicated, and several mathematical papers on the location of the end-point had been published, notably one by Langer and Stevenson.

The Rotating Platinum Electrode

By C. J. O. R. MORRIS

The dropping mercury electrode has proved so satisfactory for most purposes in polarographic analysis that there has been little incentive to search for a substitute, at least so far as cathodic reactions are concerned. There are, however, two classes of reaction for which an alternative electrode system would be valuable. First, those cathodic reductions in which mercury interferes chemically with the electrode process, and secondly in the study of anodic oxidations. The latter class has not been of very much importance in inorganic polarography, but the increasing interest in the polarographic analysis of organic compounds has more recently caused greater attention to be given to such processes. There is, however, an important limitation in the study of anodic oxidations with the dropping mercury electrode. While it is possible to carry out cathodic reductions at potentials up to about −2·7 v., on the anodic side oxidation of the mercury itself limits the range to about +0·4 v. The use of an inert electrode would extend the range up to about +1·1 v., at which point evolution of oxygen from the anode sets the upper limit of the process.

Many workers have used solid metallic electrodes in the study of diffusion currents, both cathodic and anodic, but obstacles such as electrode polarisation (as distinct from concentration polarisation) made it very difficult to obtain reproducible results, and it was not until the introduction of the dropping mercury electrode by Heyrovský that the measurement of diffusion currents became sufficiently reliable to be of value to the analyst. Nevertheless, under strictly controlled conditions, quantitative measurements can be made with solid metallic electrodes. Thus Glasstone and Reynolds[1] studied the oxidation of ferrous ions at a platinum anode, and showed that the diffusion current was proportional to the ferrous ion concentration.

The first thorough investigation of the platinum electrode from the standpoint of the analytical chemist was carried out by Laitinen and Kolthoff.[2,3] They first examined the stationary platinum electrode and derived an equation for the diffusion current, *viz.*, $i = knDC$, in which i is the diffusion current, n the number of electrons involved in the electrode reaction, D the diffusion coefficient, C the molar concentration of the diffusing substance, and k a constant. They confirmed this equation for silver, copper, thallous, lead, ferric and ferrous ions, and showed that analyses could be carried out with an accuracy of about 1 per cent. provided proper precautions were taken. In particular it was essential to prevent thermal convection near the electrode, and also to ensure absence of mechanical vibration. They found that the temperature coefficient of the diffusion current was about 4 per cent. per degree C., as compared with about 2 per cent. for the dropping mercury electrode.

The stationary platinum electrode has, however, certain disadvantages. The greatest of these is the necessity of waiting for at least 2 minutes at each value of the applied potential for equilibrium to be reached. Thus a polarographic record in the usual sense is impossible. Other disadvantages are the rigorous temperature control necessary and the possibility of electrode polarisation.

Laitinen and Kolthoff found that these difficulties could be largely overcome by rotating the platinum electrode at a constant rate. The zone of convection equilibrium attained with the stationary platinum electrode was then replaced by a zone of uniform stirring. Owing to the much greater rate of diffusion in this zone, the current density was much higher, and the observed diffusion currents were 10 to 20 times greater. In fact, under comparable conditions, the rotating platinum electrode gave larger diffusion currents than the dropping mercury electrode. The rotating electrode was less sensitive to external conditions than the stationary electrode, showed a temperature coefficient of diffusion current of about 2 per cent. per degree C. in the reactions studied, and was in general more similar in its behaviour to the dropping mercury electrode. It was found difficult, however, to get reproducible results from day to day, and this has also been our own experience with this electrode. Laitinen and Kolthoff suggested therefore that perhaps the greatest utility of the rotating platinum electrode was as an indicator electrode in amperometric titrations, where such reproducibility is not essential. Kolthoff and his collaborators have reported several such amperometric

titrations, an interesting example being the titration of –SH groups with silver nitrate solution (Kolthoff and Harris[5]). The type of electrode used by Laitinen and Kolthoff is shown in Fig. 1. Kolthoff and Harris have suggested a modified form for use in suspensions (Fig. 2). This apparently functions by minimising disturbances of the diffusion layer by solid particles.

A comparison of various electrodes for the continuous estimation of oxygen in sea-water was carried out by Giguere and Lauzier.[7] They examined the dropping mercury electrode, the stationary platinum electrode and the rotating platinum electrode as cathodes, with the mercury pool, the saturated calomel electrode and various metallic electrodes as anodes. They made the important observation that when platinum electrodes were used for long periods with either the mercury pool or the saturated calomel electrode as anode, mercury was deposited electrolytically on the cathode and led to erratic results. They were able to overcome this difficulty by surrounding the anode with a fine-mesh platinum wire

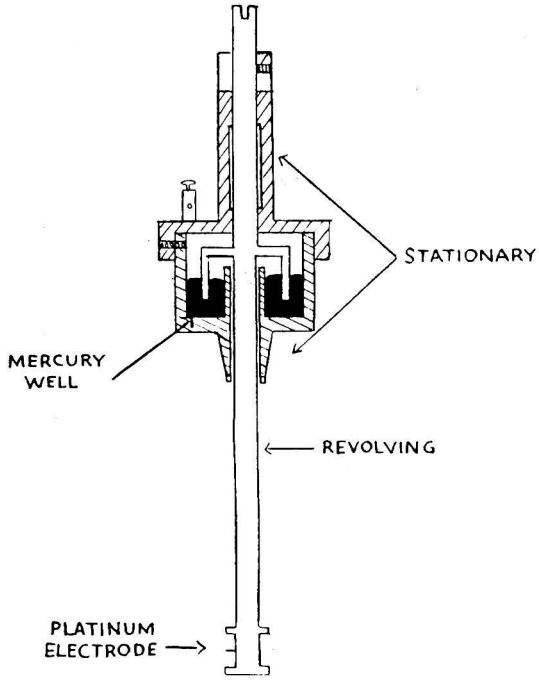

Fig. 1.

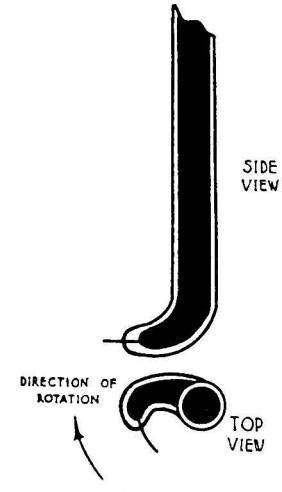

Fig. 2

(Reproduced from *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 162).

cage maintained at −0·2 v. with respect to the anode. Their apparatus is shown in Fig. 3. They found that the rotating platinum electrode gave considerably larger diffusion currents than either the stationary platinum electrode or the dropping mercury electrode, and obtained a linear relation between diffusion current and oxygen concentration over a wide range. The work of Giguere and Lauzier would appear to have wide application, especially in the measurement of oxygen uptake in biological systems, for theoretically the polarographic method should be more sensitive than the manometric methods generally used, and it offers the additional advantage of a continuous record.

An interesting attempt to overcome one of the most serious disadvantages of solid electrodes, *viz.*, electrode polarisation, has been described by S. D. Müller.[8] His apparatus is shown in Fig. 4. Two platinum electrodes are used with either a mercury pool or a solid metallic anode. When one electrode is cathodically polarised, the other is kept at anode potential. The states of polarisation and depolarisation are reversed at a constant frequency by means of a commutator. Müller used a metronome device to actuate the commutator and used polarisation times of 0·3 to 5·0 secs.; the best results were obtained at 1 to 2 secs. He examined the cathodic reduction of copper, lead, cadmium and zinc in sodium potassium tartrate solution and his published polarograms appear very similar to parallel runs with the dropping mercury electrode. We are constructing in our laboratory a similar arrangement

with electronic control of the polarisation period. This is shown in Fig. 5. The frequency is controlled by the neon tube relaxation oscillator, which operates a uniselector switch acting as commutator. We are hoping to study various organic anodic oxidations with this apparatus.

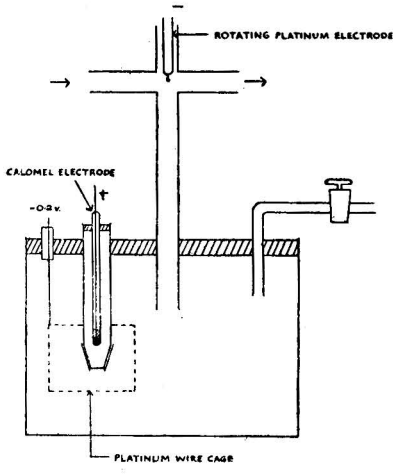

Fig. 3.

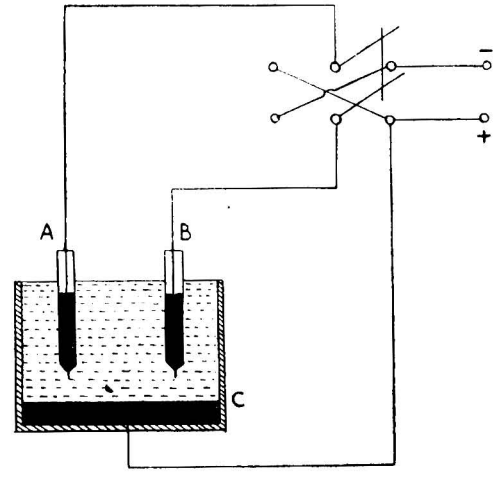

Fig. 4.

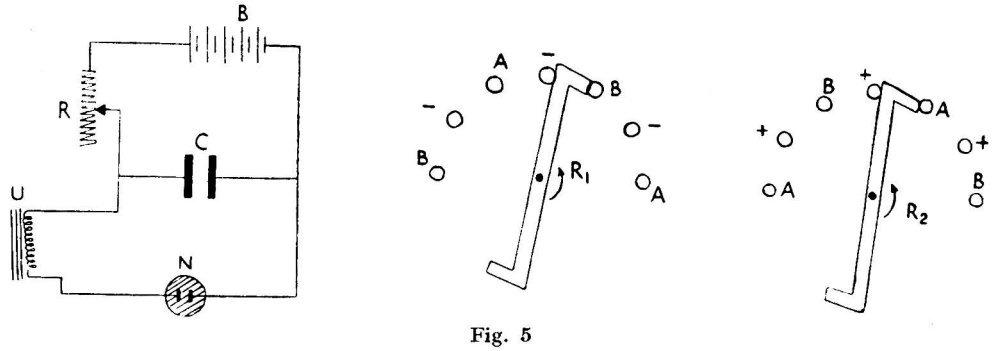

Fig. 5

The various types of metallic electrode described do not replace the dropping mercury electrode, which is still the most reliable for general analytical purposes. They are, however, of value in special circumstances, and further study of such electrode systems will doubtless extend still further the range of polarographic analysis.

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Discussion

Mr. J. T. Stock asked if, in the Müller double electrode system there was a pronounced temperature coefficient, as might be expected from Dr. Morris's remarks about stationary micro-electrodes in general, for in the fundamental equation for such electrodes the diffusion current appears as the first power and not as the square root.

Dr. J. E. Page asked if Dr. Morris had used the rotating platinum electrode to study the oxidation of organic substances, and if he knew whether its application to the measurement of oxygen concentrations had been used in respiration experiments (*cf.* R. J. Winzler, *J. Cell. and Comp. Physiol.*, 1943, **21**, 229).

Dr. J. E. B. Randles asked if any phenomena similar to those appearing with the ordinary direct mechanism had been noted. He had thought that Müller used the ordinary galvanometer for his recording.

Dr. W. Stross mentioned two arrangements described in a doctorate-thesis by W. Kaeppel (printed at Bonn in 1941) from v. Stackelberg's laboratory; these were not accessible in ordinary publications but might be of interest. The first consisted of four stationary electrodes dipping in the test liquid which was separated by a diaphragm from a calomel reference electrode. A rotating contact connected one electrode at a time in rapid succession (5 to 20 cycles per potential increase of 0·1 volt) as the cathode, whilst the other three were connected with a depolarising rail to which various potentials could be applied by means of a battery and a variable resistance. The second apparatus also consisted of four electrodes of which in turn one is dipped into the solution by an electromagnet every second, whilst the other three are kept above the liquid. Both instruments were used for experiments on thiourea.

Dr. Page said that Dr. Kaeppel's thesis contained a number of interesting points. The quinone - hydroquinone system was regarded as reversible but when it was examined by Dr. Kaeppel's technique oxidation of hydroquinone and reduction of benzoquinone occurred at different potentials. Some of the statements in the thesis were unexpected and required confirmation.

Dr. Zade asked if it would be possible to use alternating current in amperometric titration with the rotating platinum electrode; if so, contact trouble could be overcome by capacitive coupling.

Mr. G. S. Smith asked if in order to avoid difficulty with contacts it would be possible either to cause the electrode to make a circular movement in the liquid without rotating or to make the liquid rotate round a stationary electrode.

Dr. Morris, in reply to Mr. Stock, said he thought that the Müller system might have a relatively high temperature coefficient, but he imagined trouble due to convection would be reduced. In reply to Dr. Page, he said he knew of no published work on the study of oxidation of organic compounds by means of the rotating platinum electrode; he and his colleagues were mainly interested to applying it to biological analysis. Replying to Dr. Randles, he said that contact trouble was the main difficulty with the rotating electrode, but it could be overcome; the kind of galvanometer used by Müller was not clear from his reference to it. To Dr. Zade's question as to the possibility of using alternating current he replied that this could be done. He thought Mr. Smith's suggestions for avoiding contact difficulties were worth trying. Possibly, however, he had made too much of contact trouble ; there were ways of overcoming it. Rotation of the liquid by means of a solenoid had been tried but was not a success.

The Application of the Cathode Ray Oscillograph to Polarography: Underlying Principles

By J. E. B. RANDLES

The ordinary polarograph is applicable to the estimation of small quantities of many metals and electro-reducible or -oxidisable substances in general. Its use frequently gives results more accurate than are easily obtainable by purely chemical means and very often in a mere fraction of the time required for a chemical analysis. The most usual form of commercial instrument records the current - voltage curves photographically. This has some disadvantages in that an appreciable amount of time is spent in recording, developing and drying the polarograms before they can be measured. Furthermore, the actual form of the polarogram cannot be seen until the photograph is developed, so that any malformation is not perceived until the recording, or recordings, are completed. The photographic procedures are eliminated in pen recording instruments now made, and the polarogram can be seen as it is recorded, but the recording is still a comparatively slow process. Thus the idea of obtaining a current - voltage curve rapidly traced on the screen of a cathode ray tube has arisen. Work with this object in view has been carried out by Matheson and Nichols[1] and on less direct lines by Müller, Garman, Droz and Petras[2] and Heyrovský and Forejt.[3] However, in no case has any near approach to a practicable precise analytical instrument been attained.

It is believed that the apparatus described below represents a considerable development in this direction.

The basic principle of polarography is the measurement of the diffusion current passed by a micro-electrode in a state of concentration polarisation. It is usual to obtain, by one of two methods, a diffusion current, either steady or of steady average value, suitable for measurement by a galvanometer. The two methods are the use of the rotating platinum electrode and the use of the dropping mercury electrode. If, on the other hand, a cathode ray oscillograph is employed, rapidly changing currents may be followed without difficulty. This permits the measurement of diffusion currents under quite different conditions. To explain this difference of principle a brief consideration of the elementary theory of diffusion currents is necessary.

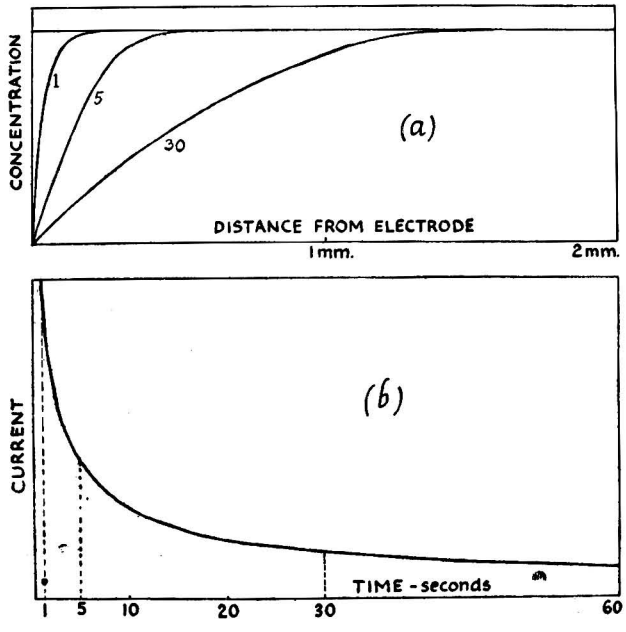


Fig. 1.

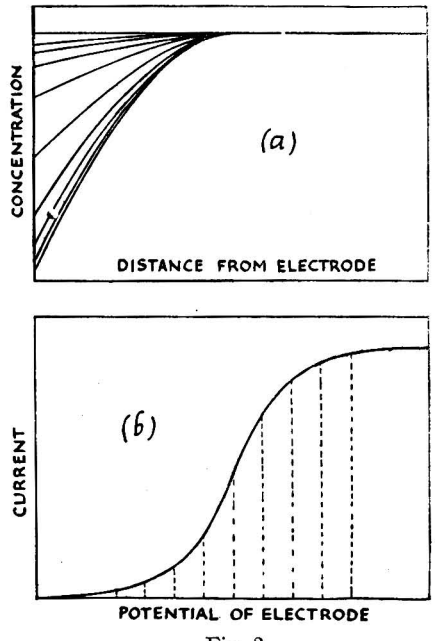


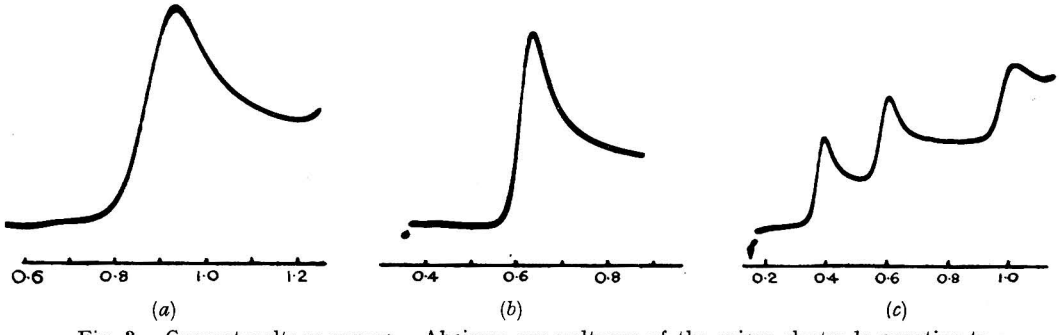
Fig. 2.

If a micro-electrode is immersed in a solution containing an electro-reducible or -oxidisable substance (and an indifferent electrolyte), and a potential, sufficient to cause reaction of the substance, is applied suddenly to the electrode, a diffusion current is obtained which changes with time as shown in Fig. 1 (*b*). The current starts at a high value and declines rapidly, being in its early stages proportional to $t^{-1/2}$, t being the time measured from the application of the electrolysing potential. This decline is due to the widening of the diffusion layer as electrolysis proceeds, which is illustrated in Fig. 1 (*a*). Ordinates are concentrations of the reacting substance in the solution and abscissae the distances from the electrode surface. Successive curves refer to the values of t shown, but the numerical values in general are only intended to show approximate magnitude. Since the current passed by the electrode is proportional to the rate of arrival of reacting substance at its surface, which is proportional to the concentration gradient of the substance, close to this surface, the reason for the decline of the current is evident from Fig. 1, (*a*).

Although the decrease of the diffusion current to a stationary electrode becomes slower as time goes on, the current becomes unsteady owing to convection effects resulting from the concentration differences near the electrode. A stationary micro-electrode, cannot, therefore, be used in conjunction with a galvanometer for polarographic purposes. The difficulty of the widening diffusion layer is ordinarily overcome in two ways, as already mentioned. The thickness of the layer may be limited by controlled stirring, as with the rotating platinum electrode. Or the electrode and the solution undergoing electrolysis may be automatically renewed at frequent intervals, as is in fact done when the dropping mercury electrode is used. The rotating electrode gives a steady current, and the dropping electrode a fluctuating current,

but of constant average value, for a given potential of the electrode. The rate at which the potential of such an electrode is changed in order to obtain a current - voltage curve (*i.e.* the ordinary polarogram) must be slow (about 1·0 volt in 5 minutes) so that there shall not be significant lag in the attainment of the true current for each potential, and in the response of the galvanometer to the changes in current. Particularly the latter applies when the dropping electrode is employed, as the galvanometer must be sufficiently damped for it not to respond appreciably to the fluctuations of current with the growth and fall of each mercury drop. The form of current - voltage curve obtained with slow change of potential of the electrode, and the condition of steady, or steady average, current for any given electrode potential, is that of the ordinary polarogram. The gradual increase of current is due to the gradual decrease of the concentration of the reacting substance at the electrode surface as the reaction becomes more complete with the change of the potential of the electrode. This is illustrated diagrammatically in Figs. 2 (*a*) and 2 (*b*) for the case of a diffusion layer of constant thickness as at the rotating platinum electrode. The concentration - distance curves in Fig. 2 (*a*) correspond to potentials of the electrode indicated by dotted lines in Fig. 2 (*b*).

We may now turn to a consideration of diffusion currents varying with time as well as with the potential of the electrode. These arise, as we have already seen, from the sudden application of a suitable potential to a micro-electrode in a solution containing an electro-reducible or -oxidisable substance; or preferably, in practice, from the application to such an electrode of a rapid potential "sweep" covering say 1·0 volt in 1 to 2 seconds. Any electro-reduction or -oxidation having a polarographic "half-wave potential" within this range gives rise to a diffusion current which rises rapidly to a peak and then declines as the diffusion layer widens. Fig. 3, (*a*) is reproduced from a photograph of a cathode ray trace showing the current - voltage curve obtained with a platinum micro-electrode in this way. In practice successive potential "sweeps" are applied to the electrode, so that the trace is repeated, but with a stationary electrode there must normally be an interval of at least 10 seconds with the electrode at its starting potential, between the "sweeps," to allow time for the surrounding solution to regain its original uniformity.



(*a*) (*b*) (*c*)

Fig. 3. Current voltage curves. Abcissae are voltages of the micro-electrode negative to a silver - silver chloride electrode.

(*a*) $1/8 \times 10^{-3}M$ $Cd^{\cdot\cdot}$ in *M* KCl. Stationary platinum electrode.
(*b*) $1/4 \times 10^{-3}M$ $Cd^{\cdot\cdot}$ in *M* KCl. Dropping mercury electrode.
(*c*) $1/16 \times 10^{-3}M$ $Pb^{\cdot\cdot}$, $Cd^{\cdot\cdot}$ and $Zn^{\cdot\cdot}$ in *M* KCl. Dropping mercury electrode.

Although a stationary electrode has some advantages, in many cases a dropping mercury electrode is preferable. A potential "sweep" applied to a dropping electrode at a late stage of drop growth produces a current - voltage curve very similar to that obtained with a stationary electrode (see Fig. 3). The repetition of the "sweep" must be synchronised with drop formation in such a way that each sweep occurs at the same stage of drop growth, otherwise successive current - voltage curves would not be identical. The method of carrying this out will be explained shortly.

The potential "sweep" is provided by the charging of a condenser. Fig. 4 shows the simplest form of circuit for this purpose; it is very similar to one used by Matheson and Nichols. The starting potential of the "sweep" is controlled by potentiometer Q, and the rate of change of potential by rheostat R_2. The potential differences between points A and B and between C and D, are amplified by direct-current amplifiers and applied to the deflector plates of the cathode ray tube giving horizontal and vertical deflection of the beam, respectively. Horizontal deflection thus gives a measure of the potential difference across the

polarographic cell and vertical deflection a measure of the current passing through it. The trace is therefore a current - voltage curve. The potential "sweep" is ended by short-circuiting the condenser by means of a relay-switch K. This relay is operated by an electronic circuit ("flip-flop" circuit) triggered by an impulse derived from the amplifier whose input is the potential difference across R_1. The impulse arises from the sudden change in current when the mercury drop falls. After a certain delay (controlled by a rheostat in the "flip-flop" circuit) to allow drop growth, the relay reopens and another potential sweep is started, to be terminated when the drop falls.

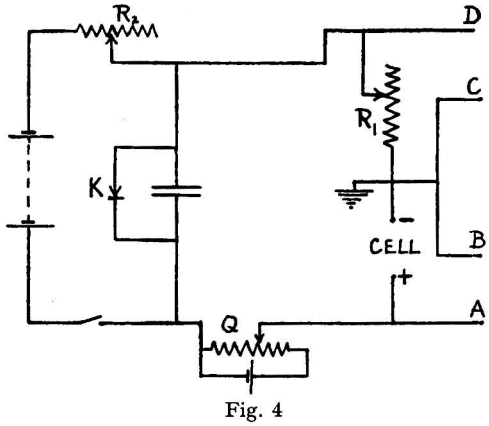


Fig. 4

In order that there shall be accurate proportionality between peak current and concentration of the reacting substance in the solution, not only is exact timing of the potential sweep required but also the rate of change of potential of the electrode must be constant and independent of the current passed. This cannot be achieved by the simple circuit of Fig. 4, for the rate of change of potential difference across the cell is inevitably affected by the sudden increase in potential drop in R_1 when the current rises. To overcome this an electronic circuit was devised which applies the potential difference to the cell in such a way that it is independent of the potential drop in R_1 and of the current. This, and the other circuits, will be described in detail elsewhere.[4]

References

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2. Müller, R. H., Garman, R. L., Droz, M. E., and Petras, J., *Ind. Eng. Chem., Anal. Ed.*, 1938, **10**, 339.
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4. Randles, J. E. B., *Trans. Faraday Soc.* (in press).

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The Application of the Cathode Ray Oscillograph to Polarography: General Lay-out and Uses of the Cathode Ray Polarograph

By L. Airey

In the present paper the apparatus of which the principles have been explained by Mr. Randles in the preceding paper is considered in more detail from the standpoint of instrumentation and uses. It is emphasised that a practical Cathode Ray Polarograph has been designed and built,* having uses both in analysis and in research. Details of construction and circuits will be published elsewhere but the general lay-out of the main parts is indicated in block diagram form in Fig. 1 and further information on them is given in the following Sections 1 to 4.

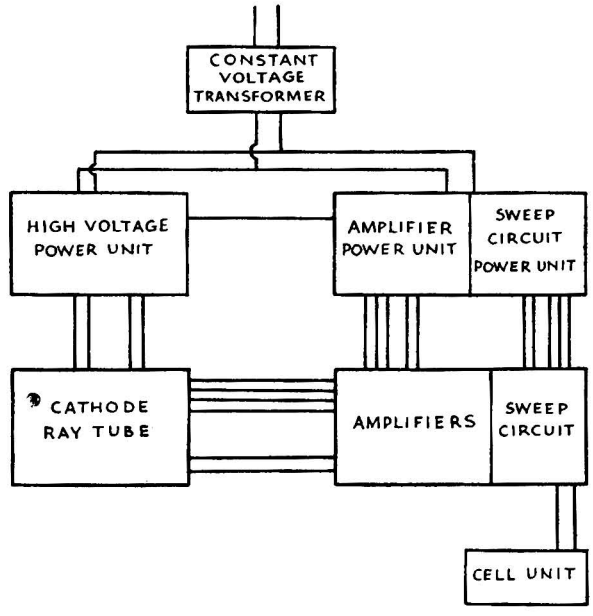
1. *Cathode Ray Tube and Power Supply*—The tube itself has a fluorescent screen showing long afterglow properties (about 10 secs.). As will be seen from the diagram, the units are separate, but they may commercially be combined in one box.

2. *Amplifiers and Voltage Sweep Circuit, etc.*—These units are mounted side by side on a common chassis and housed in one box. The lay-out of the circuits follows conventional electronic practice but emphasis must be laid on adequate electrical screening of the input leads, etc., and the provision of a satisfactory anti-vibration support for the two valves in the voltage sweep circuit.

Other circuits included on the same chassis are (*a*) an electronic time delay circuit, (*b*) an auxiliary trigger amplifier and (*c*) an electromagnetic relay to control the cathode ray

* The instrument was demonstrated at the meeting.

"spot" brightness during the quiescent condition. Circuit (*a*) is the conventional "flip-flop," with provision for converting it to a multivibrator. As the former, it consists simply of a circuit having one stable and one metastable state into which it can be thrown on receipt of a suitable impulse (from the amplifier); the duration of its stay in the metastable condition can be altered at will by variation of resistance values in circuit. As the multivibrator, the circuit possesses two metastable states, and the frequency of change from one to the other may be precisely governed by variation of resistance values.



In order to utilise to full advantage the long afterglow screen of the cathode ray tube, it is necessary that full spot brilliancy be employed. However, if the beam at this intensity should rest on one portion of the screen for any length of time permanent damage is done to the screen. To obviate this, the circuit (*c*) is introduced whereby the intensity is diminished during the quiescent part of the cycle.

3. *Power Supplies for Amplifiers, etc.*—Two independent power supplies for the amplifiers and voltage sweep circuit respectively are required. Good stabilisation by means of gas discharge tubes is necessary, and for the supply for the voltage sweep circuit additional resistance capacity decoupling after the stabilisers is desirable. The precise location of the power pack chassis with respect to the amplifiers is not important but it is desirable to keep the connecting leads as short as possible and to avoid stray alternating magnetic fields.

4. *Cell Assembly and Constant Voltage Transformer*—The cell assembly can be of any design whatsoever; the mercury column is earthed and the leads to the amplifier are electrostatically screened. A point of some importance is to ensure that the cell assembly is completely clear of stray magnetic fields from the apparatus. The constant voltage transformer is necessary only where main voltage fluctuations are greater than ± 2 per cent.

As an analytical tool the apparatus is essentially a comparison instrument and it resembles other types of polarographs in requiring calibration with known standards. It is therefore essential that the controls shall be precisely reproducible in setting. It will be convenient to consider the sequence of operations required for the production of a quantitative polarogram.

(1) *Adjustment of the drop time*—With the capillary dropping in the test solution, the multivibrator circuit is set for a repetition frequency of 7 secs. (a convenient arbitrary time). With a constant known potential applied to the cell the drop break-away is apparent as a small "blip" in the cathode ray trace. By adjusting the head of mercury, the "blip" may be obtained at the same place on the trace in each successive cycle, and then the drop time is exactly that of the multivibrator. It is important, of course, that these measurements are made at a known potential, obtainable from a calibrated potentiometer included in the circuit.

(2) *Measurement of "peak"*—The voltage sweep rate is adjusted by means of the appropriate control. The multivibrator is switched over to "time delay" and the extent of the delay varied until a polarographic peak appears on the screen. Small variations in the fine control on the time delay circuit are then made until the horizontal projection of the "tail" of the peak is some convenient arbitrary length. A note is made of the initial starting voltage and of the "horizontal" amplifier gain setting. Finally the cell series resistor, R, is varied until the peak height is some definite figure (say 7·5 cm.). The value of R is recorded. The solution is then replaced by a standard, the peak length adjusted if necessary, and R varied to produce a peak height of 7·5 cm. The solution concentration being inversely proportional to the resistance, the unknown concentration is then easily evaluated.

The description just given applies to the reduction of a single species of ion. When more than one is present in solution, the technique is modified slightly. By careful use of the controls governing the starting potential across the cell, together with those of the time delay circuit, it is possible to isolate and measure, as described above, peaks corresponding to each individual ion. The method is advantageous in the estimation of a small amount of, say, cadmium (−0·6 v. versus S.C.E.) in presence of a relatively large amount of lead (−0·4 v. versus S.C.E.). By beginning at −0·5 v., no peak is obtained for the lead ion, and it is possible to estimate cadmium in presence of 50 times as much lead and possibly more.

USES OF THE INSTRUMENT—

The instrument has so far been considered as an analytical tool, but it has other important uses. Like other types of polarographs it may be used for certain fundamental physical measurements such as those of diffusion coefficient or the number of electrons involved in a reduction or oxidation process. It is particularly useful for rapidly checking the efficiency of electrolytic separations, and the relatively short time required for an observation (this can be reduced to about 4 secs.) makes it a valuable means for following the course of rapid reactions, *e.g.*, tautomeric conversions. However a particular virtue lies in the fact that the shape of the reduction (or oxidation) peak is indicative of the nature of the reaction. Ions such as those of cadmium, thallium and lead are characterised by the fact that their reductions are thermodynamically reversible. Other ions such as those of stannic tin complexes, nickel in sulphate media, and a large number of reducible organic compounds do not show this ease of reduction but require an overvoltage to effect the change. The result is to introduce a rate-controlling process other than diffusion, and this manifests itself as a rounding and flattening of the polarographic peak. For truly reversible reactions the curves can be treated mathematically in a manner analogous to that used in deriving the Ilkovic equation, but so far little or no work has been done in attempting to treat irreversible reactions in this manner. Apart from the mathematical aspect of the matter, the appearance of the polarograms is a very useful criterion of the suitability of a reaction for analytical or electrochemical work.

It may be useful to consider briefly certain recent developments. First, it has been shown that platinum micro-electrodes may be used in the region +1 to −0·3 volt (versus S.C.E.) with some success. Secondly, it has been shown that the dropping mercury electrode may be replaced by an amalgamated silver or preferably an amalgamated platinum micro-electrode. Providing that amalgamation is good, such electrodes may be used up to −2·1 volts without difficulties due to hydrogen evolution. The technique adopted with these "film electrodes," as they may be described, is to execute a voltage sweep in the ordinary way and to observe the polarogram in a manner similar to that described above. The potential is then returned to a point below the half-wave potential of the ion in question and maintained there for about 10 seconds, during which period the deposited ion is redissolved and concentration gradients are practically eliminated. As will be apparent, it is essential that the "film" be regenerated during the latter part of each cycle and in consequence the electrode can be used only for such metals as will dissolve easily from an amalgam. In the case of reduction (or oxidation) from one valency state to another the limitation does not of course apply.

Although much work remains to be done on this type of electrode it would appear that its main advantage lies in the great simplification in the circuit of the apparatus. The electrodes may be made of constant and accurately reproducible dimensions and appear to be reliable in operation. The use of a synchronising circuit is no longer necessary and hence a much simpler polarogram-measuring technique may be employed. Moreover it becomes

possible to enhance the sensitivity of the method by increasing the surface area of the electrode beyond that normally obtainable from a capillary tube. A point of theoretical interest which has not yet been investigated is that with both types of film electrodes some maximum suppressor appears to be necessary when dealing with certain ions.

In conclusion a comparison of the three types of instrument—photographic, pen-recording and cathode ray tube polarographs—under various headings will be given.

(1) *Accuracy*—There are no very significant differences.
(2) *Sensitivity and Robustness*—It is considered that the cathode ray instrument is much superior to the other types owing to the lack of delicate moving parts. However, circuit characteristics would set a practical limit to an increased sensitivity at about one order greater than commercial equipment.
(3) *Adaptability*—As an analytical tool the instrument has the merit of speed for routine work. As with the pen-recorder type, any malformation of the polarogram may be easily detected, but the lack of a permanent record may be a drawback.
(4) *Complexity and Size*—On this score the apparatus is obviously inferior to the other types. Even with improved design it will require approximately twice the space required by a pen-recording instrument and some considerable knowledge of electronics may be required to trace and remedy any circuit faults that may develop.
(5) *Cost*—A quite satisfactory apparatus can be built with limited workshop facilities, and it is thought that the cathode ray polarograph should be no more expensive than the other types.

The authors wish to acknowledge valuable advice and criticism from Mr. S. H. Bales, Principal Scientific Officer and Mr. A. S. Nickelson, Principal Scientific Officer of the Chemical Inspection Department, Ministry of Supply.

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CHEMICAL INSPECTION DEPARTMENT, MINISTRY OF SUPPLY
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DISCUSSION

Mr. A. C. MASON asked if the apparatus described was more effective than the ordinary polarograph in separating waves whose half-wave potentials are close together.

Dr. ZADE asked if dI/dE curves had been plotted on the cathode ray oscillograph, as in the method used by Heyrovsky recently.

Dr. J. G. A. GRIFFITHS, referring to the use of "film electrodes" mentioned by Mr. Airey, asked if it was satisfactory to deposit a film of silver on a metal into which mercury does not diffuse.

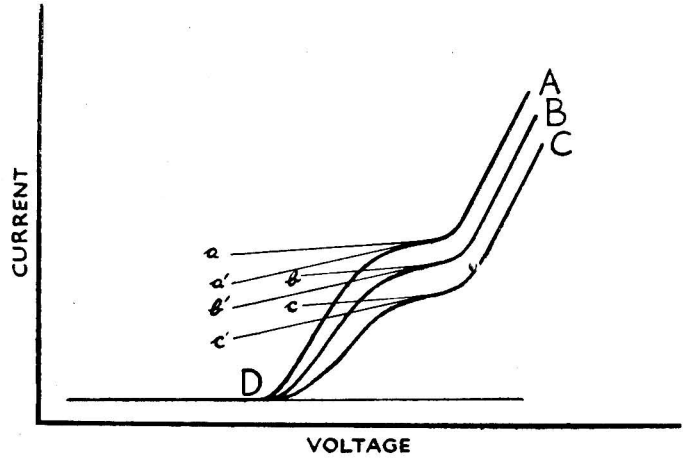
Mr. K. GOLDSCHMIDT said that Messrs. Randles and Airey had described a spectacular, but rather complicated instrument, and he was doubtful if it could be operated by an unqualified assistant. Had the authors had experience with simpler circuits in which the cathode ray oscillograph is employed as an indicator in conjunction with the usual polarographic measuring or recording equipment? He had used a modification of a method first described by Boeke and van Suchtelen (*Z. Elektrochem.*, 1939, **45**, 753), in which a small A.C. bias superimposed on the electrode potential permitted accurate detection of half-wave potential and of changes of curvature at the beginning and end of a wave. From the latter the concentration could be derived by noting the difference between the corresponding deflections of a galvanometer, or alternatively by recording the desired part of a wave on a polarograph. Complete analysis of a solution could thus be carried out very rapidly, and in some instances continuously. The cathode-ray oscillograph may be of any commercial type, and the only other components necessary are a few radio-type resistors and capacitors. Interpretation of the pattern obtained on the cathode-ray tube is very simple, and the device can be used by an unqualified investigator.

Mr. AIREY, in reply to Mr. Mason, said the instrument was an improvement, but not a great one, on the ordinary polarograph in the separation of waves that were close together. Waves 0·15 volt apart could be separated but that was about the limit. He had not yet attempted the plotting of dI/dE curves with the instrument. In reply to Dr. Griffiths he said that they had obtained satisfactory film electrodes by dipping red-hot platinum into mercury.

Notes

AIDS TO ACCURACY IN QUANTITATIVE ESTIMATION FROM SIMPLE POLAROGRAMS

In quantitative polarographic estimations it is often a matter of some difficulty to decide the exact angle at which to draw the tangent to a diffusion current curve, as, for example, the choice between *a* and *a'* in the accompanying diagram.



In the "internal standard" method of estimation this difficulty can be overcome by making a second addition of a known amount of standard solution. The usual technique according to the "internal standard" procedure is to obtain a "wave" for the unknown, say C, to add a known amount of standard solution of the substance to be estimated, and obtain another "wave" as at B. Then concentration is proportional to wave height, and the latter is found by drawing parallel tangents to B and C and a tangent to the "foot" of the curve (D), the height being measured as the intercept on a vertical drawn at the half-wave potential. It will be obvious that the correct slope of the tangents is a matter of some importance in accurate work.

If now a second addition is made, the ratios of the wave heights for the solutions (making an allowance for the approximate unknown concentration) are fixed, and tangents can be drawn to ensure these ratios. The common slope of all the diffusion current tangents is then completely determinate.

In a case producing a curve similar to that depicted, the intercepts could have been measured as follows:

C = 11 to 14 mm. (unknown concentration)
B = 17 ,, 20 ,, (10 μg. per ml. addition)
A = 24 ,, 27 ,, (20 ,, ,, ,, ,,).

From the average values of C and B, the unknown concentration was about 21·25 μg. per ml. Thus, as a first approximation, the wave-height ratios were

$$\frac{h_A}{h_B} = \frac{41{\cdot}25}{31{\cdot}25} = 1{\cdot}32 \text{ and } \frac{h_B}{h_C} = \frac{31{\cdot}25}{21{\cdot}25} = 1{\cdot}47.$$

The stipulation of both these ratios simultaneously narrows the possible intercept values (as may be seen by setting up the ratios on a slide rule) to:

C = 12·4 to 13·6; B = 18·2 to 20·0; A = 24·0 to 26·4.

Then taking C as 13·0 and drawing parallel tangents to all three curves, there is obtained:

C = 13·0; B = 19·0; A = 26·0

whence the unknown concentration is 20 μg. per ml.

For greater accuracy, successive approximations can be made for the unknown concentration, which process is facilitated by a device for laying on the polarogram, comprising a sheet of transparent material on which a series of parallel lines (*e.g.*, 1 mm. apart, with centimetres marked) is ruled, photographed, or otherwise applied.

The "internal standard" method of estimation is described in "*Polarography*," by Kolthoff, I. M., and Lingane, J. J., Interscience Publishers, New York, 1941, p. 251, and an inspection of Fig. 91 on p. 252 will show the difficulty of measurement in some instances, particularly as the half-wave intercept method mentioned above is now generally used (*cf.*, "*Polarographic and Spectrographic Analysis of High Purity Zinc and Zinc Alloys for Die Casting*," H.M. Stationery Office, 1945, p. 26).

It must be stressed that the method herein described involves a different principle from the known method of making successive additions, measuring wave heights and plotting back to the unknown concentration. At no point in that method is any wave-height accurately fixed, and systematic inaccuracies will be reflected in the final result.

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JOHN A. LEWIS

DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH
CHEMICAL RESEARCH LABORATORY
TEDDINGTON, MIDDLESEX

ERRATUM: In the issue for June, 1947, p. 253, line 5 from the bottom of the page
for "County of Essex" *read* "County of Sussex."

Ministry of Food

STATUTORY RULES AND ORDERS*

1947 876. The Pickles and Sauces Order, 1947. Dated May 7, 1947. Price 2d.

In this Order, which replaces the Pickles and Sauces Order, 1946 (*S.R. & O.*, 1946, *No.* 2216)—

"Pickles" means sweet pickles (whether mixed with any sauce or not) mixed pickles or piccalilli, or any pickled beetroot, cabbage, capers, cauliflowers, cucumbers, gherkins, mushrooms, olives, onions, pimentos, tomatoes or walnuts, or any other pickled vegetables, fruit or fungi.

"Sauce" means anchovy essence or sauce, caper sauce, chutney, fruit sauce of the type commonly used with meat or fish, mushroom ketchup, horseradish cream or sauce, mayonnaise, mint jelly or sauce, mushroom ketchup or sauce, mustard sauce, prepared mustard, salad cream or any other salad dressing, sandwich spread, tomato ketchup or sauce, walnut ketchup, Worcester sauce, or any other similar products.

No person shall sell any product under the description "tomato ketchup" or "tomato catsup" unless it contains no fruit or vegetable other than tomatoes (except onions, garlic and spices added for flavouring purposes).

— **968. The Edible Gelatin (Control) (Amendment) Order, 1947. Dated May 16, 1947.** Price 1d.

This amending Order

(*a*) *provides that edible gelatin (see S.R. & O.*, 1947, *No.* 161; ANALYST, 1947, **72**, 66) *may be manufactured by the blending together of two or more gelatins (not necessarily individually complying with the definition of "edible gelatin"); and*

(*b*) *adds jellied eels to the list of foods in the manufacture of which edible gelatin may be used.*

* Obtainable from H.M. Stationery Office. Italics signify changed wording.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

Food and Drugs

Polarographic Analysis of Streptomycin. G. B. Levy, P. Schwed, and J. Warren (*J. Amer. Chem. Soc.*, 1946, **68**, 528–529)—Under certain conditions, streptomycin gives rise to a well-defined polarographic reduction wave at high *p*H values. At 13·6° C., with 3 per cent. tetramethyl ammonium hydroxide as supporting electrolyte, experimental points over a range of 100 to 1000 units per ml. fall on a straight line with an average deviation of ±13 units. The half-wave potential is approximately −1·45 v. *versus* the mercury anode. The reaction probably involves one electron, and the diffusion coefficient is 3×10^{-6} cm.^{2} sec.$^{-1}$ The temperature coefficient of the diffusion coefficient is 3 per cent. per degree at room temperature, but erratic results are obtained at elevated temperatures. All oxygen must be removed by passing nitrogen through the solution before a polarogram is taken, and if any non-aqueous solvent is present a special calibration curve must be used.

Some streptomycin solutions contain materials that interfere with the analysis. These impurities can be precipitated by lead ions, the excess of lead being removed by precipitation with sulphuric acid.

Results obtained by the polarographic method agree closely with those obtained by microbiological assay. J. G. W.

Biochemical

Estimation of Threonine and Serine in Proteins. M. W. Rees (*Biochem. J.*, 1946, **40**, 632–640)—The method is a modification of that of Nicolet and Shinn (*J. Amer. Chem. Soc.*, 1939, **61**, 1615; 1941, **63**, 1486; *J. Biol. Chem.*, 1941, **138**, 91; 1941, **139**, 687; 1942, **142**, 139), essentially the same method being used and the same apparatus (*cf.* Abst., ANALYST, 1941, **56**, 343).

Procedure—Put about 3·5 and 2·5 ml. of sodium bisulphite solution (5 ml. of a commercial solution, sp.gr. 1·34, are diluted to 100 ml.) into the first and second receiver tubes of the apparatus and dilute to 25 ml. Pipette a sample of the protein or test solution, containing 1·5 to 7 mg. of threonine and 2 to 7 mg. of serine, into a reaction tube and neutralise to *p*H 7 with *N* sodium hydroxide. Add 1·5 ml. of potassium arsenite solution (dissolve 25 g. in water, dilute to 100 ml. and add *N* sulphuric acid until, on addition of 1 ml. of 0·5 *M* periodic acid to 1·5 ml. of the solution in 20 ml. of water, the *p*H is 7·0) followed by 6 ml. of a phosphate buffer solution of *p*H 7·16 (mix 30 ml. of a KH_2PO_4 solution containing 18·156 g. per litre with 70 ml. of Na_2HPO_4 solution containing 19·536 g. per litre), adjust the volume to approximately 18 ml. and then, when each of the sets of tubes has been prepared in this way, close the stop-cocks of the separating funnels, and into each funnel put 1 ml. of 0·5 *M* periodic acid and 3 ml. of water. Connect up the apparatus, draw air through it slowly, and open the stop-cocks. After 3 to 4 min., increase the rate of aeration to 1 litre per minute and continue for 1 hour. Stop the flow of air and wash the contents of each pair of receiving tubes into a titration flask. Stopper the flasks and set aside until the formaldehyde distillations are completed.

To estimate the acetaldehyde, oxidise the excess of bisulphite with approximately 0·1 *N* iodine and finally with 0·01 *N* iodine, using 2 ml. of a starch solution as indicator. Add about 7 ml. of *M* sodium bicarbonate and titrate the liberated bisulphite with 0·01 *N* iodine until the fading rate of the blue colour slows down. Add another 2 ml. of the bicarbonate solution, continue the titration, and then add a further 2 ml. and complete the titration; the blue colour should persist for one minute or longer.

After removal of the acetaldehyde by aeration, transfer the contents of each reaction tube quantitatively without undue delay to a micro-Kjeldahl flask into which 0·6 ml. of *N* sulphuric acid has been put. Steam-distil the contents of the flask at such a rate that the volume is about 5 ml. after 30 min. and the volume of distillate is about 250 ml. Titrate the formaldehyde in this distillate in the same way as the acetaldehyde, but use 4 ml. of starch solution, and 5 per cent. sodium carbonate solution in place of the bicarbonate solution. The recoveries of added threonine and serine were 99 ± 1 per cent. and 100 ± 2 per cent., respectively. Satisfactory agreement was obtained with the amounts of ammonia simultaneously produced. F. A. R.

Precision Method for the Quantitative Determination of Calcium in Blood Plasma. K. Salomon, B. W. Gabrio, and G. F. Smith (*Arch. Biochem.*, 1946, **11**, 433–443)—The method involves preliminary ashing of the plasma with a perchloric acid - nitric acid mixture, precipitation of the calcium as oxalate, and titration of the oxalate ion with ammonium hexanitratocerate in perchloric acid solution, nitro-*o*-phenanthroline ferrous sulphate (nitroferroin) being used as indicator.

Reagents—Ashing mixture, a 1+1 mixture of 72 per cent. perchloric acid (sp.gr. 1·69) and concentrated nitric acid (sp.gr. 1·42, analytical reagent). Triton, N.E. (1:30 dilution of original solution from Rohm and Haas Co., Washington Square, Philadelphia). Two per cent. aqueous ammonia, prepared daily (distil concentrated aqueous ammonia and prepare a 10 per cent. stock solution from which

the 2 per cent. solution is obtained). Saturated solution of ammonium oxalate analytical reagent, kept at room temperature. 0·001 *N* Sodium oxalate, primary standard, analytical reagent (the salt is dissolved in 2 *N* perchloric acid; in the absence of perchloric acid, the solution is not stable on storage at this concentration). 0·00025 *N* Nitro-*o*-phenanthroline ferrous sulphate indicator (obtained from G. Frederick Smith Chemical Co., Columbus, Ohio, in 0·025 *M* solution, to be diluted with re-distilled water). Ammonium hexanitratocerate, standard or reference purity (G. Frederick Smith Chemical Co.). Prepare a 0·01 *N* solution of the cerium salt in 2 *N* perchloric acid by adding slowly 167 ml. of 72 per cent. perchloric acid to 5·4819 g. of ammonium hexanitratocerate, stirring constantly; then add slowly, with constant stirring, re-distilled water to 1000 ml.; slow addition of 50 ml. of water with stirring for 1 min. after each addition is essential. Dilute the resulting 0·01 *N* perchloratoceric acid with 2 *N* perchloric acid to 0·001 *N* concentration, and standardise with 0·001 *N* sodium oxalate solution. Store the solution in the dark and in a refrigerator to increase its stability with age.

Procedure. Ashing—Pipette 0·5 ml. of plasma or serum into a 25-ml. Erlenmeyer flask and add 2 ml. of the ashing mixture. Heat on a hot plate, beginning the ashing at 180° C.; if a plasma of high lipid content is being ashed, the initial temperature should be 100° C. to avoid spattering. Raise the temperature slowly to 240° C.; the temperature should never exceed 250° C. Ashing is complete when there are no remaining drops of liquid and no more white fumes escaping, and when only the snow-white, dry ash remains in the flask. It is important that no traces of perchloric acid be left in the flask as the perchlorate ion interferes with quantitative precipitation of the amounts of calcium oxalate concerned in this procedure. The ashing takes about 3 to 4 hours, which may be shortened by brushing the top half of the flask with a small, colourless gas flame at the end of the digestion to minimise refluxing of the acid condensate on the inside walls of the flask, and to facilitate elimination of the excess of acid.

Precipitation of the calcium as oxalate—Dissolve the ash while it is still hot with 2 ml. of 0·04 *N* hydrochloric acid, using gentle heating over a micro-flame if necessary. Transfer the dissolved ash with re-distilled water (total about 5 ml.) to a conical, 15-ml. centrifuge tube. Add 2 ml. of saturated ammonium oxalate solution and leave for 16 hours at room temperature for complete precipitation. The *p*H is always 4·0 to 6·0, which is within the optimum range for the precipitation of calcium oxalate as indicated by Sendroy (*J. Biol. Chem.*, 1944, **152**, 539).

Treatment of the precipitated calcium oxalate—The procedure of Sendroy (*loc. cit.*) is followed, with slight modifications. To the solution containing the calcium oxalate precipitate add 0·2 ml. of Triton before centrifuging at 2600 r.p.m. for 5 min. Slowly siphon off the supernatant liquid with a hooked capillary, allowing about 0·2 ml. of the liquid to remain in the tube. Wash the precipitate with 3 ml. of 2 per cent. aqueous ammonia, allowing 1 ml. of this solution to wash down the sides of the tube slowly and adding the remaining 2 ml. more rapidly, also down the sides of the tube. Centrifuge again at 2600 r.p.m. for 5 min. Draw off the supernatant liquid as before and wash for a second time in the same way. After the third centrifugation and withdrawal of the fluid, dissolve the precipitate immediately in 4 ml. of 2 *N* perchloric acid, with stirring. Transfer the solution to a 25-ml. beaker and wash the centrifuge tube twice with a total volume of 6 ml. of 2 *N* perchloric acid.

Titration—Immediately before titration, add 0·5 ml. of 0·00025 *N* nitroferroin indicator to produce a distinct pink colour. Titrate with 0·001 *N* perchloratoceric acid to a sharp colour change from pink to colourless, using a 10-ml. micro-burette (Koch) graduated in 0·05 ml., and having a reservoir and a standard capillary tip. One ml. of 0·001 *N* perchloratoceric acid is equivalent to 20 μg. of calcium.

Standardisation of perchloratoceric acid solution—Standardise the 0·001 *N* perchloratoceric acid in 2 *N* perchloric acid in the same manner as the titration of the oxalate from calcium oxalate, using a 0·001 *N* solution of sodium oxalate in 2 *N* perchloric acid. For 10 ml., use 0·5 ml. of 0·00025 *N* nitroferroin indicator. The blank correction for the indicator thus amounts to approximately 0·1 ml. of oxidant; this correction can be duplicated precisely, and therefore introduces no error. A 0·001 *N* perchloratoceric acid solution in perchloric acid is stable for about 4 days in the dark at about 0° C.

Modification of procedure using 3 ml. of plasma—Pipette 3 ml. of plasma or serum into a 25-ml. vitreosil crucible and dry at about 100° C. Ash over a Bunsen burner until nearly all the material is white, and then complete the ashing by adding 2 ml. of ashing mixture and heating on a hot plate at about 240° C. until a dry, white ash is obtained; the total time required for ashing is about 2 hours. Dissolve the ash in 5 ml. of 0·04 *N* hydrochloric acid, transfer the solution with a total of 15 ml. of water to a 40-ml. centrifuge tube, add 4 ml. of saturated ammonium oxalate solution, and leave for 16 hours. No *p*H adjustment is necessary. Treat the precipitated calcium oxalate as above, but use 4 ml. of 2 per cent. aqueous ammonia for each washing. For the final titration, use 0·01 *N* perchloratoceric acid with the same quantity and concentration of nitroferroin indicator as above.

Evidence which suggests that the presence of

increased amounts of lipids interferes with direct precipitation methods of determining calcium is presented, and the above procedure involving preliminary ashing is recommended. E. M. P.

Determination of Serum Phosphate by the Molybdi-vanadate Method. D. G. Simonsen, M. Wertman, L. M. Westover, and J. W. Mehl (*J. Biol. Chem.*, 1946, **166**, 747–755)—The molybdi-vanadate method for orthophosphate described by Kitson and Mellon (*Ind. Eng. Chem., Anal. Ed.*, 1944, **16**, 379) is suitable for the estimation of serum phosphate.

Procedure—Add 2 ml. of serum to 6 ml. of 10 per cent. trichloroacetic acid in a 15-ml. centrifuge tube. Mix by inversion, leave for 15 min., and then centrifuge for 7 to 10 min. at 2500 to 3500 r.p.m., or until the supernatant liquid is clear. Transfer 3 ml. of the supernatant liquid to a Klett tube graduated at 5 ml. Add 0·5 ml. of diluted nitric acid (1+2), followed by 0·5 ml. of 0·25 per cent. ammonium vanadate solution (prepared by dissolving 2·5 g. of the salt in about 500 ml. of boiling water, cooling slightly, adding 20 ml. of concentrated nitric acid, allowing to cool to room temperature, and then diluting to 1 litre). Next add 0·5 ml. of 5 per cent. ammonium molybdate solution and dilute to 5 ml. with water. Leave for 5 min. and evaluate the colour in a Klett colorimeter with a No. 42 (blue) filter. Prepare a blank with 3 ml. of 7·5 per cent. trichloroacetic acid, treat this in the same way as the test solution, and subtract the reading thus obtained from the reading of the test solution. A spectrophotometer may be used for evaluating the colour. Calculate the phosphorus content from a standard curve obtained by treating a standard solution of potassium dihydrogen phosphate (0·4389 g. in 1 litre of 7·5 per cent. trichloroacetic acid) in the same way. The standard solution contains 100 μg. of phosphorus per ml., and to prepare the curve, solutions giving a range of 5 to 80 μg. of phosphorus in the final 5 ml. are used.

Recoveries of phosphate added to serum ranged from 99·3 to 101·0 per cent. of the theoretical.

F. A. R.

Yeast Microbiological Method for Estimation of Nicotinic Acid. W. L. Williams (*J. Biol. Chem.*, 1946, **166**, 397–406)—The method utilises a yeast, *Torula cremoris*, the growth of which is estimated turbidimetrically. The method is rapid, 16 to 18 hours being allowed for growth, and specific. It can be used for the differential assay of nicotinic acid, trigonelline, and N^1-methylnicotinamide.

Procedure—Maintain stock cultures on Difco malt agar for 24 hours at 37° C. for a period not exceeding one month. Make a fresh transfer 24 hours before each series of assays and incubate at 37° C. Transfer the yeast from this culture to 10 ml. of sterile saline to give a suspension with 15 to 25 per cent. absorption in an Evelyn colorimeter. Add the contents of the tube to 90 ml. of sterile saline and use the suspension for inoculating the assay tubes at the rate of 1 ml. per tube. Prepare a basal medium of the following composition

Component	Amount per 10 ml. of final medium
	mg.
Glucose, anhydrous	500
KH_2PO_4	3
$MgSO_4$	1
	ml.
Charcoal-treated peptone	1(=100 mg.)
Potassium citrate buffer (1 litre contains 100 g. of potassium citrate and 20 g. of citric acid)	0·5
	μg.
Biotin	0·25
Aneurine hydrochloride	25
Pyridoxine hydrochloride	25
Calcium pantothenate	25
Inositol	250

Weigh out an amount of sample estimated to contain 5 to 10 μg. of nicotinic acid, suspend in a minimum quantity of water, add 50 ml. of 3 N sodium hydroxide, and autoclave at 15 lb. pressure for 1 hour. Cool, adjust to *p*H 5·0 to 5·5 with 3 N sulphuric acid and dilute to 200 ml. Centrifuge, if necessary, to obtain a clear extract or, if starch is present, incubate at 100° C. for 5 min. with 2 to 4 mg. of taka-diastase and then centrifuge. Milk and milk products may become highly coloured by the alkaline treatment and, as these do not contain trigonelline or N^1-methylnicotinamide, N sulphuric acid may be substituted for the 3 N sodium hydroxide.

Put 5 ml. of the double-strength basal medium into a series of standard Evelyn test tubes and to 8 of these add 0, 0·5, 1·0, 1·5, 2·0, 2·5, 3·0, and 4·0 ml. of a standard solution of nicotinic acid containing 50 millimicrograms per ml. Into another 4 test tubes put 1·0, 2·0, 3·0, and 4·0 ml. of the solution to be tested, and adjust the volume in each test tube to 9 ml. by adding water. Plug each with cotton wool, steam for 10 min., cool, and then add 1 ml. of inoculum. Shake the tubes in an incubator at any temperature between 30 and 38° C., and measure the turbidity after 16 to 18 hours. Calculate the nicotinic acid content of the test solution from the standard curve. The recovery of nicotinic acid added to various materials was 95 to 99 per cent. of the theoretical.

Differential assays of nicotinic acid and its two derivatives can be carried out by treating the sample with: (*a*) 50 ml. of water, (*b*) 50 ml. of 2 N sulphuric acid, and (*c*) 50 ml. of 3 N sodium hydroxide, autoclaving at 15 lb. for 1 hour, neutralising, diluting, and assaying each solution as described above. The response induced by the

first solution is due to nicotinic acid and trigonelline, by the second solution to all 3 compounds, and by the third solution to nicotinic acid alone.

F. A. R.

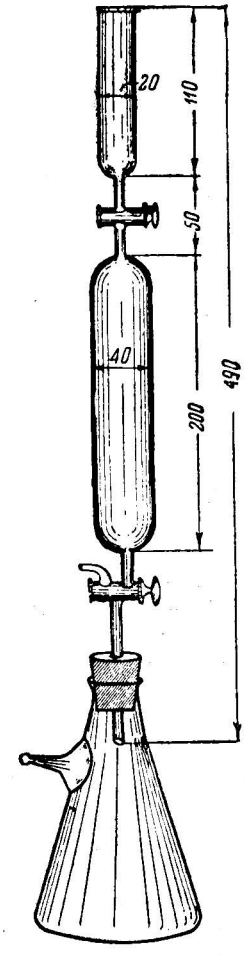
Chemical Assay Method for Penicillin G. J. C. Sheehan, W. J. Mader, and D. J. Cram (*J. Amer. Chem. Soc.*, 1946, **68**, 2407)—The method depends on the low solubility of the N-ethylpiperidine salt of penicillin G in amyl acetate - acetone mixtures. Penicillins K and F, and the degradation products of G are said not to interfere. The method is claimed to be quantitative with penicillin G contents exceeding 50 per cent., and with potencies exceeding 800 units per mg.

Procedure—Transfer, by means of a 2-ml. syringe inserted through the rubber cap, the contents of a weighed penicillin vial (100,000 or 200,000 units) to a chilled centrifuge tube, using 3 ml. of ice-cold water. Dry the bottle and weigh. Add to the aqueous solution exactly 2 ml. of ice-cold amyl acetate saturated with N-ethylpiperidine salt of penicillin G (the solubility is approximately 0·6 mg. per ml.). Add, with shaking and cooling in ice, 0·5 ml. of 20 per cent. phosphoric acid solution and centrifuge. Remove about 1·8 ml. of the amyl acetate layer, and dry with 0·1 g. of sodium sulphate. Filter through a sintered-glass, micro-filter-crucible. The *p*H of the spent aqueous layer should be about 2. Transfer exactly 1 ml. of the dried amyl acetate solution to a 10-ml. micro-beaker in an ice-bath. Add 1 ml. of acetone saturated with the N-ethylpiperidine salt of penicillin G (the solubility is approximately 2 mg. per ml.) followed by 0·5 ml. of a 10 per cent. solution of N-ethylpiperidine in amyl acetate saturated with the amine salt (about 2 mg. per ml.). After 2 hours at 0° to 5° C., filter through a weighed micro-filter stick, wash with 1 ml. of cold acetone (saturated with amine salt), and dry *in vacuo* at room temperature for 1 hour. The practically colourless N-ethylpiperidine salt of penicillin G melts, with decomposition, at 152 to 154° C., when placed in a capillary in a bath at 140° and heated at a rate of 3° per min.

W. S. W.

Organic

Determination of Low Concentrations of Ethyl Alcohol in Aqueous Solutions. N. V. Tschalov and L. P. Volskaja (*Zavod. Lab.*, 1946, **12**, 286–291)—The determination of low concentrations (0·02 to 1 per cent.) of alcohol in aqueous solutions containing large proportions (5 to 200 times that of the alcohol) of aldehydes, ketones, esters, furfural, etc. (*e.g.*, industrial solutions obtained in the preparation of alcohol from sulphited pulp) is described. The method is based on the formation of ethyl nitrite, which is highly volatile (b.p. 18° C.) (Fischer and Schmidt, *Ber.*, 1924, **57**, 693, and 1926, **58**, 679; Skrabal, *Z. anal. Chem.*, 1940, **119**, 222). All variations of this method that have been described previously depend on the removal of the nitrite from the reaction vessel by means of carbon dioxide, nitrogen, or air, and its subsequent absorption in a solvent (*e.g.*, carbon tetrachloride); the necessary apparatus is complicated, the time required is long, and the purification of the solvents is tedious. Such a method is, therefore, unsuitable under works conditions.



The modification now described consists in the retention of the nitrite in the gaseous form in the reaction vessel and the removal of the reaction liquid; the ethyl nitrite is then saponified in the reaction vessel, the nitrous acid is allowed to react with sulphanilic acid, and the diazo compound is coupled with α-naphthylamine to give an orange azo dye which can be estimated colorimetrically.

The reaction vessel is a glass cylinder, capacity 200 ± 5 ml., joined through a tap at the top with a funnel used for delivery of the reagents, and through a two-way vacuum tap at the bottom to a glass tube which passes down into a pressure

filtering flask through a rubber bung (see Figure; the dimensions given are in mm.).

Procedure—By means of a water-pump or other means reduce the pressure to about 500 mm. of mercury in the reaction vessel, and then introduce successively through the upper tap 1 ml. of 50 per cent. sodium nitrite solution, 20 ml. of the solution under examination, previously diluted to a concentration of 0·025 to 0·005 per cent. of alcohol, and 1 ml. of 20 per cent. hydrochloric acid, without admitting any air. Shake for 30 sec. Ethyl nitrite is formed and enters the space above the liquid, but oxides of nitrogen are also present and they must be removed by adding 2 ml. of 25 per cent. sodium hydroxide solution and shaking vigorously five to eight times. Connect the flask to a pump and when the pressure is less than that in the reaction vessel open the lower tap and allow all but a small quantity of liquid (3 to 5 mm. height in the tube above the tap) to flow into the flask. Purify the ethyl nitrite further by introducing simultaneously 0·5 ml. of 25 per cent. sodium hydroxide solution and 10 ml. of 2 per cent. potassium permanganate solution and shaking for 30 sec. Remove the bulk of the solution, and wash out the vessel twice with 10 ml. of distilled water. Now add to the purified ethyl nitrite 10 ml. of sulphanilic acid solution (6 g. of sulphanilic acid in one litre of 0·1 *N* hydrochloric acid), shake for 30 sec., run in 10 ml. of α-naphthylamine solution (3·2 g. of the amine in 1 litre of water containing 6 ml. of 20 per cent. hydrochloric acid), allow to stand for 2 min., add 10 ml. of 15 per cent. sodium hydroxide solution, make up to 250 ml. with distilled water, and determine the colour intensity by means of a photometer with potassium photo-electric cell. Evaluate the alcohol content of the original solution from calibration curves obtained from the use of standard solutions.

The total time for a determination is 20 min., and an accuracy of 3 parts in 100 is attainable, but the conditions as given above are fairly critical. The formation of ethyl nitrite is not quite quantitative and some loss occurs later; hence the results must be referred to those obtained with standards under the same conditions. The indicated size of the reaction vessel and volumes of solutions must be observed; some extension of the times of shaking and colour development is permissible. Liquors from hydrolysis works can be analysed directly, but those from sulphite works should be first distilled to avoid the formation of foam in the reaction vessel. Alcohols other than ethyl alcohol react similarly, but volatile products are not formed from the other components of the solutions. G. S. S.

Determination of Linoleic Acid in Cholesteryl Linoleate. J. S. Front and B. F. Daubert (*J. Amer. Chem. Soc.*, 1945, **67**, 1509–1510)—If cholesteryl linoleate could be directly isomerised in alkaline ethylene glycol solution and the spectral absorption of the isomerised product measured by the method of Mitchell, Kraybill, and Zscheile (*Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 1; *cf.* ANALYST, 1942, **67**, 310), a satisfactory method of ascertaining its purity would result. It was found that, unlike most fats, synthetic glycerides and potassium linoleate, cholesteryl linoleate cannot be isomerised directly in alkaline ethylene glycol or glycerol by the method cited. When heated with the alkaline glycol at 180° C. for 30 min. in an oil bath, it melts and rises to the surface. The average $E^{1\%}_{1\,cm.}$ value at 234 mμ. of the product, *viz.*, 46, indicates that although partial hydrolysis has occurred it represents only 12·3 per cent. of the theoretical value of 374 based on an experimentally determined $E^{1\%}_{1\,cm.}$ value of 867 for linoleic acid.

The alkaline glycol used in the method was prepared by dissolving 7·5 g. of potassium hydroxide in 100 ml. of redistilled ethylene glycol. The solution was heated before use to 190° C., cooled to room temperature, and made up to 100 ml. with the glycol. About 0·1 g. of cholesteryl linoleate was weighed in a 1″ × 6″ Pyrex test tube. One ml. of alcoholic potassium hydroxide (55 g. to 100 ml. of 99 per cent. alcohol) was added to the sample and to a blank tube. The tubes were warmed in a water-bath at 55° to 60° C. for 90 min., after which 10 ml. of alkaline ethylene glycol were added to both sample and blank, and the tubes were placed in a constant temperature bath at 180° C. for 30 min. in an atmosphere of nitrogen. At three successive 1-min. intervals the tubes were removed from the bath and shaken thoroughly for proper mixture of ester and reagent. The slight frothing that occurred during the heating process did not interfere with isomerisation. After exactly 30 min. of total heating time the tubes were removed from the bath and immediately cooled in water. The isomerised soaps and excess of reagent were transferred with 99 per cent. alcohol to volumetric flasks and further diluted until their optical densities were suitable for measurement in the spectrophotometer.

The $E^{1\%}_{1\,cm.}$ value of the linoleic acid from which the ester was prepared was 867. Comparison of the calculated theoretical $E^{1\%}_{1\,cm.}$ value of 374 for cholesteryl linoleate with the average value found experimentally, *viz.*, 373 indicated a purity of the ester of 99·7 per cent.

Absorption measurements made with a Beckman spectrophotometer of a solution of cholesteryl linoleate in *iso*-octane solution showed a total diene conjugation of less than 0·1 per cent. and the triene conjugation was negligible. Corrections for preformed conjugation were, therefore, not made in the final $E^{1\%}_{1\,cm.}$ values.

The ester used in the investigation was prepared

from cholesterol and linoleyl chloride by the method of Page and Rudy (*Biochem. Z.*, 1930, **220**, 304) with the following modifications. After reaction, the cooled liquid ester was dissolved in 300 ml. of ethyl ether and the solution was washed with a 5 per cent. potassium carbonate solution and with water. The ether was removed from the dried and filtered liquid under nitrogen, and the liquid residue was dissolved in a small volume of ether. Sufficient ethyl alcohol was then added to impart a slight turbidity to the solution and crystallisation of the ester occurred on cooling overnight at 0° to 5° C. Recrystallisation several times from the same mixture of solvents yielded a product melting sharply at 42·5° C. The ester was also prepared by the following method. Cholesterol (5 g.) dissolved in a mixture of 20 ml. of chloroform and 5 g. of quinoline was refluxed on a steam-bath for 3 hr. with 3·9 g. of linoleyl chloride, and, when cold, the product was dissolved in 300 ml. of ethyl ether. The solution was washed successively in a separating funnel with 30-ml. portions of 0·5 *N* sulphuric acid, 5 per cent. potassium carbonate solution, and water. After being dried over anhydrous sodium sulphate the solution was treated in the manner already described. The m.p. of the crystallised product was 42° to 42·5° C.

The purity of cholesteryl linoleate deduced from its linoleic acid content as determined by this method is more reliable than that based on the iodine value. The method should be equally applicable to the determination of the purity of cholesteryl linolenate. A. O. J.

Iodimetric Micro-titration for Mustard Gas. V. E. Kinsey and W. M. Grant (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 794–797)—The method is based on the reaction of dichloramine-T with mustard gas. The residual chlorine in the dichloramine, after reaction with the mustard gas, is determined by titration of the iodine liberated from potassium iodide in presence of acetic acid, the end-point being sufficiently clear to render the use of starch indicator unnecessary. Under the conditions specified, about 10 equivalents of chlorine are used per molecule of mustard gas.

The stability of dichloramine-T was found to be satisfactory in purified kerosene, technical carbon tetrachloride, *cyclo*hexane, benzene, and chloroform, but there was appreciable loss of chlorinating power in xylene and in light petroleum. Accordingly, the dichloramine-T was dissolved (0·2 per cent.) in carbon tetrachloride and the mustard gas, in concentrations of 50 and 150 μg. per ml., in purified kerosene or in *cyclo*hexane. One ml. of the chlorinating solution was then added to 1 ml. of the mustard gas solution and samples for titration were withdrawn periodically during 30 min. The reaction was found to be essentially complete in 20 min. and this reaction period was adopted in all subsequent experiments. Since the amount of mustard gas is represented by a difference in the amounts of sodium thiosulphate required to titrate a sample and a blank determination, it is desirable to keep the amount of dichloramine-T present at a minimum value. As the degree of chlorination of the mustard gas increases slightly over the concentration range of 0·1 to 0·2 per cent. of dichloramine-T, 0·2 per cent. was chosen as the standard concentration. The extent of the reaction was found to be dependent upon the nature of the solvents employed. Carbon tetrachloride was ultimately selected as the best solvent for the dichloramine-T, and the mustard gas was dissolved in either *cyclo*hexane or in purified kerosene. Results vary somewhat with different lots of *cyclo*hexane and, consequently, standard curves should be constructed for each new supply of solvent unless C.P. grade *cyclo*hexane is available. The latter gives a reproducible equivalent of 0·67 ml. of 0·01 *M* sodium thiosulphate per 100 μg. of mustard gas. During a search for the impurity in "practical" (Eastman Kodak) *cyclo*hexane responsible for the variation mentioned, it was found that the presence of *cyclo*hexanol greatly increased the sensitivity of the test, but, although the sensitivity could be increased twenty-fold, the effect was evident only over an extremely narrow concentration range of *cyclo*hexanol in pure *cyclo*hexane or purified kerosene. Advantage is taken of the sensitising action of *cyclo*hexanol in Procedure II (*infra.*).

Procedure I—Allow 1 ml. of mustard gas solution to react at 27° C. with 1 ml. of a 0·2 per cent. solution of dichloramine-T in carbon tetrachloride for 20 min. Add 4 drops of saturated potassium iodide solution and 4 drops of glacial acetic acid, shake the mixture, and add 0·01 *M* sodium thiosulphate until the colour of iodine no longer persists. Vigorous shaking is necessary, as the end-point is approached, and the whole procedure should be carried out away from direct sunlight, best results being obtained by maintaining uniform temperature conditions.

Interference of derivatives of mustard gas was negligible, especially when the solution of the sample in the organic solvent was first extracted with water. Cornea and rabbit blood (representing biological material) exerted no interfering action provided that purified kerosene, xylene, or *cyclo*hexane was used for the extraction. When the other layer is water, most of the mustard gas (98 to 99 per cent.) remains in the purified kerosene or *cyclo*hexane; when the other layer is blood, *cyclo*hexane retains most of the mustard gas (97 per cent.), but kerosene retains only 88 per cent.

Procedure II—This procedure provides the greatest sensitivity in the range 0·2 to 5 μg. of mustard gas. To 1 ml. of a 5 per cent. by volume

solution of *cyclo*hexanol in pure *cyclo*hexane or in purified kerosene, add 1 ml. of *cyclo*hexane containing up to 5 μg. of mustard gas, and place the mixture in a test tube in a water-bath thermostatically controlled to ±0·1° C. in the range 25° to 28° C. Add 1 ml. of a 0·1 per cent. dichloramine-T solution in carbon tetrachloride and, exactly 20 min. later, add 4 drops of saturated potassium iodide solution and 4 drops of glacial acetic acid. Titrate the liberated iodine with 0·01 *M* sodium thiosulphate to a colourless end-point. A. O. J.

Chromatography of Sugars and their Derivatives. L. W. Georges, R. S. Bower, and M. L. Wolfrom (*J. Amer. Chem. Soc.*, 1946, **68**, 2169–2171)—A synthetic hydrated calcium acid silicate, known as Silene EF, has been found suitable for the chromatographic separation of sugars and their derivatives. To develop the columns, dioxan and alcohols of low molecular weight were used, after addition of varying amounts of water. The columns are extruded from the tubes, and the positions of the zones are ascertained by "streaking" with

TABLE I

GROUP I—Sugars, sugar alcohols, and glycosides: (0·5 ml. of 90 per cent. dioxan followed immediately by a solution of 2 mg. of the substance in 0·2 ml. of 90 per cent. dioxan).

Class 1—(10 ml. of 90 per cent. dioxan.)
α-*d*-Galacturonic acid
Lactose monohydrate
Lactitol
Dulcitol
Melezitose, raffinose pentahydrate, gentiobiose, *d*-gluco-*d*-gulo-heptose
Sucrose, maltose monohydrate, cellobiose, *d*-glucitol (sorbitol)
d-Galactose, *d*-mannitol
d-Glucose, *d*-fructose, *d*-mannose, *l*-sorbose
l-Fucitol
l-Arabinose

Class 2—(5 ml. of 90 per cent. dioxan.)
l-Fucose
d-Xylose, *l*-rhamnose monohydrate
Methyl α-*d*-glucopyranoside

GROUP II—Acetylated sugars and acetylated sugar alcohols: (0·5 ml. of benzene followed immediately by a solution of 2 mg. of the substance in 0·5 ml. of benzene)

Class 1—(15 ml. of 250:1 v/v benzene - absolute ethanol)
Raffinose hendeca-acetate
β-Melibiose octa-acetate, sucrose octa-acetate
β-Maltopyranose octa-acetate

Class 2—(15 ml. of 500:1 v/v benzene - absolute ethanol)
Keto-*d*-fructose penta-acetate
d-Glucitol (sorbitol) hexa-acetate, *d*-mannitol hexa-acetate
β-*d*-Glucopyranose penta-acetate
α-*d*-Arabinopyranose tetra-acetate

Class 3—(15 ml. of 1000:1 v/v benzene - absolute ethanol)
α-*l*-Fucose tetra-acetate

GROUP III—Methylated sugars: (0·5 ml. of benzene followed immediately by a solution of 2 mg. of the substance in 0·5 ml. of absolute chloroform)

Class 1—(15 ml. of 100:1 v/v benzene - absolute ethanol)
2:3-Dimethyl-*d*-glucose
2:3:6-Trimethyl-*d*-glucose

Class 2—(12·5 ml. of 250:1 v/v benzene - absolute ethanol)
2:3:4:6-Tetramethyl-*d*-glucose

TABLE II

Substance	Adsorbate soln.	Size of column, cm.	Developer	Distance of zones from top, cm.
100 mg. *d*-Glucitol	30 ml.	5·1 × 24·0	1800 ml.	5 –10·5
100 ,, *d*-Mannitol	90% dioxan		92% dioxan	15·0–21·5
800 ,, Raffinose, $5H_2O$	33 ml.	5·1 × 25·5	500 ml.	1·5– 4·7
800 ,, Sucrose	90% abs. ethanol		95% propanol-2	11·2–21·0
500 ,, *d*-Galactose	20 ml.	4·4 × 19·5	218 ml.	2·3– 4·4
500 ,, *l*-Rhamnose, H_2O	90% dioxan		92% dioxan	13·7–18·0
500 ,, Maltose, H_2O	20 ml.	4·4 × 17·0	300 ml.	1·0– 3·3
500 ,, *d*-Glucose	85% dioxan		92% dioxan	4·5– 6·8
250 ,, *d*-Glucitol hexa-acetate	33 ml.	5·1 × 26·0	1800 ml.	9·0–14·8
250 ,, *d*-Mannitol hexa-acetate	Benzene		benzene - abs. ethanol (1000:1)	16·5–20·0

alkaline potassium permanganate solution. With this adsorbent, sugars can be chromatographed directly without the necessity of preparing derivatives. Table I gives the chromatographic adsorption series of some sugars and their derivatives arranged in decreasing order of adsorptive strength. Columns, 11 cms. long and 0·9 cm. in diameter, of a 5:1 mixture of Silene EF and Celite were used. The nature of the adsorbate solution is recorded under the Group headings and that of the developer solution under the Class headings.

The reagent used for "streaking" the column was a 1 per cent. solution of potassium permanganate in 2·5 *N* sodium hydroxide; this formed brown zones on reaction with the adsorbed sugars. In Table I, substances on the same line are not separated from one another; separation can sometimes be effected by using different developers as shown in Table II. W. S. W.

Inorganic

Determination of Boric Oxide in Glass. M. Hollander and W. Rieman (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 788–789)—The method gives results with a mean error of 0·04 per cent. of boric oxide with glasses containing less than 15 per cent. *Procedure*—(*A*) *Using a pH meter*—Fuse 0·5 g. of glass with 3 g. of sodium carbonate for 10 min. Dissolve the melt in 20 ml. of 6 *N* hydrochloric acid and add 6 *N* sodium hydroxide until the *p*H is between 5·0 and 5·5. Aluminium, iron, and similar elements and most of the silica are precipitated. Sweep out carbon dioxide by bubbling air (purified by passing through concentrated sulphuric acid and Ascarite) through the solution at 60° ± 5° C. for 0·5 hr. Filter, and wash the precipitate with warm water until the volume of the filtrate is 250 ml. Cool the solution and add carbonate-free 0·05 *N* sodium hydroxide until the *p*H is 6·30. Add 40 g. of mannitol and titrate with the 0·05 *N* sodium hydroxide until the *p*H is again 6·30. The amount of alkali used in the titration, corrected as described below, is a measure of the boric oxide. A double precipitation is sometimes necessary to recover co-precipitated boric oxide. Dissolve the precipitate in 10 ml. of 6 *N* hydrochloric acid, repeat the above procedure, and titrate the second filtrate separately.

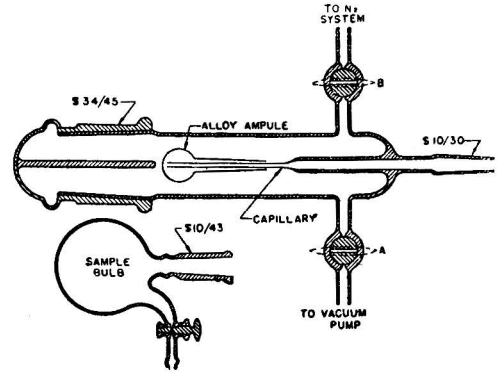
(*B*) *Using an indicator*—Add 20 drops of 0·04 per cent. bromocresol purple solution to the solution of the melt. Add 6 *N* sodium hydroxide until the first distinct colour change (yellow to dirty green) can be seen. This indicates a *p*H of about 5·5. Continue by the above method, adjusting the *p*H to 6·30 with the aid of a phosphate comparison buffer solution.

Titration corrections—In the first adjustment of the *p*H to 6·30, a small amount of boric acid is neutralised and in the second the equivalence point is not quite reached. Under the above conditions, which are selected to give the minimum correction, the amounts of boric acid shown in the table must be added to the amount titrated. These corrections are based on potentiometric titrations of boric acid under the conditions described.

H_3BO_3 found, mg.-equivalents	Correction, mg.-equivalents	H_3BO_3 found, mg.-equivalents	Correction, mg.-equivalents
0·0	0·000	1·2	0·024
0·1	0·005	1·4	0·026
0·2	0·008	1·6	0·029
0·4	0·013	1·8	0·031
0·6	0·016	2·0	0·033
0·8	0·019	2·4	0·036
1·0	0·022	2·8	0·040

Small amounts of carbon dioxide and silica do not interfere with the titration as the *p*H is the same before and after it. Larger amounts make the end-points indeterminate, but are removed if the above method is followed. Aluminium, iron, calcium, barium, magnesium, potassium, lithium, lead, zinc, manganese, antimony, arsenic, titanium, zirconium, sulphur, and fluorine do not interfere. Phosphate causes high results unless the melt is dissolved in 6 *N* nitric acid and the phosphate precipitated by adding a slight excess of silver nitrate at *p*H 5·5. L. A. D.

Routine Analysis of Sodium-potassium Alloys. S. L. Walters and R. R. Miller (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 468–469)—The method is rapid and accurate for alloys containing little or no impurities; its main difficulty lies in obtaining a clean, oxide-free sample of known weight.



Apparatus and procedure—To obtain a weighed, oxide-free sample with the apparatus shown, draw about 30 g. of the alloy from its container into the sample bulb by applying suction at the stop-cock, attach the sample bulb to the sampling device, which contains a thin-glass ampoule of known weight, and is supported to allow rotation; withdraw the air through A and refill through B with

re-purified nitrogen. Warm the sample bulb and sampler to a temperature above the melting-point of the alloy, and tilt the apparatus so that, on applying suction at A, a small portion of the alloy is drawn into the ampoule. Re-admit nitrogen to force the excess of alloy from the capillary back to the sample bulb. Return the apparatus to the horizontal position, withdraw the ampoule and seal it in a flame; weigh after cooling. With a glass rod, break the ampoule under neohexane that has been washed first with sulphuric acid and then with alkali before distillation over sodium. Add ethyl alcohol dropwise to decompose the sample, the vessel being submerged in a water-bath to minimise over-heating. When decomposition is complete, transfer the liquid to a 250-ml. Erlenmeyer flask, and titrate with N hydrochloric acid to the phenolphthalein end-point. Then evaporate off the hexane and continue the titration to the methyl orange end-point. By this means the danger of over-titrating is minimised, and the time of titration accordingly reduced.

Calculation—If the potassium content of a W-g. sample of the alloy is X g., and the titre to methyl orange is $M.E.$ mg.-equivalents, then

$$M.E./1000 = X/39{\cdot}1 + (W-X)/23,$$

whence $$X = 2{\cdot}43W - 0{\cdot}0559M.E.$$

The method gives an accuracy of 0·1 per cent. of potassium, and less than an hour is required for a titration. Errors are incurred by the use of impure hexane, by the presence of an oxide film on the sample, and by loss of sample during the transference.

Solid alloys—The freezing point may be conveniently used for identifying the composition of alloys containing more than 92 per cent. of potassium. Using a magnesia cooling-bath and plotting freezing point against the alloy composition as determined by the above volumetric method, a straight line, represented by the equation $Y = 0{\cdot}259t + 83{\cdot}5$, where Y is the percentage by weight of potassium, and t is the Centigrade temperature, was obtained in the region 92 to 100 per cent. of potassium. The freezing point of potassium of purity greater than 99·0 per cent. has been determined as 63·7° C. M. E. D.

Determination of Rates of De-oxidation of Iron Oxide Materials in Reducing Gases. E. P. Barrett and C. E. Wood (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 285–286)—The rate of reduction of iron oxide is of importance in work such as a study of the relation between the porosity of an iron ore, its relative reducibility, and the size to which it should be crushed for efficient use in a blast furnace (T. L. Joseph, *A.I.M.M.E., Tech. Publ.*, 688, 1936). It is usual to determine the rate by heating a sample in a tube furnace in a stream of hydrogen, absorbing the water produced, and weighing it at regular intervals (T. L. Joseph, E. P. Barrett, and C. E. Wood, *Blast Furnace Steel Plant*, 1933, **21**, 147–150, 207–210, 260–263, 321–323, and 336). The new method is claimed to have several advantages. The sample is heated in a current of hydrogen or other reducing gas while it is suspended from a balance, so that the loss in weight may be determined at any time during the process.

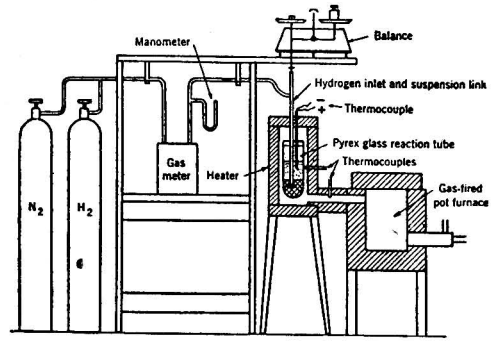


Fig. 1.

A hydrogen inlet tube of heat-resisting alloy, with a distributor on its lower end, extends to the bottom of the sample tube, which resembles a test-tube, 1·7″ × 12″, of Pyrex, or heat-resisting alloy.

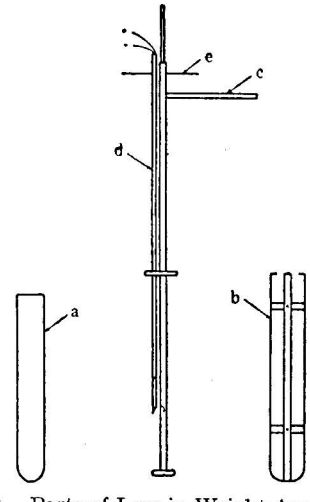


Fig. 2. Parts of Loss-in-Weight Apparatus.

a. Reaction tube
b. Stainless Steel Basket
c. Hydrogen inlet and connection to balance
d. Thermocouple
e. Heat deflector

A 2″-layer of −10 to +14 mesh mullite grog in the bottom of the tube serves to heat and further to distribute the hydrogen. The sample tube is supported by a stainless steel basket fastened to the inlet tube. The assembly is suspended from one side of a balance by a rod and is enclosed by a

furnace. A water-cooled plate is fitted between the furnace and the balance. Gas is fed to the inlet tube through a gas meter and a manometer is also fitted.

Preparation of the sample—Sieve through a 0·525″ mesh, crush oversize material to pass the sieve, and add it to the undersize product. Wet-screen on 20-mesh, dry the oversize at 105° C. and separate into four sizes:—0·525″ to +3 mesh; −3 to +6 mesh; −6 to +10 mesh; and −10 to + 20mesh. Make a 227-g. (0·5 pound) composite sample of the four portions.

Procedure—Suspend the charge in the furnace and pass nitrogen through the inlet tube. When the desired temperature is reached stop the nitrogen flow and pass in hydrogen, *e.g.*, at a rate of 0·25 cu.ft. per min. Record the loss in weight at 5-min. intervals until constant weight is obtained. The loss of weight during the passage of nitrogen represents the loss on ignition at the temperature of the test. Determine the iron and manganese contents of a duplicate composite sample to check the available oxygen. L. A. D.

Colorimetric Determination of Antimony. A. I. Kokorin (*Zavod. Lab.*, 1946, **12**, 64–68)—Tervalent antimony in dilute sulphuric acid gives with phosphomolybdic acid a yellow-green colour that turns blue (*cf.* Feigl and Neuber, *Z. anal. Chem.*, 1923, **62**, 376) when sufficient sulphuric acid is added to decompose the excess of reagent. Application of the reaction to the determination of small amounts of antimony in, *e.g.*, copper and tin, is now described.

With 0·15 mg. of antimony and 2 ml. of a 5 per cent. aqueous solution of phosphomolybdic acid in a final volume of 30 ml., which includes 8 ml. of 7 *N* sulphuric acid used for destroying the excess of reagent, the acidity during the 10- to 15-minute reduction (over boiling water) must not exceed 0·5 *N*. An antimony-free solution becomes colourless when the final acidity exceeds 1·5 *N*, but this acidity must not exceed 2 *N* in actual determinations or the colour intensity of the blue reduction product will be lowered. Excess of phosphomolybdic acid has no effect. With an acidity of 0·4 *N* during the reduction, the colour intensity of the final solution attains its full value when the time of heating on the water-bath has been not less than 10 min. Quantities of stannic tin up to twice the amount of antimony present, and of cupric copper up to 1 mg. in 50 ml., have no effect. Iron in any form interferes. Bismuth up to 50 mg. in 50 ml. has no effect. Large quantities of bismuth tend to counteract the adverse effect of higher acidities on the reduction, which may then be carried out in *N* acid without loss of colour intensity in the final solution.

Procedure—Prepare standard solutions for use with a photo-colorimeter as follows:—Dissolve 0·1 g. of A.R. antimony metal in 50 ml. of concentrated sulphuric acid by heating, pour into 500 ml. of water in a 1-litre graduated flask, add 50 ml. of sulphuric acid, cool, and make up to the mark. By means of a micro-burette, run various volumes (0·5 ml. and greater) into conical flasks. To each add sufficient 7 *N* sulphuric acid or alkali to make the acidity 0·4 *N* on dilution with water to 30 ml. After dilution, add 2 to 3 ml. of a saturated aqueous solution of sulphur dioxide, boil to remove sulphur dioxide, dilute again to 30 ml., add 2 ml. of freshly prepared, 5 per cent. phosphomolybdic acid solution, and heat for 10 min. over boiling water. Cool to room temperature, add 8 ml. of 7 *N* sulphuric acid, shake periodically for 5 min., transfer to a 50-ml. measuring flask, and make up to volume with water. Measure the colour intensity by means of a compensation photo-colorimeter, yellow filters being used. In solutions so obtained, 0·05 to 0·5 mg. of antimony in 50 ml. can be accurately determined.

To determine antimony in tin and copper, take 0·1 to 1 g. of tin or 2·5 g. of copper and deposit the antimony on a copper foil spiral (*cf.* Clarke, ANALYST, 1928, **53**, 373), place the washed foil in a small beaker, add 25 ml. of 0·5 *N* sodium hydroxide and 15 drops of perhydrol, and boil for 5 min. Transfer the solution to a conical flask, rinse the foil and the beaker two or three times with the minimum amount of water, add 1·5 ml. of concentrated sulphuric acid, and 3 ml. of saturated sulphur dioxide solution, and evaporate until fumes just appear. Add 15 ml. of water, 14 ml. of 2 *N* sodium hydroxide, then 2 ml. of phosphomolybdic acid, and proceed as above. Results are accurate to about 1 part in 10. G. S. S.

Amperometric Titration of Cyanide with Silver Nitrate, using the Rotating Platinum Electrode. H. A. Laitinen, W. P. Jennings, and T. D. Parks (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 574–575)—With a rotating platinum electrode, the end-point of the amperometric titration of cyanide by silver nitrate can be obtained with an accuracy of 0·1 to 0·2 per cent. for cyanide concentrations as low as 0·002 *N*, which is comparable in accuracy with the visual (Dénigès) end-point. The visual end-point becomes indistinct at 2×10^{-4} *N* cyanide, and fails at higher dilutions, whereas the amperometric end-point is still observed when 4×10^{-6} *N* cyanide is titrated with 5×10^{-4} *N* silver nitrate.

The normality of the silver nitrate solution should be, in general, approximately five times that of the cyanide solution, so as to avoid the necessity of correcting for the dilution effect, but should be not less than 5×10^{-4} *N*. Between titrations, silver must be removed from the platinum electrode either by anodic polarisation or by means of nitric acid. If nitric acid is used, the electrode must

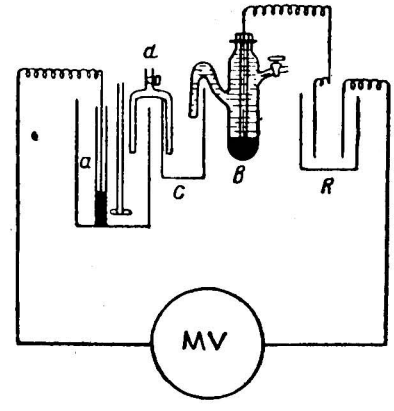
stand in aqueous ammonia solution for a few minutes and be rinsed with water before use.

Titration results are accurate to 0·5 per cent. in the presence of a fifty-fold excess of chloride or bromide, or in 1 *N* salt solutions. High alkali concentrations tend to give high results. Compounds that form very insoluble silver salts, *e.g.*, iodides or sulphides, or sulphydryl compounds, or materials that form, with silver, complexes more stable than that formed by cyanide will, in general, interfere with the method. J. G. W.

Potentiometric Methods of Determining Aluminium in Magnesium Alloys of Elektron Type. L. J. Poljak (*Zavod. Lab.*, 1946, **12**, 268–275)—Potentiometric methods of titrating aluminium with (*a*) sodium fluoride, using a platinum electrode in a ferrous - ferric solution, and (*b*) sodium hydroxide, using an antimony electrode, are studied.

With (*a*), aluminium forms a complex $[AlF_6]'''$ that is much more stable than the corresponding iron complex, so that as long as aluminium ions are present the sodium fluoride added should go to form Na_3AlF_6 and the oxidation-reduction potential determined by the ratio $Fe''' : Fe''$ should scarcely change until all the aluminium ions are removed and the iron complex starts to form. At this point, the potential should change suddenly. The method, as described by Treadwell and Bernasconi (*Helv. Chim. Acta*, 1925, **13**, 500) is, however, unsatisfactory, since the potential change at the end-point is poorly defined and the electrode process is so slow that the titration takes 40 to 50 min. A modification (the addition of a larger amount of ferric salt) due to Tarajan (*Ibid.*, 1939, **8**, 3) gives a sharper change and reduces the titration time to 10 to 15 min. The method has now been further improved and applied satisfactorily to the analysis of magnesium alloys containing 2 to 10 per cent. of aluminium. The addition of dilute acid at the stage in the titration where the potential begins to fail, *i.e.*, when about 1/3 to 1/4 of the aluminium has reacted, causes the potential of the indicator electrode to return to its earlier value and to remain constant until the equivalence point is reached, as shown by a sharp fall. Aluminium may be determined thus in presence of iron, titanium, silica, zinc, manganese, small quantities of nickel and cadmium, and a ten-fold excess of magnesium. Copper interferes, but it may be separated from aluminium by precipitation of the latter with aqueous ammonia, which serves also to remove the bulk of magnesium.

Procedure for (*a*)—Dissolve 1 g. of the alloy in 30 ml. of diluted hydrochloric acid (1 + 1), precipitate aluminium hydroxide with aqueous ammonia using methyl red as indicator, filter but do not wash, transfer the precipitate to the original beaker, wash the filter with 30 to 40 ml. of hot, diluted hydrochloric acid (1+1), and heat to dissolve the hydroxides. Cool, make up to 100 ml. in a measuring flask, and pipette 25 ml. into the titration beaker, *a*. This is fitted with an ebonite cover through which pass the indicator electrode (*cf.* Treadwell, *loc. cit.*), the burette jet, a glass stirrer, a glass tube for introducing carbon dioxide from a cylinder, and one arm of an inverted U-tube containing saturated potassium chloride solution, which serves to connect the solution in the beaker with a saturated calomel electrode. A millivoltmeter, MV (reading 18 millivolts, internal resistance 840 cm.) is connected on the one side to the indicator electrode, and on the other to the calomel electrode, *B*, through a variable



resistance, *R* (0·05 *N* copper sulphate in a beaker containing one fixed copper wire and one movable copper wire in a glass sheath, arranged so that the resistance may be regulated before the test by moving the latter in or out of the solution). Add to the solution under test 0·5 ml. of 0·05 *N* ferric chloride or sulphate, neutralise to tropaeolin OO with aqueous ammonia (the optimum *p*H for the reaction is 2 to 2·5), saturate the solution with sodium chloride, and add an equal volume of ethyl alcohol. Then pass carbon dioxide gas for 2 to 3 min., introduce 2 to 3 drops of cold, freshly prepared, saturated ferrous sulphate solution, and titrate with *N* sodium fluoride, standardised potentiometrically on aluminium chloride, without stopping the current of carbon dioxide during the whole of the titration. When the potential starts to fall, add a few drops of very dilute acid and continue the titration to the sharp end-point. To standardise the fluoride solution, take 10 ml. of aluminium chloride solution containing 3 g. of aluminium per litre, add 0·5 ml. of 0·05 *N* ferric sulphate, dilute with water to 25 to 30 ml., neutralise, and titrate as described above. Results are satisfactory, *e.g.*, with Elektron type alloys (chemical results in brackets), the values 7·47, 7·44, 7·53 (7·43) and 3·46, 3·46, 3·55 (3·50), were obtained.

With (*b*), in spite of adverse criticisms by previous workers (Treadwell *et al.*, *loc. cit.*; Stefanovski,

J. Applied Chem. Russ., 1932, **5**, 92, etc.), it is shown that the method may be made suitable for routine analysis of magnesium alloys. Experimental errors do not exceed 3 per cent., the method is economical in materials, and it takes only 20 min. in all.

Procedure for (*b*)—Place a suitable weight of the sample (3 g. for 2 per cent., 1·5 g. for 4 per cent., 1 g. for 6 to 8 per cent., or 0·5 g. for 10 to 12 per cent. of aluminium) in a 250-ml. conical flask, cover with 25 ml. of water, and add in small portions, avoiding unnecessary excess, 8 to 10 ml. of concentrated hydrochloric acid. After dissolution of the alloy, add 3·5 g. of ammonium chloride per g. of alloy taken, dilute somewhat with water, and neutralise to bromophenol blue (2 to 3 drops of 0·2 per cent. aqueous solution). (Potentiometric titration of the acid is not satisfactory since the potential change at neutrality is indefinite). Transfer the solution to the titration beaker (the apparatus set-up is the same as for the fluoride titration, except that an antimony electrode, in the form of a cast rod, polished with fine emery paper and then with filter paper, the polishing being repeated after every 3 to 4 tests, takes the place of the platinum), and titrate with 0·5 to 0·7 *N* sodium hydroxide, carbonate-free, that has been standardised potentiometrically against oxalic acid or aluminium chloride, adding the alkali in 0·2-ml. portions close to the equivalence point, which is indicated by a sudden potential rise. Copper interferes, but in Elektron type alloys it can normally be ignored. If an insoluble residue appears after the acid attack on the alloy, filter before adding ammonium chloride to avoid dissolving more than traces of copper. G. S. S.

Agricultural

Chromatographic Determination of Carotene J. B. Wilkes (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 702–707)—The chromatographic technique has been extensively used for the quantitative separation of carotene from other plant pigments; powdered sugar, soda-ash, and mixtures of magnesium oxide with soda ash or kieselguhr have been proposed as adsorbents for this purpose. Heat-treated kieselguhr, especially Johns–Manville Hyflo Super-Cel, has been found to be an excellent adsorbent and its use is now recommended. Any of the well-known methods of extracting plant pigments with hydrocarbon solvents may be used, but polar solvents must be removed before chromatography. The method is suitable for the preparation of carotene from kryptoxanthin, xanthophyll esters, and acid-decomposition products of xanthophyll. It does not enable carotene to be separated from lycopene.

Procedure—Extraction of carotene—Weigh into a 125-ml. conical flask 1 to 2 g. of finely ground (40-mesh) alfalfa meal, and cover with a mixture of 20 ml. of redistilled acetone and 40 ml. of light petroleum (b.p. 30° to 60° C.) Stopper tightly and leave the flasks in the dark for 16 to 70 hours. Decant the liquid into a 300-ml. beaker, rinse out the flask with two 50-ml. portions of light petroleum, and evaporate to 10 to 15 ml. on a water-bath. Add a further 50 ml. of light petroleum, again evaporate to 10 to 15 ml., and add a further 40 ml. of light petroleum. This procedure removes all traces of acetone and the solution is ready for chromatography. For routine control work, a simplified method may be used. Weigh a 1-g. sample of the finely-ground meal into a 125-ml. conical flask. Add 100 ml. of light petroleum (b.p. 30° to 60° C.), stopper tightly, and leave in the dark for at least 16 hours.

Chromatographic Separation of Carotene—Fill a glass column, 3·3 cm. in diameter, to a height of 15 to 25 cm. with Hyflo Super-Cel. The most rapid method is to insert a plug of cotton wool in the bottom of the tube and then force the tube into a container of the adsorbent. When sufficient adsorbent has been introduced, press it firmly down with a plunger. For more exacting separations, add the adsorbent in small quantities at a time and press the material firmly with a plunger after each addition. Apply suction to the column and pour the extract, without filtering it, on to the top of the column. Wash the carotene through the column with light petroleum until the filtrate is colourless. Make up the solution to 200 ml. and estimate the carotene colorimetrically. When the material contains appreciable quantities of fat, a longer column is required.

Estimation of vitamin A—Vitamin A ester, like carotene, is only feebly adsorbed on Hyflo Super-Cel whereas free vitamin A is more strongly adsorbed. The two forms of vitamin A give separate bands that fluoresce when exposed to ultra-violet light. The method appears to have a possible application in the estimation of vitamin A.

Extraction with a mixture of light petroleum and purified acetone gave higher results than extraction with light petroleum alone, but with impure acetone low results were obtained, owing to loss by oxidation; it is suggested that addition of dimethylaniline or hydroquinone would prevent this. If a trace of acetone is left in the petroleum extract, a grey band, having an orange-red fluorescence in ultra-violet light, will pass into the carotene band; this grey band is normally retained at the top of the column. A 4 per cent. solution of acetone in light petroleum may be used to separate the non-carotenoid pigments from the chromatogram, if desired. W. S. W.

Gas Analysis

Determination of Oxides of Sulphur in Flue Gas. E. W. F. Gillham (*J. Soc. Chem. Ind.*, 1946, **65**, 370–372)—Existing methods for the determination of sulphur dioxide and sulphur trioxide are

discussed. A common disadvantage is the use of alkaline absorbents in which sufficient oxidation of sulphite occurs to cause large errors in the trioxide result, even when inhibitors are used. The method described uses distilled water as absorbent and gives more accurate results.

Apparatus—This consists of two bubblers connected in series, a gas volume meter, and any suitable pump. The first bubbler is equipped with a sintered-glass dispersion disc of No. 1 porosity and contains 50 ml. of distilled water. This absorbs all the sulphur trioxide up to about 0·2 grain per cu.ft., some dioxide, and other acid constituents such as oxides of nitrogen. The second bubbler is a plain one and is charged with 10 ml. of neutral, sulphate-free, 20-volume hydrogen peroxide, and 40 ml. of water. The water-sealed gas meter is placed after the absorption train. Flue gas is drawn through the apparatus at a rate of 0·5 to 1 cu.ft. per hour (15 to 30 litres per hour), preferably through a sampling tube of not less than 5 mm. bore to avoid carrying grit into the absorbers.

Reagents—Screened methyl red indicator—Dissolve 0·4 g. of methyl red in 15·2 ml. of 0·1 *N* sodium hydroxide: dissolve 0·2 g. of methylene blue in water. Mix and dilute to 1 litre. *Phenolphthalein - thymol blue indicator*—Dissolve 0·2 g. of thymol blue in 250 ml. of water and 250 ml. of alcohol: dissolve 1·25 g. of phenolphthalein in 250 ml. of alcohol. Mix and dilute to 1 litre with water. *Standard potassium palmitate solution*—Dissolve 25·63 g. of palmitic acid by warming with 250 g. of glycerol and 400 ml. of alcohol. Add 5 ml. of phenolphthalein solution, and alcoholic potassium hydroxide solution (34 g. per litre) until the solution is just coloured pink. Cool, dilute to 1 litre with 90 per cent. alcohol, and standardise against 0·1 *N* barium chloride.

Procedure—Rinse the contents of the bubblers into flasks. Titrate the solution from the first bubbler with 0·1 *N* sodium hydroxide (*a* ml.), using screened methyl red indicator. Add a few drops of hydrogen peroxide and titrate again (*b* ml., representing the sulphur dioxide in the first bubbler). Titrate the contents of the second bubbler (*c* ml.). Add a few drops of nitric acid to each solution, boil, and add a measured volume of 0·1 *N* barium chloride sufficient to precipitate all the sulphate and leave an excess of about 10 ml. Boil for 10 min., cool rapidly, and determine the excess of barium chloride by titrating with the palmitate solution as follows. Add 1 ml. of phenolphthalein - thymol blue indicator and then add *N* sodium hydroxide until the colour changes to green. Add 0·1 *N* sodium hydroxide until the colour just becomes violet and discharge this colour by adding a drop of 0·1 *N* nitric acid. Titrate with the palmitate reagent until the violet colour again appears. If the palmitate equivalent of the sulphate in the first bubbler is *d* ml. of 0·1 *N* solution and of the second bubbler *e* ml. then:

Sulphur trioxide absorbed $\equiv d-2b$ ml. of 0·1 *N* solution
Sulphur dioxide " $\equiv e+2b$ " "
Other acids " $\equiv a+b-d+c-e$ "

L. A. D.

Automatic Determination of Sulphur Trioxide in Flue Gas. E. W. F. Gillham (*J. Soc. Chem. Ind.*, 1946, **65**, 405–409)—The flue gas is scrubbed by being passed through a wetted sintered-glass disc. All the sulphur trioxide and some sulphur dioxide are absorbed. The solution of sulphur oxides is then scrubbed with flue gas that has been freed from acid gases and the sulphur dioxide is thereby removed. The electrical conductivity of the solution is then determined and used as a measure of the sulphur trioxide content.

Apparatus is described in which these operations are carried out continuously so that a record of sulphur trioxide concentration is obtained. Devices are incorporated to ensure constant conditions in the apparatus under all conditions of plant load and flue-gas pressure. The original paper should be consulted for full details and diagrams.

L. A. D.

Water Analysis

New Simple Titration Methods for Determination of Hardness of Water. G. Schwarzenbach, W. Biedermann, and F. Bangerter (*Helv. Chim. Acta*, 1946, **29**, 811–818)—In earlier communications (*Idem.*, 1945, **28**, 181, 377, **828**, 1133; 1946, **29**, 364), it was stated that certain derivatives of imino-diacetic acid are suitable for determinations of hardness of water. Four methods for the application of these derivatives are now described.

Method A—The derivative used in this method is disodium ethylenediamine tetra-acetate, and the standard solution (0·1 *M*) is made by dissolving 37·21 g. of the salt ($Na_2C_{10}H_{14}O_8N_2.2H_2O$) in a litre of water. Each millilitre of this solution is equivalent to 1 mg.-ion of calcium. A saturated solution of murexide (ammonium purpurate) serves as an excellent indicator for the calcium ion in alkaline solution. Treat 200 ml. of the water with 10 ml. of *N* sodium hydroxide and with 0·3 ml. of the indicator and titrate immediately with the standard disodium ethylenediamine tetra-acetate solution. It is inadvisable to allow the alkaline solution to stand long before titration since calcium carbonate may then be precipitated. The end-point occurs within three drops and the best results are obtained by taking the mid-point of the colour change. Murexide, although affected by zinc, cadmium, mercuric, and cupric ions, as well as by the calcium

ion, is not affected by the magnesium ion, and calcium can therefore be determined in presence of magnesium.

Method B—In this method the same standard solution of the complex salt as in Method A is used. The indicator is a mixture of 2 parts of methyl red and 3 parts of bromocresol green dissolved to a concentration of 0·2 per cent. in alcohol. To 200 ml. of the water add 15 drops of the indicator and with very dilute hydrochloric acid adjust the colour to the turning point of the indicator, which is at *p*H 5. As a comparison solution use 190 ml. of distilled water with 10 ml. of the standard complex salt solution and 15 drops of indicator. To the sample solution now add 10 ml. of the standard complex salt solution and titrate the mixture, now become acid to the indicator, back to *p*H 5 with 0·1 *N* sodium hydroxide which has been standardised against the derivative, each g.-molecule of the complex salt being equivalent to 2 g.-molecules of sodium hydroxide. This method has the advantage that the temporary hardness and permanent hardness may be determined successively. The temporary hardness is found by titrating the water sample to *p*H 5. The carbon dioxide is then expelled, the standard complex salt solution is added, and the sample is titrated back to *p*H 5 to obtain the remaining hardness. The method is subject, however, to a slight error due partly to the somewhat indistinct end-point and partly to the stoicheiometric end-point's being at *p*H 5·3 and not at *p*H 5. With practice, however, good results can be obtained. The method cannot be used in presence of magnesium.

Method C—In this method the trisodium salt of ethylenediamine tetra-acetic acid is used. To prepare the standard solution dissolve 37·21 g. of disodium ethylenediamine tetra-acetate in water, add 0·1 molecular equivalent of sodium hydroxide and dilute to 1 litre. The end-point is at *p*H 8·5 and the indicator used is 0·1 per cent. alcoholic phenolphthalein. To prepare the comparison solution add 10 ml. of the standard complex salt solution to 200 ml. of boiled and cooled distilled water, add 10 drops of indicator, and titrate to a faint permanent pink colour with 0·1 *N* sodium hydroxide. Record the number of millilitres required. Slightly acidify 200 ml. of the water to be tested, remove carbon dioxide by boiling, cool, add 10 drops of indicator and titrate to the pink colour of the comparison solution. Now add 10 ml. of the complex salt solution and again titrate to the colour of the comparison solution. From the titre of the sample deduct the titre of the comparison solution. Each g.-equivalent of base corresponds to 1 g.-ion of calcium or magnesium. By this method the sum of the calcium and magnesium contents is titrated; the procedure is thus useful for the determination of total hardness. Removal of carbon dioxide sharpens the end-point, but is not absolutely necessary.

Method D—The complex salt used in this method is the neutral sodium salt of uramil diacetic acid. To prepare the standard solution dissolve 27·71 g. of pure uramil diacetic acid in water, neutralise with sodium hydroxide solution to methyl red indicator, and dilute to 1 litre. To prepare the comparison colour solution at *p*H 5·5 add to 200 ml. of distilled water 14 ml. of *N* sodium acetate solution, 2 ml. of *N* acetic acid and 10 drops of the indicator, which is the methyl red and bromocresol green mixture used in Method B. Treat 200 ml. of the water to be tested with 10 drops of indicator and with hydrochloric acid or sodium hydroxide until the colour is that of the comparison solution. Add 10 ml. of the sodium uramil diacetate solution and titrate with 0·1 *N* sodium hydroxide solution to the comparison colour. When magnesium is present this method gives the sum of the calcium and magnesium contents and is thus useful for the determination of total hardness. It has the advantage over Method C that, since the determination is in an acid medium (*p*H 5·5), carbon dioxide has no effect.

Methods A and C are to be recommended since they give the lime hardness and the total hardness, respectively. Method B is somewhat less accurate and is suitable only when magnesium is absent. Method D gives good results, but uramil diacetic acid is not available commercially. The complex substances mentioned are obtainable from the firm of Siegfried in Zofingen.

A. O. J.

Physical Methods, Apparatus, etc.

Most Economical Sampling for Chemical Analysis. C. W. Churchman (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 267–268)—The method of sequential analysis developed by A. Wald (*Ann. Math. Stat.* XVI, 1945, 118, and *J. Amer. Stat. Assoc.*, 1945, **40**, 277) is applied to the problem of discriminating with as few analyses as possible between alternative formulae for organic compounds. In the simplest case only one element, α, is being estimated and, on the hypotheses that the compound has the formula 1 or 2, the percentage of this element will be α_1 or α_2. It is assumed that σ_α^2, the error variance of a single determination, is already known. $\sum^n \alpha$ denotes the sum of the first n determinations of α. Calculate $h = \dfrac{4{\cdot}595}{\alpha_2 - \alpha_1}\sigma_\alpha^2$ and $s = (\alpha_1+\alpha_2)/2$, where $\alpha_2 > \alpha_1$. The hypothesis that the true percentage α is α_1 can be accepted as soon as $\sum^n \alpha \leq sn - h$, and the alternative hypothesis that the true percentage is α_2 can be accepted as soon as $\sum^n \alpha \geq sn + h$. For $\sum^n \alpha$ lying

between $sn - h$ and $sn + h$, it is necessary to take further observations. The constant 4·595 used above refers to the 0·01 probability; this implies that the probability of accepting possibility X when Y is actually true is 0·01, *i.e.*, we go wrong in this way one time in 100. Similarly, the chance of accepting possibility Y when possibility X is actually true is 0·01: for the 0·001 and 0·05 levels of probability, the corresponding constants are 6·907 and 2·944.

The more complicated case of two elements α and β being determined is also dealt with. If n and m are the numbers of determinations of α and β, respectively, the critical function is

$$\left[\frac{\alpha_2-\alpha_1}{\sigma_\alpha^2}\right]\overset{n}{\Sigma}\alpha + \left[\frac{\beta_2-\beta_1}{\sigma_\beta^2}\right]\overset{m}{\Sigma}\beta - n\left[\frac{\alpha_2^2-\alpha_1^2}{2\sigma_\alpha^2}\right] - m\left[\frac{\beta_2^2-\beta_1^2}{2\sigma_\beta^2}\right]$$

and should this be less than or equal to $-4{\cdot}595$ we accept the hypothesis that $\alpha = \alpha_1$, and $\beta = \beta_1$; should it be greater than $+4{\cdot}595$ we accept $\alpha = \alpha_2$, and $\beta = \beta_2$; for intermediate values more observations are necessary. The procedure depends on our previous knowledge of the error variances, and is then the most efficient available.

An example of the application of this method in deciding between the two formulae $C_{19}H_{12}O_6Br_4S$ and $C_{19}H_{10}O_5Br_4S$ from experimental determinations of the sulphur and bromine contents of a compound is given. K. A. B.

Improvement of Precision by Repeated Measurements. Application to Analytical Control Methods. J. Mandel (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 280–284)—The practice of duplicating analyses is well established among analysts, but it is well known that frequently the apparent closeness of the duplicate gives a grossly misleading idea of the accuracy of the analysis. Moran (*Ibid.*, 1943, **15**, 361–364) ascribes this to the fact that duplicate analyses made at the same time are not truly random, but are rather influenced by slight variation in technique, or surrounding conditions.

The methods of the analysis of variance are applied to estimating a quantity "the coefficient of improvement by N-fold replication" (*i.e.*, by carrying out the analysis N times on a particular occasion) defined by $(CI)_N$ = (variance of a single determination)/(variance of the average of N parallel determinations).

The larger the variability between series, the lower will be $(CI)_N$: when there is no improvement by replication on a given occasion, it will have the limiting value 1; and when there is complete independence of the replication on a given occasion, it will be equal to N. It is shown how to lay down confidence limits for the estimated value for $(CI)_N$.

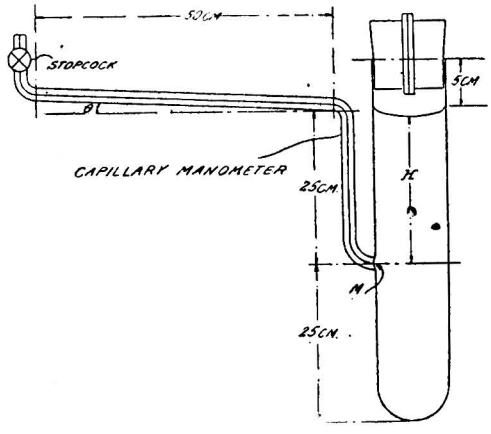
These methods are applied to discussing the effect on the over-all error of varying M and N where we have M series (at different times) of N analyses (at the same time). The case is also discussed where a blank determination on a standard is done at the same time.

Data obtained in routine determinations over a period of one year for the specific gravity of carbon tetrachloride, and the data obtained by Williams and Haines (*Ibid.*, 1944, **16**, 157) in their new method of determining sodium in potassium hydroxide are used to illustrate the application of the methods indicated above. K. A. B.

Measuring the Distribution of Particle Size in Dispersed Systems. W. M. Dotts (*Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 326–328)—The method is based on the micro-manometric procedure, whereby the change in concentration of the dispersed internal phase during sedimentation is traced by the change in pressure at a fixed point below the surface of the suspension. The simplified procedure described is suitable for all systems from concentrated emulsions to dilute suspensions. Systems of mixed pigments where photographic or colorimetric methods give dubious results are readily tested by this method, which is also suggested for measurements upon emulsions of all concentrations at various ages to determine the effects of ageing upon the distribution of globule size. In order to avoid the effects of hindered settling, concentrated suspensions or emulsions should be diluted before measurement to a concentration of about 5 per cent. by volume of internal phase. According to the author, most emulsions having a concentration of internal phase not greater than the close-packing ratio for equal spheres (74·048 per cent.) do not break on dilution with excess of the pure external phase, and there is little evidence that dilution affects the size of the dispersed globules. The testing of a series of similar emulsions under approximately the same conditions will always tend to increase the significance of the comparative results.

Constructional details—The apparatus is shown in the figure. The capillary should have a uniform inside diameter of about 0·75 mm. and an outside diameter of 8 mm. for rigidity. Tubing of smaller bore should not be used owing to the effects of capillarity. The length of the manometer gauge should be 50 cm., and sin θ, where θ is the angle with the horizontal, should lie between 0·01 and 0·05 for most emulsions, while a value of about 0·1 is suitable for most dispersions. If any difficulty arises in obtaining the desired value of sin θ, a trial run can be made during which the clamps may be adjusted to bring the manometer as close as possible to the horizontal as necessary to obtain the desired accuracy. The position of the meniscus

may conveniently be read by means of a millimetre scale fixed to the manometer tube. Since this position may be measured to the nearest 0·1 mm. with simple equipment, a precision of 1 per cent. is obtained on all but 2·5 per cent. or less of the dispersed phase. The diameter of the sedimentation vessel should not exceed 16 mm. and it should be long enough to contain 50 ml. below and above M, the point of junction of the manometer. The height H of the liquid surface above the point M should also be known to the nearest millimetre.



Procedure—Set up the apparatus in a room or bath where a fixed temperature can be maintained to ± 0·5° C., and fill the manometer tube with dispersion medium, leaving the main tube empty. Pour the suspension under test, suitably diluted, into the main tube to a pre-determined mark such that the meniscus in the manometer tube is supported near the beginning of the scale close to the stop-cock. Start the timer immediately, and simultaneously open the stop-cock. Take the first few readings at close intervals so that the position of the meniscus at zero time may be obtained by a short extrapolation. The rate of recession of the meniscus due to evaporation *via* the stop-cock and the main tube should be determined for each liquid used. Volatile liquids will give a recession of only a fraction of a millimetre per hour. Continue to read the position of the meniscus at suitable intervals until the rate of recession approaches this value.

Computation of results—If Y represents the total recession of the meniscus in the manometer during a run, y the recession after time t, and P the percentage of the total mass of internal phase settling or rising past the point M during the same time, it can be shown that $P = 100y/Y$. The actual material represented by any value of P is a heterogeneous system consisting of (*a*) particles in size groups the whole of which have passed M during the time t, and (*b*) smaller particles belonging to all the other size groups present. The rate of sedimentation (up or down) of all these groups is dP/dt, and $t.dP/dt$ gives the percentage of dispersed phase in these latter size ranges. Therefore, if S represents the percentage of internal phase in class (*a*) above,

$$P = S + t.dP/dt.$$

If P is plotted against t, the ordinate intercept on the P axis of any tangent to the resulting curve will be a value of S (Fischer and Odén, *Proc. Roy. Soc. Edinburgh*, 1924, **44**, 98), and the difference between the ordinate intercepts of any two tangents to this P, t curve gives the percentage ΔS of the total dispersed phase corresponding to the size range $r \ldots\ldots r_n$ calculated from Stokes's Law for the times in question. The distribution of particle size can now be tabulated for all the material in the dispersed phase.

The following form of Stokes's law should be used for this calculation:

$$r^2 = \frac{9\eta H}{2(d_i - d_x)gt}$$

where r = radius of particles moving with constant velocity; η = viscosity of dispersion medium; H = sedimentation depth; d_i = density of internal phase; d_x = density of external phase; g = acceleration due to gravity; t = time of sedimentation.

Experimental curves are given for three runs on identical suspensions of 1·80 per cent. of Fe_3O_4 in diethylene dioxide. The ordinate deviations of the points from the curve are 1 per cent. or less in all but the initial values. This appears to be the limiting accuracy of the method owing to the influence of temperature variations, inertia, evaporation, and contamination of the walls of the manometer.

B. A. S.

Reviews

INDEX TO THE LITERATURE ON SPECTROCHEMICAL ANALYSIS, PART II, 1940 TO 1945. By BOURDON F. SCRIBNER and WILLIAM F. MEGGERS. Pp. 180. Philadelphia: American Society for Testing Materials. 1947. Price $3.00.

In 1940 the American Society for Testing Materials issued an Index to the Literature on Spectrochemical Analysis covering the years 1920 to 1939, in which references were given to a wide range of subjects such as light sources, photographic materials, progress in quantitative analysis and so forth.

The present volume, Part II, is a continuation of this work and in one important respect is of considerably more value in that each reference is now followed by a useful abstract of

the original paper. This expansion is particularly welcome since many of the journals in which the originals appeared are difficult of access owing to the War.

A detailed subject index is included and although no author index is provided, this omission is to some extent off-set by the arrangement of the material in alphabetical order of the authors, for each year under review. The compilers and their staff at the National Bureau of Standards are to be congratulated on the production of this useful work of reference.

B.S. COOPER

SUPPLIES OF LABORATORY CHEMICALS

Letters received from members of the Society setting out difficulties they have encountered in obtaining supplies of laboratory chemicals have been forwarded to the Board of Trade.

A reply has been received to the effect that the information supplied has proved of great assistance. It has enabled the Board to take the matter up with chemical suppliers with a view to ameliorating the position. It is understood that in the first place the Board will obtain a fairly detailed statement of the suppliers' difficulties and that subsequent action will depend on what the Board thus learns.

K A. WILLIAMS, *Hon. Secretary.*

REPRINTS OF PROFESSOR HEYROVKÝ'S LECTURE

Reprints of Professor Heyrovský's Lecture on The Fundamental Laws of Polarography, from the June (1947) ANALYST, will shortly be obtainable. Orders should be sent, with remittances, to the Editor, THE ANALYST, 7–8 Idol Lane, London, E.C.3. Price, to members of the Society 1s. 6d.; to non-members 2s.

MICROCHEMISTRY AND PHYSICAL METHODS GROUPS

A Joint Meeting of these two Groups will be held in the University Chemical Laboratories at Cambridge, on Friday, September 26th, 1947. After visits to the Colloid Science Department and Radiochemistry Laboratory a meeting will be held at 4 p.m. in The Chemistry Lecture Theatre, Pembroke Street, when the following short papers on Micro-physical Methods of Analysis will be read and discussed: "Micro-methods for Molecular Weight Determination," by Cecil L. Wilson, M.Sc., Ph.D., F.R.I.C.; "Turbidimetric Methods used in Agricultural Analysis," by J. Tinsley, B.Sc., Ph.D., F.R.I.C.; "Microchemical Applications of Potentiometric Methods," by J. T. Stock, M.Sc., F.R.I.C.; "Micro-analysis, using X-ray Diffraction Technique," by H. P. Rooksby, B.Sc., F.Inst.P.

Further particulars may be obtained from Dr. J. E. Page, Glaxo Laboratories, Greenford, Middlesex.

PHYSICAL METHODS GROUP

The Third Annual General Meeting of the Physical Methods Group will be held in the rooms of the Chemical Society, Burlington House, London, W.1, at 6 p.m., on Tuesday, November 25th, 1947, and will be followed by a lecture on "Electron Microscopy," by Mr. B. S. Cooper.