THE ANALYST

Some Analytical Problems Involved in Determining the Structure of Proteins and Peptides

A Review*

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Summary of contents

Investigators have available today a variety of refined techniques that can be used for elucidating protein structure. These methods originate from the pioneer work on insulin, carried out by Sanger and his colleagues at Cambridge University. It is now accepted that the qualitative techniques so elegantly employed by Sanger, while adequate for a protein of the size of insulin, are not easily applicable to larger molecules. Emphasis has therefore shifted to the quantitative methods more recently introduced by Hirs, Moore and Stein at the Rockefeller Institute.

It is our intention not to present a complete account of all current methods, but rather to emphasise some of the problems that have been encountered in the quantitative approach. Many of the techniques were developed during studies on the amino-acid sequence of ribonuclease, of which the complete sequence[1,2,3,4] is presented in Fig. 1. on p. 90.

Amino-acid analysis

Quantitative analyses of the amino-acid compositions of proteins and peptides occupy a central rôle in the determination of their structure. Difficulties are present at two stages, that of performing the hydrolysis under optimum conditions and that of detecting and determining amino-acids in the hydrolysate.

For the most accurate results, proteins and peptides should be de-salted by dialysis, gel-filtration, or ion-exchange chromatography before hydrolysis. Losses of serine and threonine, owing to their conversion to esters of inorganic acids, and losses of cystine and tyrosine may be excessive when hydrolysis is performed in the presence of inorganic salts or carbohydrates. Further, considerable distortion of peaks and displacement from normal elution positions may occur when ion-exchange chromatography of amino-acid mixtures containing large amounts of salt is used.

Glass tubes to be used for hydrolysis should be rigorously cleaned with chromic acid solution, distilled water, and N hydrochloric acid. The sample should be hydrolysed in 6 N hydrochloric acid (analytical-reagent grade), and air must be excluded as far as possible. For this purpose, the hydrolysis tube is immersed in a solid carbon dioxide - ethanol mixture and the pressure is reduced to 60 microns; the tube is then removed from the coolant and the acid cautiously thawed to permit slow and thorough de-gassing. The tube is sealed under the reduced pressure.[5,6] This de-aeration procedure has proved essential for accurately determining carboxymethylcysteine[6] and *S*-succinylcysteine,[7] and advantageous for determining serine, threonine, tyrosine and cystine. Hydrolysis is performed for 22 hours at

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110° C, preferably in an oven with a forced air-draught to ensure a uniform temperature. For consistent results with the labile amino-acids, careful control of temperature is important. The hydrochloric acid is then removed under reduced pressure on a rotary evaporator. This rapid procedure has proved more satisfactory than the slow removal of acid in a vacuum desiccator containing sodium hydroxide pellets, which results in slight losses of serine and threonine and the appearance of new ninhydrin-positive derivatives.[6] Even with the improved procedures, some unavoidable losses of serine and threonine take place, and it is necessary to obtain a series of results at different hydrolysis times to permit determination of these amino-acids by extrapolation.[8] Alternatively, empirical correction factors may be used; for serine, the recovery at 22 hours may be divided by 0·90, and for threonine the recovery may be divided by 0·95.[9]

Leucine, isoleucine and valine are released at a particularly slow rate from peptides in which these amino-acids are consecutive. Periods up to 72 hours may be necessary to ensure total hydrolysis. The same extended hydrolysis is necessary for the formation of *S*-succinylcysteine from proteins modified by *N*-ethylmaleimide.[7] On the other hand, tryptophan is destroyed by acid hydrolysis, but can be determined by alkaline hydrolysis[9] with subsequent chromatography on a starch column[10] or on Amberlite IR-120 (G. R. Stark in a personal communication). Alternatively, spectrophotometric methods may be used on the intact protein.[11] Cystine is decomposed to a variable extent during acid hydrolysis and is best determined as cysteic acid after vigorous oxidation of the protein with performic acid[12]; in this procedure, methionine is converted to the sulphone, and some destruction of tyrosine and histidine occurs. Methionine may be present as the sulphoxide before hydrolysis. During acid hydrolysis, the sulphoxide largely reverts to the parent thio-ether,[13,14] and therefore the determination of the relative amounts of methionine and methionine sulphoxide in a protein or peptide requires a special method.[13,15] Tyrosine can give low recoveries on hydrolysis, because of the ease with which it undergoes conversion to chlorotyrosines and some unidentified products,[16] but these losses are particularly significant only when unusually small amounts of protein are hydrolysed. Glutamic acid, under the usual conditions of acid hydrolysis, forms an equilibrium pair with pyrrolidone carboxylic acid[17] The position of this equilibrium is approximately 98 to 2 in favour of glutamic acid, and so a small correction may be applied during careful quantitative determination of this amino-acid.

The determination of glutamine and asparagine in a protein is complicated by their conversion to the corresponding acids on hydrolysis. A recent method of total enzymic hydrolysis of proteins[18] permits these two amino-acids to be liberated from proteins or peptides without loss of their amide groups, and their determination by column chromatography is then possible.[19] The presence of an amide group on one of these amino-acids in a peptide can often be demonstrated simply by electrophoretic analysis of the peptide.[20] A peptide with an asparagine or glutamine residue has a smaller negative charge than the corresponding peptide with a residue of aspartic or glutamic acid. Quantitative determination of the total number of amide residues in a protein or a peptide[3] is best performed in the manner whose description follows. Contaminating ammonium salts are first removed by dialysis or by gel-filtration on a column of Sephadex. Two equal portions of the de-salted solution are treated separately with the same volume of concentrated hydrochloric acid; one sample is hydrolysed under the standard conditions described above, and the other (a control) is immediately evaporated to dryness under reduced pressure with the minimum of heating. The ammonia content of each solution is determined by quantitative amino-acid analysis, and the control value is subtracted from the ammonia content of the hydrolysed sample. A further subtraction must be made to correct for the presence of adventitious ammonia derived from acid decomposition of serine and threonine.

Quantitative determination of the amino-acids in a hydrolysate is performed with the greatest accuracy by ion-exchange chromatography with an automatic amino-acid analyser.[19] Reproducible yields (±3 per cent.) can be obtained with mixtures containing at least 0·5 μmole of each amino-acid. Proline, which gives less colour with ninhydrin than do the other amino-acids, must be present in relatively larger amounts to ensure the same degree of accuracy. When a mixture contains only a few amino-acids that are easily resolved from each other, shorter columns may be successfully used; there is a small increase in sensitivity, owing to the sharpness of the eluted peaks, and the analysis is completed in a shorter time.[4,17,21] With peptides, reasonably satisfactory analyses can be obtained at a level of 0·05 μmole of each

amino-acid. In the analysis of proteins for amino-acid compositions, the most important feature is that of precision, so that small variations in composition may be detected[22]; on the other hand, in the analysis of amino-acid compositions of biologically active peptides, the limiting consideration is often the scarcity of material. Sensitivity of analysis is then vital, and semi-quantitative or qualitative paper methods may be the only techniques practicable. Although these methods are simple and economic to perform, they lack the precision of methods involving column chromatography. A detailed study of amino-acid analysis by paper chromatography has been made by Hanes[23] and an extensive bibliography has been compiled by Lederer and Lederer.[24] Micro methods for determining amino-acids by high-voltage paper-electrophoresis have been presented by Naughton and Hagopian,[25] by Atfield and Morris[26] and by Blackburn and Lee.[27]

Other methods of determining amino-acids involve their conversion to derivatives. Examples of these are the fluorodinitrobenzene method,[28] in which dinitrophenylamino-acids are separated by column chromatography and estimated by light-absorption methods, and also the gas-chromatographic method,[29,30] in which the amino-acids are converted to volatile acylated amino-acid esters. Both these methods are capable of considerable sensitivity, but they are not yet preferable to ion-exchange chromatography.

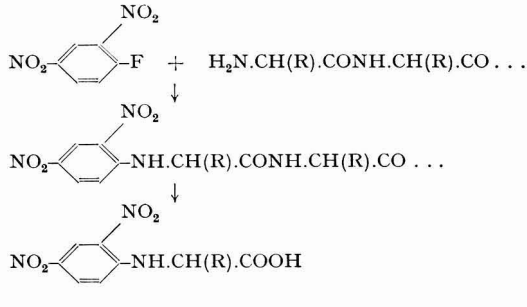
The accuracy with which a determination must be carried out depends on the number of amino-acid residues in the protein or peptide. For a large protein, no available analytical method is capable of defining the exact composition. Tristram[31] has pointed out that an analysis must define the amount of a particular residue to within $\pm 0{\cdot}4$ of an integral number of that residue. For example, since the error in amino-acid analysis by the method of Spackman, Stein and Moore[19] is ± 3 per cent., it is just possible to define the integral number of aspartic acid residues in ribonuclease as 15, because the experimental values obtained would not be less than 14·55 or more than 15·45. Clearly, the larger the number of residues of a particular amino-acid in a protein, the greater must be the accuracy needed for defining that integral number. At the same time, amino-acid analysis of a high order may be necessary to establish the *purity* of even a small peptide, particularly when the contaminant contains amino-acids common to both peptides.

Amino-terminal analysis

A quantitative amino-terminal analysis of a protein provides information on the minimum number of peptide chains in the molecule. It can also provide an index of purity by indicating the presence, or absence, of amino-terminal groups derived from contaminating peptides. The yield of the terminal residue (or residues) obtained from a pure protein should be close to an integral number. It should also be noted that several proteins possess blocked amino-terminal residues. Impurities can arise by lysis of the protein itself during isolation and these too are revealed by amino-terminal analysis.

Fluorodinitrobenzene method—

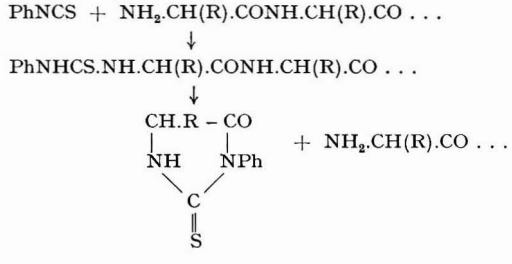
The classical reagent for amino-terminal analysis is fluoro-2,4-dinitrobenzene.[32] The method has proved invaluable in structural investigations, and has formed the subject of extensive reviews.[28,33,34] The procedure involves formation of a dinitrophenyl derivative of a polypeptide, and then hydrolysis to release the dinitrophenyl derivative of the amino-acid originally present in the amino-terminal position, as shown in the diagrammatic reaction scheme given below—



The principal difficulty of this method lies in obtaining an accurate measure of the amount of end-group. The addition of fluorodinitrobenzene to a protein is often performed as a heterogeneous reaction, and the reaction products also are frequently insoluble. Thus, reactions may not go to completion, particularly if precipitation of an ϵ-dinitrophenyl derivative should occur before reaction has taken place at the α-amino group. The reaction mixtures are separated into fractions by paper chromatography, and the individual dinitrophenylamino-acids are eluted from the paper for determination by light-absorption methods. Losses inevitably occur during these operations. A further source of difficulty is provided by the instability of dinitrophenylamino-acids to light. Refinements of the method are available, however, in the separation of dinitrophenylamino-acids by counter-current distribution[35] or by column chromatography.[36] Perhaps the most serious drawback of the method is that extensive decomposition occurs on acid hydrolysis of the dinitrophenyl derivatives of glycine, serine, threonine, cystine, and tyrosine[37,38]; complete decomposition of dinitrophenyl proline and dinitrophenyl tryptophan also occurs. The use of correction factors may permit the determination of some of these; for others, milder conditions of hydrolysis have proved advantageous.[39] Exploratory experiments are usually necessary for finding the optimum conditions. When the hydrolysis is performed without prior removal of dinitrophenol (derived from the reagent), artifacts appear on paper chromatograms, and these can cause misleading interpretations.[40] During investigations on the amino-acid sequence of ribonuclease,[1] similar difficulties led to an incorrect identification of dinitrophenyl serine.

PHENYLISOTHIOCYANATE METHOD—

Many of the above-mentioned problems are overcome in the method of Edman,[41,42] as modified by Eriksson and Sjoquist.[43] The amino-terminal residue of the protein is coupled with phenylisothiocyanate, and the phenylthiocarbamyl peptide can be made to undergo cyclisation under mild acidic conditions to release the terminal amino-acid as its phenylthiohydantoin, as shown in the diagrammatic reaction scheme given below—

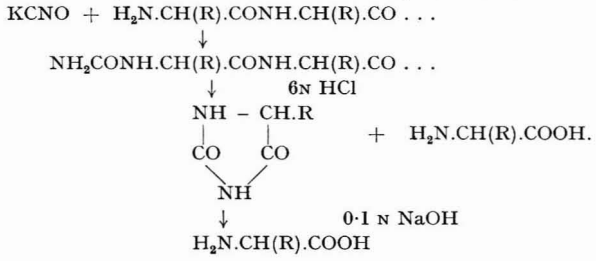


Good yields are obtained with amino-acids whose dinitrophenyl derivatives are labile under the vigorous hydrolytic conditions used in the dinitrophenyl method. The phenylisothiocyanate method has been extensively applied for structural studies of small peptides.[1,3,4,44] After the sequence of reactions has been performed, a portion of the residual peptide can be hydrolysed and analysed; comparison with the analysis of the peptide before degradation reveals a loss of the amino-acid that was initially in the amino-terminal position. The subtractive method clearly cannot be applied to proteins in which the changes in composition would be too slight to be detected.

The phenylisothiocyanate method, though not yet widely tested on proteins, is potentially promising. Of the three methods described in this review, it is the only one that can be used in a stepwise fashion (see under "Stepwise Degradation," p. 89), and that can distinguish between the amides and the acidic forms of aspartic and glutamic acids in the amino-terminal position. It has, however, some inherent difficulties. The cyclisation reaction may have a competitive side reaction that results in a loss in yield.[44] (G. R. Stark in a personal communication.) Paper chromatography can be used for separating phenylthiohydantoins which are eluted from the paper and estimated by optical methods. Quantitative results are difficult to obtain in these circumstances. Again, solubility problems can be encountered in the reaction of a polar compound such as a protein or peptide, with an essentially non-polar reagent such as phenylisothiocyanate.

CYANATE METHOD—

Many of the difficulties mentioned above are absent from the cyanate method,[17] which is illustrated diagrammatically in the reaction scheme given below—



The initial addition of cyanate to a protein takes place in 8 M urea, in which almost all proteins are soluble, and in which the carbamylated products usually remain dissolved. The carbamyl protein undergoes cyclisation in 6 N hydrochloric acid, releasing the hydantoin of the amino-acid initially present in the amino-terminal position of the protein. The hydantoin is isolated and then hydrolysed, releasing the corresponding amino-acid, which is determined quantitatively by automatic amino-acid analysis.[19] The cyanate method is accurate and reproducible; paper chromatography is avoided and, except with serine and threonine, quantitative yields are obtained. Slight losses of proline and tryptophan also occur. The procedure has the advantage of being highly specific, in that negligible amounts of amino-acids derived from non-terminal residues appear in the final analysis.

Many proteins have recently been found, in which the amino-terminal group is blocked by acetylation.[45] Early amino-terminal analysis of cytochrome C by the dinitrophenyl method led to the erroneous report that the terminal residue was histidine,[46] or arginine.[47] Proof of the complete absence of an α-amino group was delayed until the acetylated amino-terminal peptide had been isolated.[48] Application of the cyanate method has confirmed that cytochrome C has no free α-amino group.[17] Similarly, structural studies on peptides from ribonuclease[1] erroneously suggested the presence of amino-terminal serine at position 11 (residues 11 to 18, and 11 to 31; see Fig. 1) by both the dinitrophenyl and Edman techniques. This peptide was later shown to have a pyrrolidone carboxylyl residue in the terminal position.[3] During isolation of the peptide, the pyrrolidone carboxylyl residue had arisen by cyclisation of the amino-terminal glutamine initially present in position 11 of the protein. Application of the cyanate method correctly showed complete absence of an α-amino group.[17] The freedom from artifacts and the precision obtainable by the cyanate method make it suitable for determining the molecular weight or the purity of a protein preparation.

The main problem in all end-group methods is associated with the size of the protein. With a protein of molecular weight 100,000, it is preferable to use about 50 mg, *i.e.*, about 0·5 μmole, for terminal analysis. This amount of protein will provide a maximum amount of 0·5 μmole of the derivative corresponding to its amino-terminal residue. In the cyanate method, 0·02 μmole of amino-acids, derived from contaminants, can be detected with certainty. Thus the method is sensitive to contamination by an amino-acid present to the extent of 1 part in 25 parts of a protein on a molar basis, or 1 part in about 20,000 by weight. For large proteins, the problem of purity of the preparation sets a limit to the successful interpretation of results obtained by amino-terminal analysis.

In general, all structural studies should be performed on pure substances, but the removal of the last traces of contaminating substances is often extremely difficult. In particular, amino-terminal analysis of a proteolytic enzyme is complicated by the problem of autodigestion, which liberates new α-amino groups. It was found expedient to render trypsin and chymotrypsin inactive by reaction with di-isopropylphosphofluoride,[49] and to crystallise the products before proceeding with amino-terminal anslysis. With due care in experimental techniques, amino-terminal analysis may provide the most sensitive test of the purity of a protein.

CLEAVAGE OF DISULPHIDE BONDS

The disulphide groups of cystine residues in proteins can cause difficulties of two kinds. Firstly, since two peptide chains may be held together by each disulphide bond, as in insulin,

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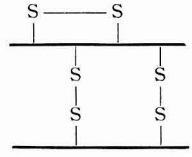
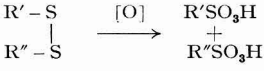
complex mixtures of peptides may arise in degradative experiments performed directly on the intact molecule. A diagrammatic arrangement of the disulphide bonds of insulin is given below—



Disulphide bonds are labile under various hydrolytic conditions used in structural work,[2,50] and they readily undergo re-arrangements that can further increase the complexity of peptide mixtures (see under "Identification and Pairing of Disulphide Bridges," p. 92). Secondly, native proteins are seldom extensively split by enzymes, and some form of denaturation may be necessary before attempting fragmentation. Cleavage of the disulphide bridges will generally render the protein more susceptible to enzymic digestion. Three methods for cleaving disulphide bonds have been successfully used: oxidation, reduction and reaction with sulphite.

PERFORMIC ACID OXIDATION—

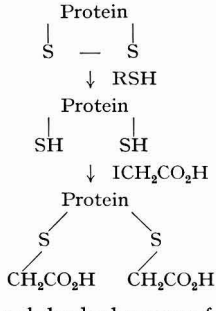
This reaction has been used in the classical studies on insulin[51] and ribonuclease.[52] The cystine residues are converted to residues of cysteic acid, and methionine is converted quantitatively to the stable sulphone.



Since the yield of cysteic acid is usually about 90 per cent., the possibility of complications caused by side reactions may have to be considered. The oxidation reaction can cause some destruction of tyrosine, and conditions should be selected to ensure maximum oxidation of cystine with minimum side reactions.[52] Tryptophan residues are destroyed in a complex manner, and after performic acid oxidation, therefore, only limited peptide sequences can be determined from a protein containing this amino-acid. It should be noted that the method of Moore[12] (described under "Amino-acid Analysis," p. 81), is an analytical procedure and not suitable for the cleavage of disulphide bonds under mild conditions.

REDUCTION—

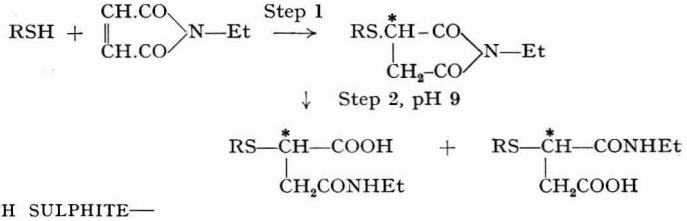
Reagents used successfully include ethanethiol[53,54] and thioglycollic acid in the presence of 8 M urea. It has now been shown that sodium borohydride, used in earlier studies, promotes hydrolysis of some peptide bonds,[6,55] and is therefore unsuitable.



In the reductive method, the two sulphydryl groups formed from each cystine residue are susceptible to re-oxidation and must be blocked by a suitable reagent. Iodoacetic acid has been used extensively,[6] and *N*-ethylmaleimide has also been applied.[7,56] The use of iodoacetic acid suffers from a number of disadvantages, in that it will react, though more slowly, with methionine,[57] lysine,[58] and histidine.[6,59,60] Large amounts of inorganic iodide are released after the reaction of iodoacetate with the sulphydryl groups of the excess of ethanethiol.

In the separation of carboxymethylated protein from iodide, care must be taken to exclude light and thereby prevent formation of iodine which would react readily with tyrosine, tryptophan and histidine residues. The introduction of an additional negative charge at each cysteine residue of a reduced protein sometimes results in insolubility of the carboxymethylated product. As mentioned under "Amino-Acid Analysis" p. 81, rigorous exclusion of air is necessary during acid hydrolysis of carboxymethylated proteins to ensure a good yield of carboxymethyl cysteine. The procedure of Crestfield, Moore and Stein[6] overcomes many of these disadvantages.

The use of *N*-ethylmaleimide as a blocking agent is disadvantageous, since the addition reaction results in the formation of two diastereoisomers (the second asymmetric centre being denoted by the asterisk in the diagram below).[7] These may exhibit different physical properties and duplication of peptides may occur when paper or ion-exchange chromatography is used. Besides its reaction with sulphydryl groups, *N*-ethylmaleimide will react slowly with α-amino groups and with imidazole[61]; under acid conditions, however, it exhibits a high degree of specificity for sulphydryl groups. A potential problem with this reagent is associated with hydrolysis of the imide ring after the addition reaction. Since the hydrolysis could occur at either of two positions, the possibility exists of the formation of four more reaction products. This hydrolytic reaction is of significance only when *N*-ethylmaleimide-modified proteins or peptides are maintained at pH values of 9 or more, but the multiplicity of *N*-ethylmaleimide derivatives of proteins and peptides suggests the need for caution when using this reagent.



REACTION WITH SULPHITE—

The method of Swan[62,63] is based on the reaction—

$$R\text{-}S\text{-}S\text{-}R + HSO_3^- \longrightarrow R\text{-}S\text{-}SO_3^- + RSH$$

The reaction is driven to completion by cupric ions present in the solution. These oxidise the thiol to more disulphide, and further cleavage by sulphite then takes place. The reaction has been applied to protein disulphide bridges and appears to be both quantitative and specific.[64] A disadvantage of the method is that the $S\text{-}SO_3^-$ groups are unstable to extremes of pH, and therefore total acid hydrolysis cannot be used for detecting and determining modified cysteine residues in peptides produced by subsequent degradation.

A serious disadvantage in the two methods of reductive cleavage is that peptide fragments contain unaltered methionine residues that are susceptible to oxidation, and are partially converted to the more acidic sulphoxide during column or paper chromatography.[6] These peptides may be obtained in poor yields as a result of "tailing" throughout the chromatographic separations. In the oxidative method, however, the methionine residues are converted to stable sulphone derivatives.

FRAGMENTATION

As described earlier (under "Cleavage of Disulphide Bonds," p. 85), the protein must first be rendered susceptible to enzymic digestion by cleaving the disulphide bonds, and the resulting peptide chains must then be separated before fragmentation. The problem of an insoluble "core" containing aggregated peptides has been solved by performing subsequent separations involving chromatography on an ion-exchange column or on a Sephadex column in the presence of 8 M urea.[64] Trypsin, though possessing a small, inherent chymotryptic activity, is a highly specific enzyme,[65] and is generally used for the initial fragmentation at the carboxyl group of lysine and arginine residues. It should be noted that when these residues are immediately followed in sequence by a proline or an acidic residue, cleavages occur slowly (*e.g.*, see Fig. 1, positions 39 and 40 in oxidised ribonuclease) or not at all (positions 41 and 42). The quality of trypsin preparations may be tested by using the reduced and carboxymethylated phenylalanine chain of insulin and an amino-acid analyser for automatic monitoring.[6]

Peptides resulting from tryptic digestion are separated into fractions by ion-exchange chromatography on columns of Dowex 50-X2[66] or Dowex 1-X2,[67] with the aid of volatile buffers; the use of pyridine buffers prevents bacterial decomposition of peptides during their isolation. Fractionation by gel-filtration on columns of Sephadex may be advantageous, either before or after ion-exchange chromatography. The resulting peptides should be examined for purity by quantitative amino-acid analysis, and by monitoring with paper electrophoresis. Additional purification in a different system may be necessary. As a general rule, not more than about 20 peptides can be separated successfully in a single chromatographic system. If the tryptic peptides contain more than about six amino-acid residues, further cleavage with a suitable enzyme should be performed. When the peptide contains tyrosine, phenylalanine, or tryptophan, cleavage at the peptide bonds involving the carboxyl groups of these residues can be effected by chymotrypsin.[16,40,68] Peptide bonds involving the imino group of proline are completely resistant to chymotryptic digestion. It has been found that peptide bonds formed from certain non-aromatic amino-acids are also cleaved by chymotrypsin (see Fig. 1), and the reactivity of such linkages may be affected by the general environment along the polypeptide chain. For example, the peptide bond between a serine and an alanine residue in ribonuclease (see Fig. 1, positions 18 and 19) is readily cleaved when the tryptic peptide containing residues 11 to 31 is digested with chymotrypsin.[3] When the peptide containing residues 1 to 20 is digested with chymotrypsin, negligible cleavage occurs at position 18 (F. M. Richards, personal communication to the authors). Similar results have been reported by Hill and Konigsberg[69] during their study of the action of pepsin on the chains of haemoglobin. Although successful in this particular instance, pepsin has not been widely used because of its lack of specificity. Papain also exhibits a wide specificity, but has proved useful in splitting bonds not susceptible to the action of trypsin and chymotrypsin (*e.g.*, papain digestion of peptides, from ribonuclease resulted in cleavages at peptide bonds from the original positions 13 and 14, 58 and 59, 68 and 69, 70 and 71, 88 and 89, 95 and 96, 101 and 102, 109 and 110, and 113 and 114; see Fig. 1). In all instances, products are separated and checked for purity before amino-acid sequence studies are attempted.

In order to establish the relative alignment of peptides obtained by tryptic hydrolysis, other peptides containing amino-acid residues that overlap the sites of tryptic digestion must be separated. Such peptides can be obtained by the direct action of chymotrypsin on the protein,[70] or more elegantly by blocking the ϵ-amino groups of lysine with cyanate,[3,71] carbobenzoxychloride[72] or trifluoroacetic anhydride,[73] to render them immune to tryptic digestion. The action of trypsin is then restricted to the cleavage of peptide chains at the carboxyl group of arginine residues. Difficulty may be experienced, however, in the separation and isolation of large peptides. In general, Dowex 50-X2 columns cannot be used successfully for resolving peptides containing more than 20 to 25 amino-acid residues.

The action of trypsin in initiating transpeptidation reactions[74,75] has not significantly affected the use of this enzyme in structural studies. A greater problem is excessive contamination of trypsin by chymotrypsin, which may result in the production of a large number of reaction products, making problems of separation and purification unsurmountable. Trypsin, thrice crystallised, is obtainable commercially (Worthington Chemical Corporation), and has less chymotryptic properties than trypsin obtained by auto-activation of crystalline trypsinogen.[6] Trypsin is relatively stable, and chymotrypsin is unstable, in the presence of acid, so the enzyme should be dissolved in 0·01 N hydrochloric acid immediately before use.[65]

The method of partial acid hydrolysis to produce fragments, successfully used with insulin, has been largely superceded by the above methods. It is now known that inversions of dipeptides can occur in acid solution,[76] and further, the multiplicity of reaction products produced by this non-specific method greatly complicates problems of subsequent separation. It may be noted, however, that successful use has been made of a combination of radioactive labelling techniques and partial acid hydrolysis.[77,78]

Specific chemical methods for fragmenting peptide chains have been restricted in their use by the poor yields obtained. Cleavages at serine and threonine residues, induced by sulphuric acid[79] and orthophosphoric acid,[80] and cleavages at tryptophan and tyrosine, by *N*-bromosuccinimide,[81,82] have received limited success. Hunt and Ingram[83] have successfully used 0·25 M acetic acid to cleave specifically at each side of an aspartic acid residue, but this method may be applicable only to peptides lacking serine and threonine residues. Cyanogen bromide, which has been used for cleaving peptides at methionine residues,[84] appears

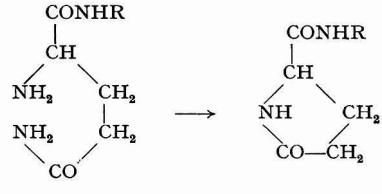
to be a promising reagent. Recently, application of this reagent to the intractable core of myoglobin[85] resulted in the production, in good yield, of three soluble peptides that were easily separated for further study. More recently, ethylenimine has been used for reacting with cysteine residues, resulting in the introduction of an amino group. The modified residues serve as substrates for trypsin digestion and good yields of peptide fragments have been obtained from the β-chain of insulin.[86] The successes achieved with these reagents may stimulate a search for further chemical methods capable of cleaving peptides.

Stepwise degradation

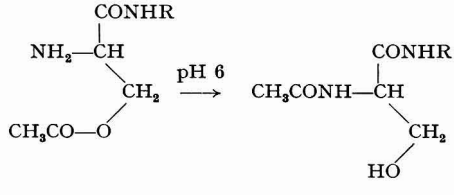
Edman degradation—

As well as being a valuable method for determining amino-terminal residues, the Edman degradation can be used for stepwise degradation, either by the subtractive technique or by direct chromatographic determination of the phenylthiohydantoins. Cyclisation takes place under comparatively mild conditions, *i.e.*, by heating at 100° C for 1 hour in glacial acetic acid saturated with dry hydrogen chloride.[1] Since the residual peptide is not hydrolysed, the method is used in a sequential fashion. The cyclisation conditions mentioned above, however, can cause complete blockage of sequential degradation on account of three side reactions[4]—

(*i*) Release of a glutamine residue at the amino-terminal position leads rapidly to its cyclisation to a pyrrolidone carboxylic acid residue, as shown in the diagrammatic reaction scheme given below—

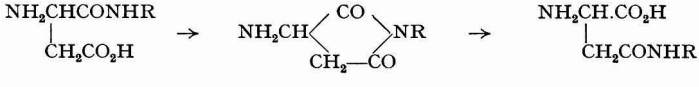


(*ii*) Internal serine and threonine residues become quantitatively acetylated. As soon as the *O*-acetylated residue becomes amino-terminal, an $O \rightarrow N$ shift of the acetyl group occurs, as indicated below—



Both reactions (*i*) and (*ii*) result in complete disappearance of free α-amino groups.

(*iii*) Peptides containing aspartic acid or asparagine undergo imide formation and then hydrolysis of the imide to form a β-peptide, as indicated below—



This linkage will not undergo cleavage in subsequent degradative steps.

Because of these side reactions, the Edman degradation cannot be successfully used under the above cyclisation conditions with peptides containing glutamine, serine, threonine, asparagine, or aspartic acid residues. It should be noted that these difficulties still occur when cyclisation is performed in anhydrous solvents for longer periods at lower temperatures; the half-life of reactions (*i*) and (*ii*) is about 1 hour at 50° C.

Analyses of residual peptides without purification, after a cycle of reaction, give poor yields of serine, threonine or tyrosine. Thus, when the degradation has been blocked by one of the three side reactions, the apparent loss of serine, threonine or tyrosine could cause it to be erroneously placed in the sequence. For example, a peptide of sequence

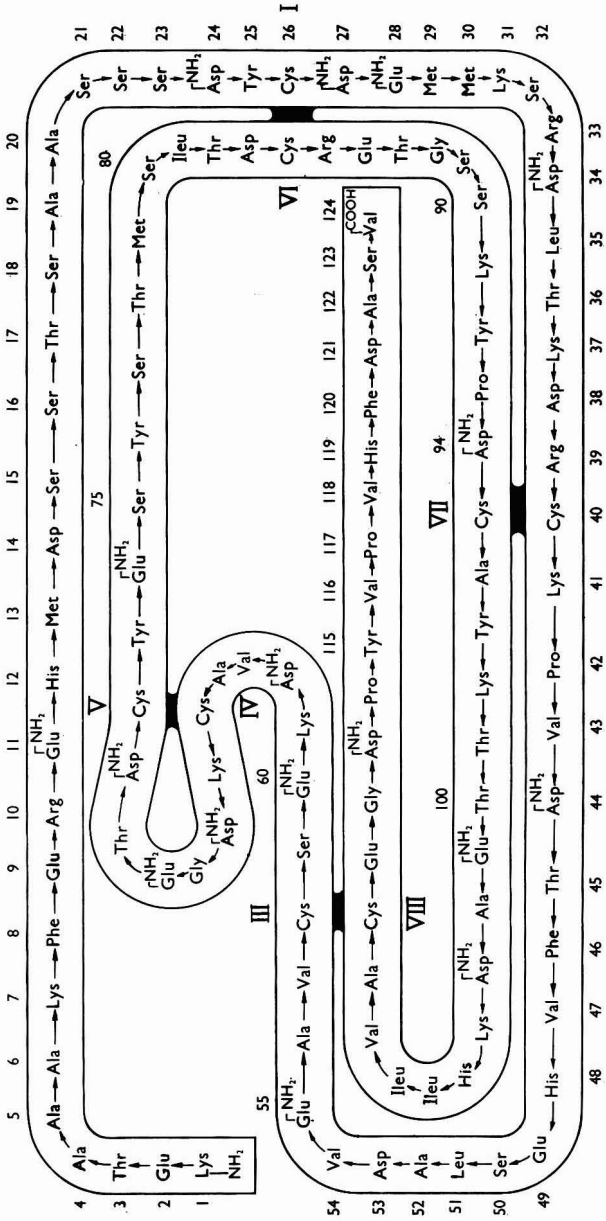


Fig. 1. The sequence of amino-acids in bovine pancreatic ribonuclease A, from Smyth, Stein and Moore.[4]

This structure is based on the experiments of Hirs, Moore and Stein,[1] and of Spackman, Stein and Moore,[2] and of Smyth, Stein and Moore[3,4]. Trypsin cleavages occur on the carboxyl side of all lysine and arginine residues, except at positions 1 and 41. Chymotrypsin cleavages occur on the carboxyl side of all tyrosine and phenylalanine residues, and also at positions 18, 35, 62, 79, 103 and 105

Ala.Glu-NH_2.Ser.Tyr.Lys., when analysed by the subtractive method, could be mistakenly identified as Ala.Ser.Tyr.Glu-NH_2.Lys. Difficulties of this kind were the cause of many errors in the originally published amino-acid sequence of ribonuclease.[1]

These difficulties have been overcome by performing the cyclisation reaction in trifluoroacetic acid[3,4,87,88] at room temperature for 1 hour. Under these conditions, the blocking reactions do not occur.[4] Moreover, purification of the resulting reaction mixtures by careful column chromatography provides residual peptides that, on hydrolysis, give integral values for the constituent amino-acids.[4] The Edman degradation, under the improved conditions, is an invaluable technique, particularly for small peptides resulting from enzymic degradation of larger molecules. The paper-strip modification[28] is a valuable alternative in experienced hands, and has been used successfully in studies on the structure of foetal haemoglobin.[89,90]

THE USE OF LEUCINE AMINOPEPTIDASE—

The pure enzyme cleaves amino-acids sequentially from the amino-terminus of a peptide.[91] Commercial preparations however, are not pure, and possess carboxypeptidase activity.[3,4] If the amino-acid at the amino terminus of a peptide has a slow rate of cleavage, as does aspartic acid, glutamic acid, cysteic acid, serine, threonine or proline, then the carboxypeptidase action of crude leucine aminopeptidase is sufficient to give misleading results. This accounts for the apparent ability of leucine aminopeptidase to cleave amino-acids on either side of a proline residue in a peptide without releasing free proline. It was previously thought that the released proline formed a diketopiperazine.[1]

Some results obtained by treatment of peptides from ribonuclease (see Fig. 1) with leucine amino-peptidase are given in Table I. In each instance, amino-acids have been released from the carboxyl terminus of the peptide.

It should be noted that leucine aminopeptidase is particularly useful for liberating glutamine and asparagine from a peptide under mild conditions when hydrolysis to the corresponding acidic amino-acids does not occur.

TABLE I

THE ACTION OF LEUCINE AMINOPEPTIDASE ON PEPTIDES OBTAINED FROM RIBONUCLEASE

Peptide	Residues*	Amino-acids released,[1] μmoles per molecule of peptide	Sequence of amino-acids[4]	
			at amino terminus	at carboxyl terminus
O-Tryp 4 Chy 1	11 to 18	Ser, 0·12; Thr, 0·06	Pyr.† His. Met . . .	. . . Ser. Thr. Ser.
O-Tryp 9 Chy 5	40 to 46	Phe, 0·30; Thr, 0·28; Asp-NH_2, 0·24; $CysSO_3H$, 0·22; Val, 0·19; Pro, 0·10; Lys, 0·03	$CysSO_3H$. Lys. Pro . . .	. . . Asp-NH_2. Thr. Phe.
O-Tryp 6	86 to 91	Glu, 0·54; Ser, 0·27; Thr, 0·04; Gly, 0·04; Lys, not determined	Glu. Thr. Gly.	Ser. Ser. Lys.
O-Tryp 14	92 to 98	Tyr, 0·86; Asp-NH_2, 0·84; $CysSO_3H$, 0·46; Ala, 0·46; Pro, 0·38; Lys, not determined	Tyr. Pro. Asp-NH_2.	$CysSO_3H$. Ala. Tyr. Lys.
O-Tryp 16 Chy 5	116 to 120	Val, 0·18; Phe, 0·16; His, 0·14; Pro, 0·04	Val. Pro. Val.	His. Phe.

* See Fig. 1.

† The amino-terminal residue in *O*-Tryp 4 Chy 1 is pyrrolidone carboxylic acid, which possesses no free amino group and should, therefore, be completely resistant to leucine aminopeptidase.

THE USE OF CARBOXYPEPTIDASE—

This enzyme cleaves amino-acids in a stepwise fashion from the carboxyl terminus of a peptide,[28] carboxypeptidase B releasing C-terminal lysine and arginine, and carboxypeptidase A releasing neutral amino-acids. As with leucine aminopeptidase, carboxypeptidase cleaves acidic amino-acid residues slowly, and sometimes stops acting when an acidic amino-acid residue forms the penultimate residue in the chain. A proline residue at the end of a chain is completely unaffected by the enzyme. Difficulty can be experienced in the interpretation of results when a number of residues of the same amino-acid are present near the carboxyl

terminus. Moreover, long incubation periods must be avoided, because amino-acids are released from the enzyme itself by auto-digestion. The best commercial preparations of carboxypeptidase have been treated with di-isopropylphosphofluoride to eliminate unwanted trypsin and chymotrypsin activity. In general, carboxypeptidase is a satisfactory reagent for use in sequence studies on small peptides, but conclusive interpretations should be restricted to location of not more than two or three amino-acids at the carboxyl terminus. It is essential that amino-acids released either by leucine aminopeptidase or by carboxypeptidase should be determined quantitatively, and a time - course study of the rate of release is advantageous.

Identification and pairing of disulphide bridges

Many proteins possess sulphydryl groups that are rendered inaccessible by the folding of the peptide chains and are therefore unreactive towards sulphydryl reagents. Such sulphydryl groups could easily be mistaken for disulphide groups, particularly since the latter undergo slow hydrolysis to release new sulphydryl groups under alkaline conditions. To clarify the position, the determination of cysteine residues should be performed by at least two procedures, preferably different in character. Suitable methods are the technique of Boyer[92] involving the use of *p*-chloromercuribenzoate titrations, amperometric titration,[93] or the technique of Crestfield, Moore and Stein,[6] in which the reaction of the protein with iodoacetate in 8 M urea precedes hydrolysis and determination of carboxymethylcysteine.

The deduction of the pairing of half-cystine residues in a protein is complicated by the fact that disulphide interchange reactions occur under neutral and alkaline conditions.[2,50] Under these conditions, some hydrolysis of a disulphide bond takes place, as indicated below—

$$\begin{matrix} A{-}S \\ | \\ B{-}S \end{matrix} \xrightarrow{OH^-} A{-}S^- + B{-}S.OH$$

The sulphydryl group thus formed can take part in an exchange reaction with a different disulphide group, forming a new disulphide linkage and releasing another sulphydryl group to catalyse further changes, as indicated below—

$$A{-}S^- + \begin{matrix} C - S \\ | \\ D - S \end{matrix} \longrightarrow \begin{matrix} A - S \\ | \\ D - S \end{matrix} + CS^-$$

For the production of fragments containing two half-cystine residues without exchange, it is essential that hydrolyses be performed under acidic conditions. Early attempts to discover the pairing of the eight half-cystine residues in ribonuclease gave incorrect results because hydrolyses were performed at pH 8 with subtilisin.[94] In another laboratory, successful use was made of pepsin digestion[2] at pH 2. The various cystine peptides were separated from one another and were then further digested with trypsin and chymotrypsin to reduce their size. These small fragments, still containing the sequence around each cystine residue, were oxidised by performic acid. The products were isolated and their amino-acid composition determined. By reference to the total sequence of ribonuclease, it was possible to deduce the sequence proximal to each half of the original cystine peptide, and this allowed an unequivocal allocation of the disulphide bridges. As with all methods of structural analysis, the problems become more acute as the size of the molecule increases. This is specially true in the elucidation of disulphide pairings in a highly cross-linked protein; in such circumstances, present methods do not permit unequivocal interpretations.

General conclusions

Proteins or peptides must be pure before structural studies are commenced. Purity should be established by a selection of appropriate methods. Disulphide bonds are split either by oxidation or by reduction; if more than one peptide is formed, the products must be completely separated and purified. The denatured polypeptide is digested with trypsin, and the fragments are separated by ion-exchange chromatography. The tryptic fragments are subjected to digestion by other proteolytic enzymes to produce smaller peptides that are more suitable for investigating amino-acid sequence. These smaller peptides are also amenable to ion-exchange chromatography, which serves both for their isolation and as an indication of purity. The examination of peptides by paper electrophoresis serves as an additional

check on their purity, and can demonstrate the presence or absence of amide residues. The amino-acid sequence of peptides resulting from successive enzymic degradation is determined by the Edman method, and with carboxypeptidase. To reduce the possibility of error, at all stages after either chemical reagents or enzymes have been used, quantitative methods are obligatory.

The methods described in this review are adequate for studying proteins of molecular weights up to 20,000. Attempts to extend these methods to larger proteins will involve many more problems, for which there is no immediate solution. In particular, the degree of resolution that can be achieved by ion-exchange chromatography may not permit the separation of the large number of peptides resulting from enzymic degradation of an extremely large molecule. The problem of obtaining overlapping sequences that would permit the correct alignment of the tryptic fragments presents an equally difficult task. So far, trypsin is the only enzyme known to possess the high degree of specificity required for predictable fission of the polypeptide chain. Perhaps the further development of chemical reagents may fulfil the need for new methods of producing specific cleavage.

References

1. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1960, **235**, 633.
2. Spackman, D. H., Stein, W. H., and Moore, S., *Ibid.*, 1960, **235**, 648.
3. Smyth, D. G., Stein, W. H., and Moore, S., *Ibid.*, 1962, **237**, 1845.
4. ——, ——, ——, *Ibid.*, 1963, **238**, 227.
5. Moore, S., and Stein, W. H., *in* Colowick, S. P., and Kaplan, N.O., *Editors*, "Methods in Enzymology," Academic Press Inc., New York and London, Volume VI, in the press.
6. Crestfield, A. M., Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1963, **238**, 622.
7. Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W., *Biochem. J.*, in the press.
8. Smith, E. L., and Stockwell, A., *J. Biol. Chem.*, 1954, **207**, 501.
9. Hirs, C. H. W., Stein, W. H., and Moore, S., *Ibid.*, 1954, **211**, 941.
10. Moore, S., and Stein, W. H., *Ibid.*, 1949, **178**, 53.
11. Beaven, G. H., and Holiday, E. R., *Adv. Protein Chem.*, 1952, **7**, 319.
12. Moore, S., *J. Biol. Chem.*, 1963, **238**, 235.
13. Ray, W. J., and Koshland, D. E., *Ibid.*, 1962, **237**, 2493.
14. Njaa, L. R., *Acta Chem. Scand.*, 1962, **16**, 1359.
15. Neumann, N. P., Moore, S., and Stein, W. H., *Biochemistry*, 1962, **1**, 68.
16. Hirs, C. H. W., *J. Biol. Chem.*, 1960, **235**, 625.
17. Stark, G. R., and Smyth, D. G., *Ibid.*, 1963, **238**, 214.
18. Hill, R. L., and Schmidt, W. R., *Ibid.*, 1962, **237**, 389.
19. Spackman, D. H., Stein, W. H., and Moore, S., *Anal. Chem.*, 1958, **30**, 1190.
20. Naughton, M. A., Sanger, F., Hartley, B. S., and Shaw, D. C., *Biochem. J.*, 1960, **77**, 149.
21. Spackman, D. H., *Fed. Proc.*, 1963, 499.
22. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, 1943, **37**, 92.
23. Hanes, C. S., *Canad. J. Biochem. Physiol.*, 1961, **39**, 119.
24. Lederer, E., and Lederer, M., "Chromatography," Second Edition, Elsevier Publishing Co., Amsterdam; Clever-Hume Press Ltd., London; D. van Nostrand Co. Inc., New York, 1957, p. 306.
25. Naughton, M. A., and Hagopian, H., *Anal. Biochem.*, 1962, **3**, 276.
26. Atfield, G. N., and Morris, C. J. O. R., *Biochem. J.*, 1960, **74**, 37.
27. Blackburn, S., and Lee, C. R., *Ibid.*, 1963, **87**, 1P.
28. Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., *in* Glick, D., *Editor*, "Methods of Biochemical Analysis," Interscience Publishers Inc., New York and London, 1955, Volume II, p. 359.
29. Graff, J., Wein, J. P., and Winitz, M., *Fed. Proc.*, 1963, 500.
30. Johnson, D. E., Scott, S. J., and Meister, A., *Anal. Chem.*, 1961, **33**, 669.
31. Tristram, G. R., *Biochem. J.*, 1946, **40**, 721.
32. Sanger, F., *Ibid.*, 1945, **39**, 507.
33. Porter, R. R., *in* Gerard, R. W., *Editor*, "Methods in Medical Research," Year Book Publishers Inc., Chicago, Volume III, 1950, p. 256.
34. Thompson, E. O. P., and Thompson, A. R., *in* Zechmeister, L., *Editor*, "Progress in the Chemistry of Organic Natural Products," Springer-Verlag, Vienna, 1955, Volume XII, p. 270.
35. Eaker, D. L., Doctoral dissertation, The Rockefeller Institute, 1962.
36. Hamilton, P. B., *Anal. Chem.*, 1960, **32**, 1779.
37. James, A. T., and Synge, R. L. M., *Biochem. J.*, 1951, **50**, 109.
38. Seanes, F. S., and Tozer, B. J., *Ibid.*, 1956, **63**, 282.
39. Hanes, C. S., Hird, F. J. R., and Isherwood, F. A., *Ibid.*, 1952, **51**, 25.
40. Gish, D. T., *J. Amer. Chem. Soc.*, 1961, **83**, 3303.
41. Edman, P., *Acta Chem. Scand.*, 1950, **4**, 283.
42. Thompson, E. O. P., *Adv. Org. Chem.*, 1960, **1**, 149.
43. Eriksson, S., and Sjoquist, J., *Biochem. Biophys. Acta*, 1960, **45**, 290.
44. Konigsberg, W., and Hill, R. J., *J. Biol. Chem.*, 1962, **237**, 2547.
45. Narita, K., *Biochem. Biophys. Acta*, 1958, **30**, 352.

46. Margoliash, E., *Nature*, 1955, **175**, 293.
47. Matsubara, H., Hagihara, B., Horio, T., and Okunuki, K., *Ibid.*, 1957, **179**, 250.
48. Margoliash, E., Smith, E., Kreil, G., and Tuppy, H., *Ibid.*, 1961, **192**, 1125.
49. Rovery, M., Fabre, C., and Desnuelle, P., *Biochem. Biophys. Acta*, 1953, **12**, 547.
50. Ryle, A. P., and Sanger, F., Smith, L. F., and Kitai, R., *Biochem. J.*, 1955, **60**, 541.
51. Sanger, F., *Ibid.*, 1949, **44**, 126.
52. Hirs, C. H. W., *J. Biol. Chem.*, 1956, **219**, 611.
53. White, F. H., *Ibid.*, 1960, **235**, 383.
54. Anfinsen, C. B., and Haber, E., *Ibid.*, 1961, **236**, 1361.
55. Kimmel, J. R., and Parcells, A. J., *Fed. Proc.*, 1960, 341.
56. Sekine, T., Barnett, L. M., and Kielley, W. W., *J. Biol. Chem.*, 1962, **237**, 2769.
57. Gundlach, H. G., Moore, S., and Stein, W. H., *Ibid.*, 1959, **234**, 1761.
58. Gundlach, H. G., Stein, W. H., and Moore, S., *Ibid.*, 1959, **234**, 1754.
59. Crestfield, A. M., Stein, W. H., and Moore, S., *Ibid.*, 1963, **238**, 2413.
60. Barnard, E. A., and Stein, W. D., *J. Mol. Biol.*, 1959, **1**, 339.
61. Smyth, D. G., Nagamatsu, A., and Fruton, J. S., *J. Amer. Chem. Soc.*, 1960, **82**, 4600.
62. Swan, J. M., *Nature*, 1957, **180**, 643.
63. Bailey, J. L., and Cole, R. D., *J. Biol. Chem.*, 1959, **234**, 1733.
64. Hartley, B., paper presented at the Symposium on Enzyme Models and Enzyme Structure, Brookhaven, 1962.
65. Young, J. D., and Carpenter, F. H., *J. Biol. Chem.*, 1961, **236**, 743.
66. Hirs, C. H. W., Moore, S., and Stein, W. H., *Ibid.*, 1956, **219**, 623.
67. Braunitzer, G., Gehring-Muller, R., Hilschmann, N., Hilse, K., Hoborn, G., Rudloff, V., and Wittmann-Liebold, B., *Z. physiol. Chem.*, 1961, **325**, 283.
68. Ramachandran, L. K., and Gish, D. T., *J. Amer. Chem. Soc.*, 1959, **81**, 884.
69. Hill, R. J., and Konigsberg, W., *J. Biol. Chem.*, 1962, **237**, 3157.
70. Hirs, C. H. W., Stein, W. H., and Moore, S., *Ibid.*, 1956, **221**, 151.
71. Stark, G. R., Stein, W. H., and Moore, S., *Ibid.*, 1960, **235**, 3177.
72. Anfinsen, C. B., Sela, M., and Tritch, M., *Arch. Biochem. Biophys.*, 1956, **65**, 156.
73. Goldberger, R. F., and Anfinsen, C. B., *Biochemistry*, 1962, **1**, 401.
74. Waley, S. G., and Watson, J., *Biochem. J.*, 1953, **55**, 328.
75. Levin, Y., Berger, A., and Katchalski, E., *Ibid.*, 1956, **63**, 308.
76. Sanger, F., and Thompson, E. O. P., *Biochem. Biophys. Acta*, 1952, **9**, 225.
77. Sanger, F., paper presented at the Symposium on Microstructure of Proteins, New York, 1960.
78. Gladner, J. A., and Laki, K., *J. Amer. Chem. Soc.*, 1958, **80**, 1264.
79. Elliott, D. F., *Biochem. J.*, 1952, **50**, 542.
80. Lucas, F., Shaw, J. T. B., and Smith, S. G., *Ibid.*, 1957, **66**, 468.
81. Ramachandran, L. K., and Witkop, B., *J. Amer. Chem. Soc.*, 1959, **81**, 4028.
82. Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B., *Ibid.*, 1960, **82**, 5923.
83. Hunt, J. A., and Ingram, V. M., *Biochem. Biophys. Acta*, 1961, **41**, 520.
84. Gross, E., and Witkop, B., *J. Biol. Chem.*, 1962, **237**, 1856.
85. Edmundson, A. B., *Nature*, 1963, **198**, 354.
86. Raftery, M. A., and Cole, R. D., *Biochem. Biophys. Res. Commun.*, 1963, **10**, 467.
87. Elmore, D. T., and Toseland, P. A., *J. Chem. Soc.*, 1956, 188.
88. Edman, P., *Proc. Roy. Australian Chem. Inst.*, 1957, **24**, 434.
89. Schroeder, W. A., Jones, R. T., Shelton, J. R., Shelton, J. B., Cormick, J., and McCalla, K., *Proc. Nat. Acad. Sci. Wash.*, 1961, **47**, 811.
90. Schroeder, W. A., Shelton, J. R., and Shelton, J. B., *Anal. Biochem.*, 1961, **2**, 87.
91. Hill, R. L., Spackman, D. H., Brown, D. M., and Smith, E. L., *in* Vestling, C. S., *Editor*, "Biochemical Preparations," John Wiley and Sons, New York and London, 1958, Volume V, p. 35.
92. Boyer, P. D., *J. Amer. Chem. Soc.*, 1954, **76**, 4331.
93. Benesch, R. E., Lardy, H. A., and Bensech, R., *J. Biol. Chem.*, 1955, **216**, 663.
94. Ryle, A. P., and Anfinsen, C. B., *Biochem. Biophys.Acta*, 1957, **24**, 633.

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Arylhydroxylamines

Part IV.* Their Colorimetric Determination

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A method is needed for determining small amounts of arylhydroxylamines in biological fluids; arylhydroxylamines are carcinogenic, and are produced *in vivo* during carcinogenesis caused by aromatic amines. Coloured complexes are formed between arylhydroxylamines or their salicylidenearylamine *N*-oxides (nitrones) and ferrocyanide or pentacyano-ammine ferroate in aqueous solutions. The pentacyanoferroate complexes of *N*-phenyl-, *N*-(3,4-dimethylphenyl)-, and *N*-(2-carboxyphenyl)- hydroxylamines have been isolated and characterised as their potassium, ferric, or cupric salts. The effect of variation of time, pH, and reagent concentration on complex formation has been determined. The lower limit of detection is 1 to 3 μg of arylhydroxylamine per ml of solution. It has been found that nitrosobenzene interferes with the determination of *N*-phenylhydroxylamine, but other known urinary metabolites of aniline do not.

The process of *N*-hydroxylation as a metabolic stage in carcinogenesis caused by aromatic amines, leading to the excretion of arylhydroxylamines or their conjugates in urine, has been described by several workers.[1,2,3] A sensitive method is described for determining these metabolites in biological fluids.

Method

Apparatus—

Spectrophotometer—Visible and ultraviolet spectra were determined by using a Perkin-Elmer (Model 137) ultraviolet spectrophotometer.

Reagent—

Light petroleum—Boiling-range 60° to 80° C.

Preparation of hydroxylamines—

All melting-points are uncorrected.

N-*Phenylhydroxylamine*—This was prepared by reducing nitrobenzene with zinc dust - ammonium chloride mixture as described by Kamm,[4] forming colourless crystals (m.p. 82° C) that were recrystallised from benzene - light petroleum mixture.

N-*Methylhydroxylamine*[5]—This was similarly prepared from the corresponding nitro analogue, and had a melting-point of 42° C.

N-(3,4-*Dimethylphenyl*)*hydroxylamine*[6]—This was similarly prepared from the corresponding nitro analogue, forming colourless needles (m.p. 101° C) that were recrystallised from an ether - light petroleum mixture.

N-(2,4,6-*Trimethylphenyl*)*hydroxylamine*[7]—This was similarly prepared from the corresponding nitro analogue, forming colourless needles (m.p. 116° C) that were recrystallised from an ether - light petroleum mixture.

N-(2-*Carboxyphenyl*)*hydroxylamine*[8]—This was similarly prepared from the corresponding nitro analogue, forming pale yellow needles (m.p. 144° C) that were recrystallised from an ethanol - chloroform (1 + 1) mixture.

N-(4-*Biphenylyl*)*hydroxylamine*[9]—This was prepared by reducing the corresponding nitro analogue with aluminium amalgam, forming pale yellow plates (m.p. 152° to 154° C) that were recrystallised from benzene, followed by re-solidification and re-melting at 180° to 190° C.

* For details of earlier parts of this series, see reference list, p. 102.

N-(2-*Naphthyl)hydroxylamine*[10]—This was prepared by reducing the corresponding nitro analogue with aluminium amalgam, forming colourless plates (m.p. 135° to 137° C) that were recrystallised from chloroform.

PREPARATION OF COMPLEXES—

A solution containing 5 g of *N*-phenylhydroxylamine and 5 g of sodium pentacyano-ammine ferroate in 100 ml of 30 per cent. v/v aqueous ethanol was set aside for 16 hours at 23° C, and then extracted with three 20-ml portions of chloroform. The aqueous fraction was evaporated to dryness under reduced pressure, and the residual purple solid was extracted with 100 ml of methanol. The methanolic extract was treated with 15 ml of ether, set aside for 16 hours at 0° C, and filtered. The filtrate was evaporated under reduced pressure to half the original volume and treated with a solution of 4 g of potassium acetate in 50 ml of methanol. The purple precipitate was collected by centrifugation and washed, successively, with 50 ml of methanol, 15 ml of water, 10 ml of methanol and 10 ml of ether, and yielded 1·6 g of potassium *N*-phenylhydroxylamine pentacyanoferroate dipotassium acetate trihydrate as a purple powder that decomposed without melting at temperatures above 200° C. The composition of the powder is given below—

Element	C	H	Fe	K	N	Water
Found, per cent.	27·4	3·0	8·8	29·5	12·1	8·0
$C_{15}H_{19}N_6FeK_5O_8$ requires, per cent.	27·2	3·3	8·4	29·5	12·7	8·2

In a similar preparation, in which cupric acetate was used instead of potassium acetate and the precipitated cupric salt was washed thoroughly with water, 3·6 g of cupric *N*-phenylhydroxylamine pentacyanoferroate pentahydrate was obtained as a bluish-purple solid that decomposed without melting at temperatures above 240° C. The composition of the solid is given below—

Element	C	H	Fe	N	Water
Found, per cent.	31·2	2·2	11·9	19·0	10·9
$C_{22}H_{24}N_{12}Cu_3Fe_2O_7$ requires, per cent.	30·4	2·7	12·8	19·3	10·4

A solution containing 0·5 g of *N*-(3,4-dimethylphenyl)hydroxylamine and 0·8 g of sodium pentacyano-ammine ferroate in 150 ml of 30 per cent. v/v aqueous ethanol was set aside for 16 hours at 23° C, and then extracted with two 30-ml portions of ether. The aqueous fraction was evaporated to dryness under reduced pressure, and the bluish-purple residue was extracted with 120 ml of methanol. This extract was treated with 20 ml of ether, set aside for 16 hours at 0° C and then filtered. The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 30 ml of water and treated with a solution of 0·7 g of ferric chloride in 50 ml of water, and yielded a blue precipitate that was collected by centrifugation, washed with 50 ml of water and then with 50 ml of ethanol. This yielded 0·4 g of ferric *N*-(3,4-dimethylphenyl)hydroxylamine pentacyanoferroate pentahydrate as a blue powder that decomposed without melting at temperatures above 200° C. The composition of the powder is given below—

Element	Fe	N	Water
Found, per cent.	23·8	18·2	19·8
$C_{13}H_{21}N_6Fe_2O_6$ requires, per cent.	23·8	17·9	19·2

As in the preparation of the *N*-phenylhydroxylamine analogue, a solution containing 0·5 g of *N*-(2-carboxyphenyl)hydroxylamine and 0·8 g of sodium pentacyano-ammine ferroate in 150 ml of 30 per cent. v/v aqueous ethanol was set aside for 4 hours at 23° C, and gave 0·75 g of cupric *N*-(2-carboxyphenyl)hydroxylamine pentacyanoferroate dicupric acetate octahydrate as a bluish-purple powder that decomposed without melting at temperatures above 240° C. The composition of the powder is given below—

Element	C	H	Fe	N	Water
Found, per cent.	27·8	3·3	8·8	12·1	10·2
$C_{32}H_{42}N_{12}Cu_5Fe_2O_{22}$ requires, per cent.	27·9	3·1	8·1	12·2	10·5

PROCEDURES—

(*i*) *Salicylaldehyde - potassium ferrocyanide procedure*—The arylhydroxylamine was dissolved in aqueous ethanol (1 + 1) or urine - ethanol (1 + 1) containing 0·25 per cent. v/v of salicylaldehyde. After being set aside for 3 hours at 23° C, 5-ml portions were treated with 0·1 ml of a 5 per cent. w/v solution of potassium ferrocyanide in water. The resulting

solutions were mixed and heated at 70° C for 20 minutes, cooled in an ice - water mixture, and the optical densities measured at the appropriate wavelength (see Table I) against the appropriate blank solutions in 1-cm cells.

(*ii*) *Potassium ferrocyanide procedure*—The arylhydroxylamine was dissolved in ethanol and 1-ml portions were added to 9-ml portions of a 1 per cent. w/v solution of potassium ferrocyanide in urine or water. The solutions were mixed, heated at 60° C for 5 minutes, stored at 23° C overnight and the optical densities were measured as in procedure (*i*).

(*iii*) *Sodium pentacyano-ammine ferroate procedure*—The arylhydroxylamine was dissolved in ethanol, and 1-ml portions were added to 5-ml portions of a 0·05 per cent. w/v solution of sodium pentacyano-ammine ferroate in water or urine. Although the optical densities of the solutions remained constant between pH 6·0 and pH 8 (see under "Results"), the stock solutions of the reagent in water or in urine were adjusted to pH 7·0 with N hydrochloric acid or N sodium hydroxide before the arylhydroxylamine solution was added. The solutions were mixed and their optical densities measured as in procedure (*i*), 2 to 3 hours after mixing (unless otherwise stated).

Results

Dependence on pH—

Steady conditions of pH were maintained by using the buffer solutions listed below: 0·2 M acetate for pH 3·4 to 5·8, 0·2 M phosphate for pH 5·8 to 8·0, and 0·2 M borate for pH 8·0 to 10·0. *N*-Phenylhydroxylamine, or *N*-(2-naphthyl)hydroxylamine, was dissolved in ethanol and 1-ml portions were added to 9-ml portions of the appropriate buffer containing 0·05 per cent. w/v of sodium pentacyano-ammine ferroate, so that the final solutions contained 20 μg of arylhydroxylamine per ml. After the solutions had been set aside for 16 hours at 23° C, their optical densities were measured at the appropriate wavelengths (see Table I) in 1-cm cells. Both complexes showed maximum stability between pH 6·0 and pH 10, although the *N*-(2-naphthyl)hydroxylamine - pentacyanoferroate complex decomposed slightly between pH 8 and pH 10 (see Fig. 1).

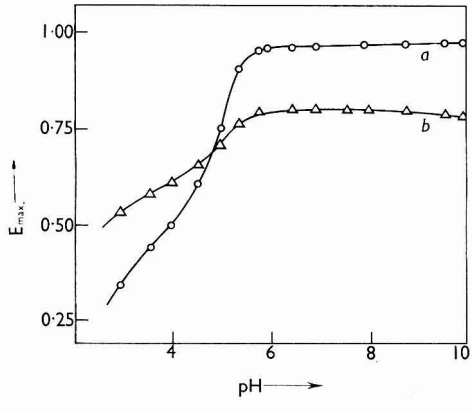

Fig. 1. Dependence of the reaction between arylhydroxylamines and pentacyano-ammine ferroate on pH: curve *a*, *N*-phenylhydroxylamine; curve *b*, *N*-(2-naphthyl) hydroxylamine

Dependence on time—

N-Phenylhydroxylamine, *N*-(2-naphthyl)hydroxylamine, *N*-(3,4-dimethylphenyl)-hydroxylamine or *N*-(2-carboxyphenyl)hydroxylamine was dissolved in ethanol, and 1-ml portions of the solution were added to 9-ml portions of a 0·05 per cent. solution of sodium pentacyano-ammine ferroate in 0·05 M phosphate buffer (pH 7) or to 9-ml portions of a 1 per cent. w/v solution of potassium ferrocyanide in the same buffer. The final concentrations of arylhydroxylamines in all the solutions were 15 μg per ml. The optical densities were

measured as described for procedures (*i*) to (*iii*) at the appropriate wavelengths (see Table I) at varying time intervals in a 1-cm cell. There was a gradual increase in intensity of colour in all solutions containing potassium ferrocyanide; this may be contrasted with the rapid (2 to 5 minutes) attainment of maximum colour by all solutions containing sodium pentacyano-ammine ferroate (see Fig. 2).

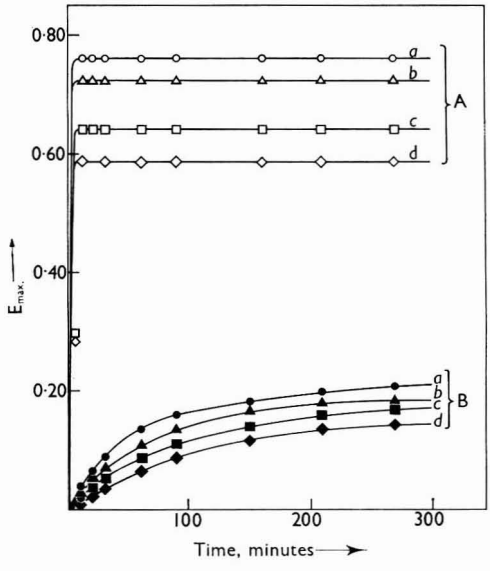

Fig. 2. Dependence of procedures (*ii*) and (*iii*) on time of reaction: curves *a*, *N*-(3,4-dimethylphenyl)hydroxylamine; curves *b*, *N*-phenylhydroxylamine; curves *c*, *N*-(2-carboxyphenyl)hydroxylamine; curves *d*, *N*-(2-naphthyl)hydroxylamine. Group A, determined by using procedure (*iii*); group B, determined by using procedure (*ii*)

TABLE I

COLOURS, ABSORPTION MAXIMA AND OPTICAL DENSITIES OF THE PENTACYANOFERROATE COMPLEXES

Pentacyanoferroate complex of	Colour*	λ_{max}, mμ	$E_{max.}$
o-Aminophenol	green	735	0·17
p-Aminophenol	greenish-blue	705	0·74
Nitrosobenzene	magenta	530	0·80
Aniline	green	680	0·12
N-Phenylhydroxylamine	magenta	535	0·94
N-(3,4-Dimethylphenyl)hydroxylamine	bluish-purple	560	1·02
N-(2,4,6-Trimethylphenyl)hydroxylamine	red	490	0·32
N-(2-Carboxyphenyl)hydroxylamine	bluish-purple	570	0·87
N-(4-Biphenylyl)hydroxylamine	bluish-purple	565	0·70
N-(2-Naphthyl)hydroxylamine	bluish-purple	560	0·79

* Colours were developed in a urine solution of the compounds, according to procedure (*iii*).

DEPENDENCE ON REAGENT CONCENTRATION—

One-millilitre portions of 0·001 M ethanolic solutions of *N*-phenylhydroxylamine and *N*-(3,4-dimethylphenyl)hydroxylamine were treated with 9-ml portions of varying concentrations of sodium pentacyano-ammine ferroate solutions in water to give 0·0001 M solutions of arylhydroxylamine containing varying molar proportions of the reagent. After the solutions had been set aside for 2 hours at 23° C, their optical densities were measured at the

appropriate wavelengths against a water blank solution in 1-cm cells. The results (see Fig. 3) indicated that (*a*) a (1 + 1) molar complex of arylhydroxylamine - reagent was formed, (*b*) the reaction was quantitative and (*c*) an eight-fold excess of the reagent did not affect the intensity of the colour.

COMPARISON OF THE SENSITIVITIES OF PROCEDURES (*i*), (*ii*) AND (*iii*)—

Solutions of *N*-phenylhydroxylamine, *N*-(2-naphthyl)hydroxylamine or *N*-(4,biphenylyl)-hydroxylamine in ethanol, containing 20 μg of arylhydroxylamine per ml, were determined by procedures (*i*), (*ii*) and (*iii*). The results showed that the sensitivities of the three procedures, in decreasing order, are (*iii*) > (*ii*) > (*i*), in either water or urine (see Table II).

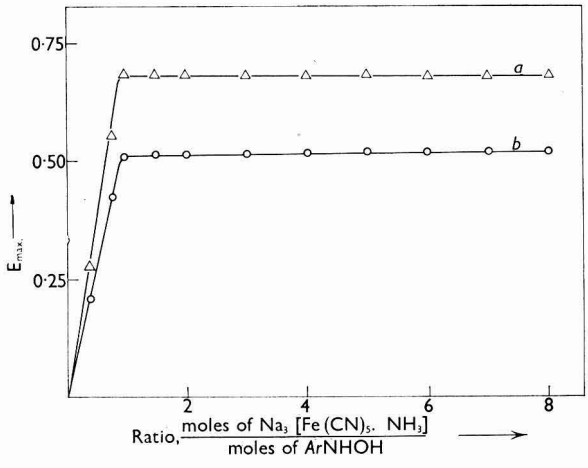

Fig. 3. Dependence of the reaction between arylhydroxylamines and pentacyano-ammine ferroate on concentration of reagent: curve *a*, *N*-(3,4-dimethylphenyl)hydroxylamine; curve *b*, *N*-phenylhydroxylamine

The coloured complexes formed by all of the procedures have identical absorption maxima for each arylhydroxylamine and are soluble in water, but insoluble in ether, benzene and chloroform. Hence, the sensitivity of all three methods may be increased if the final solutions are shaken with any of these solvents to concentrate the aqueous layer by removing the bulk of the ethanol.

INTERFERING SUBSTANCES AND ABSORPTION MAXIMA OF THE PENTACYANOFERROATE COMPLEXES—

The blank solutions obtained from normal human urine in procedures (*i*), (*ii*) or (*iii*), when compared with the corresponding water blank solutions, showed optical densities of 0·05 to 0·25, within the range 490 to 570 mμ; most of the arylhydroxylamine-pentacyanoferroate complexes absorb in this range. The colours, absorption maxima, and optical densities of solutions of some *N*- and *O*-substituted hydroxylamines and some of the known metabolites of aniline at a final concentration of 20 μg per ml in urine, obtained by procedure (*iii*), are shown in Table I. Hydroxylamine, *N*- and *O*-methylhydroxylamine, *N*-acetyl-*N*-phenylhydroxylamine and *N*-phenylhydroxylamine-*N*-sulphonic acid gave no appreciable colour after being set aside for 6 hours at 23° C. The known or possible metabolites of aniline that are listed below gave no colours, or slowly developed faint green or blue colours under similar conditions: *o*- and *p*-aminophenyl phosphates, *o*- and *p*-aminophenyl sulphates, *o*- and *p*-aminophenylglucosiduronic acids, aniline-*N*-glucosiduronic acid and *o*- and *p*-aminophenyl mercapturic acids. A comparison of the absorption spectra in the visible range of the pentacyanoferroate complexes of aniline and *o*- and *p*-aminophenols with that of

the corresponding *N*-phenylhydroxylamine complex shows almost no absorption by the first three in the 520 to 550 mμ region. Hence, *N*-phenylhydroxylamine may be determined in urine in the presence of all the known metabolites of aniline, but not in the presence of nitrosobenzene.

TABLE II

COMPARISON OF THE SENSITIVITIES OF THE PROCEDURES

(*i*) Salicylaldehyde - potassium ferrocyanide
(*ii*) Potassium ferrocyanide
(*iii*) Sodium pentacyano-ammine ferroate

Arylhydroxylamine	$E_{max.}$ in water			$E_{max.}$ in urine		
	Procedure			Procedure		
	(*i*)	(*ii*)	(*iii*)	(*i*)	(*ii*)	(*iii*)
N-Phenylhydroxylamine	0·32	0·42	0·96	0·30	0·40	0·94
N-(2-Naphthyl)hydroxylamine	0·25	0·38	0·79	0·23	0·36	0·79
N-(4-Biphenylyl)hydroxylamine	0·20	0·32	0·73	0·19	0·30	0·70

DETERMINATION OF ARYLHYDROXYLAMINES IN WATER OR IN URINE BY PROCEDURE (*iii*)—

The graphs of optical density *versus* concentration, for six arylhydroxylamines in water and in urine, are given in Figs. 4 and 5. Beer's law is obeyed over a wide range of concentrations; the lower limit of sensitivity is about 1 to 3 μg of arylhydroxylamine per ml, and

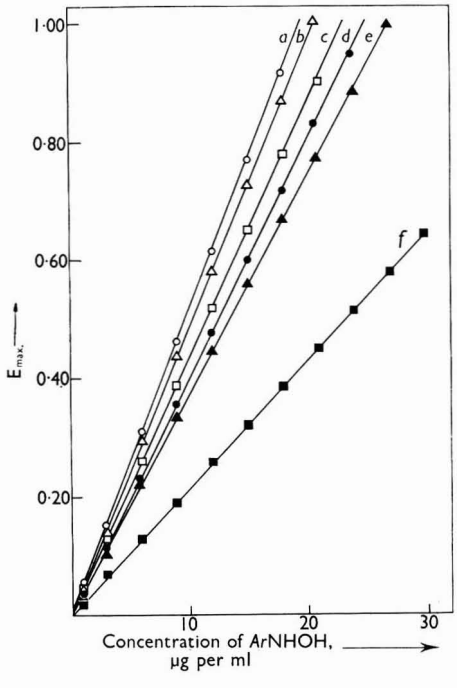

Fig. 4. Determination of arylhydroxylamines in water by using pentacyano-ammine ferroate, procedure (*iii*): curve *a*, *N*-(3,4-dimethylphenyl)hydroxylamine; curve *b*, *N*-phenylhydroxylamine; curve *c*, *N*-(2-carboxyphenyl)-hydroxylamine; curve *d*, *N*-(2-naphthyl)hydroxylamine; curve *e*, *N*-(4-biphenylyl)hydroxylamine; curve *f*, *N*-(2,4,6-trimethylphenyl)hydroxylamine.

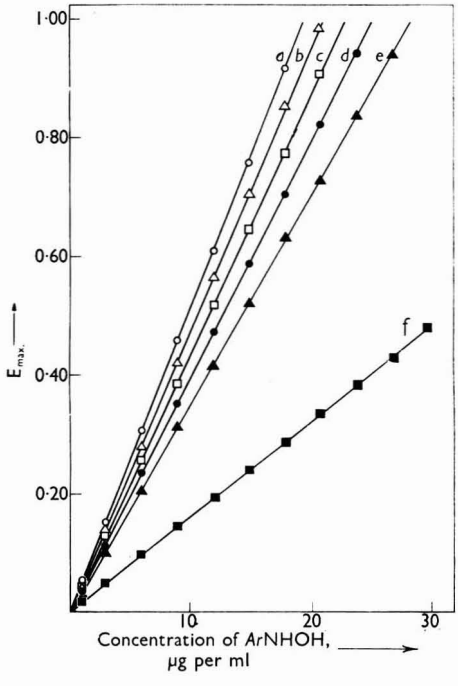

Fig. 5. Determination of arylhydroxylamines in urine by using pentacyano-amine ferroate, procedure (*iii*); curve *a*, *N*-(3,4-dimethylphenyl)hydroxylamine; curve *b*, *N*-phenylhydroxylamine; curve *c*, *N*-(2-carboxyphenyl)hydroxylamine; curve *d*, *N*-(2-naphthyl) hydroxylamine; curve *e*, *N*-(4-biphenyly)hydroxylamine; curve *f*, *N*-(2,4,6-trimethylphenyl)-hydroxylamine

the differences in sensitivities of urine and water solutions for each arylhydroxylamine, except for *N*-(2,4,6-trimethylphenyl)hydroxylamine, are very small. The sensitivity of the procedure for the arylhydroxylamines shown decreases in the order given below: *N*-(3,4-dimethylphenyl)- > *N*-phenyl- > *N*-(2-carboxyphenyl)- > *N*-(2-naphthyl)- > *N*-(4-biphenylyl)- > *N*-(2,4,6-trimethylphenyl)- hydroxylamines, and is unrelated to the molarity of the final solutions.

COLOURS PRODUCED WHEN AQUEOUS ETHANOLIC SOLUTIONS OF *N*-PHENYLHYDROXYLAMINE ARE HEATED WITH OTHER COMPLEX-FORMING SALTS—

One-millilitre portions of ethanolic solutions containing 240 μg of *N*-phenylhydroxylamine per ml were added to 5-ml portions of 1 per cent. w/v aqueous solutions of the salts listed below: (*a*) potassium ferricyanide; (*b*) potassium cobaltothiocyanate; (*c*) potassium cobalticyanide; (*d*) sodium cobaltinitrate; (*e*) sodium nitroprusside; and (*f*) potassium cyanide. The resulting solutions were mixed and heated at 70° C for 10 to 20 minutes. A reddish-purple colour was produced with (*a*) and (*e*), and an orange colour was produced with (*d*). No significant colour change was observed in the other solutions.

DISCUSSION OF RESULTS

The ferrocyanides and pentacyanoferroates are known to form coloured complexes with aromatic compounds,[11,12,13] primary aromatic amines and thioureas[14,15,16] and aromatic nitro compounds after reduction with zinc dust.[17] Ishidate and Sakaguchi[18] have shown that pentacyano-ammine ferroate, in the presence of hydroxylamine and aniline, forms a red complex having the structure $[Fe(CN)_4C(NH_2):NOH.C_6H_5NH_2]^{3-}$. Murati and Cupahin[13] have demonstrated that complex formation between ferrocyanide and nitrosobenzene involves a primary breakdown of ferrocyanide to the pentacyanoferroate ion, which is the effective complexing agent, and also that the reaction is catalysed by mercuric ions. Similar observations have been made for the reaction of ferrocyanide with thiourea in which the breakdown of ferrocyanide is catalysed by light[16]; the activation energy of the breakdown (reaction 1, below) is about 19·8 kcal per mole.[19] Complex formation between pentacyanoferroates and nitrosobenzene is also catalysed by light.[11] The reaction of ferrocyanide or a pentacyanoferroate with nitrosobenzene leads to the same end-product, *i.e.*, a nitrosobenzene pentacyanoferroate (see product **II** below, where **A** = C_6H_5NO). These complex-forming reactions may thus be represented by the general formula—

$$[Fe(CN)_6]^{4-} \xrightarrow{(1)} \underset{(\mathbf{I})}{[Fe(CN)_5]^{3-}} \xrightarrow{\mathbf{A}} \underset{(\mathbf{II})}{[Fe(CN)_5\mathbf{A}]^{3-}}$$

In the reactions of pentacyanoferroates (product **I** = $[Fe(CN)_5\mathbf{B}]^{3-}$, where **B** = NH_3, H_2O, etc.) with amino- or nitroso-aryls, **B** is replaced by a molecule of **A** to yield product **II**, where **A** = ArNO or ArNH$_2$. In the reactions described in this work, **A** = ArNHOH. Additional support for this hypothesis is provided by (*a*) the isolation of the complex salts of product **II**, where **A** is an arylhydroxylamine (see under "Preparation of Hydroxylamines"), (*b*) the demonstration that *N*-phenylhydroxylamine or *N*-(3,4-dimethylphenyl)hydroxylamine forms a 1 + 1 complex with sodium pentacyano-ammine ferroate (see Fig. 3) and (*c*) the instability of the arylhydroxylamine pentacyano-ferroates in acid media (see Fig. 1), which resembles that of the amminoprussides,[20] which have similar structures to the organic complexes.

Pentacyanoferroates having the general formula $[Fe(CN)_5\mathbf{A}]^{3-}$, where **A** = C_5H_5N, CH_3NH_2, or H_2O, have characteristic absorption bands in the 350 to 400 mμ and 700 mμ regions, but ferrocyanides do not absorb in the 700 mμ band.[20,21] Hence these reagents do not interfere with the determination of arylhydroxylamines that give complexes with ferrocyanide or pentacyano-ammine ferroate, and that absorb mainly in the 490 to 570 mμ region (see Table I).

Arylhydroxylamines decompose in aqueous solutions to yield aminophenols and the corresponding nitroso-, amino- and azoxy-derivatives.[22,23] Their decomposition is catalysed by acids, alkalis, and light. The reaction of arylhydroxylamines with sodium pentacyano-ammine ferroate is quick (see Fig. 2) and quantitative (see Fig. 3), and the coloured complexes formed are stable within broad limits of pH and time (see Figs. 1 and 2).

Procedure (*i*), though least sensitive, may be useful in those instances when an arylhydroxylamine is being liberated during a reaction, *e.g.*, during chemical or enzymic hydrolysis

of a conjugate; the nitrone formed is more stable than the parent hydroxylamine, which is again liberated when the former is heated with the reagent.[24]

We thank the following members of this Institute: Dr. D. Manson for a gift of *N*-(2-naphthyl)hydroxylamine; Mr. P. L. Grover for *N*-(2-carboxyphenyl)hydroxylamine; Mr. J. W. Gorrod for *N*-(4-biphenylyl)hydroxylamine; and Mr. C. L. Day for the micro-determination of potassium and iron. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

References

1. Cramer, J. W., Miller, J. A., and Miller, E. C., *J. Biol. Chem.*, 1960, **235**, 885.
2. Nelson, N., and Troll, W., *Fed. Proc.*, 1961, **20**, 41.
3. Boyland, E., Manson, D., and Nery, R., *Biochem. J.*, 1963, **86**, 263.
4. Kamm, O., *in* "Organic Syntheses," Collective Volume I, J. Wiley & Sons Inc., New York and London, 1941, p. 445.
5. Beckmann, E., *Leibigs Ann.*, 1909, **365**, 204.
6. Bamberger, E., and Rising, A., *Ibid.*, 1901, **316**, 284.
7. Bamberger, E., *Ber. dtsch. chem. Ges.*, 1900, **33**, 3626.
8. Bamberger, E., and Pyman, F. L., *Ibid.*, 1909, **42**, 2297.
9. Bell, F., Kenyon, J., and Robinson, P. H., *J. Chem. Soc.*, 1926, 1243.
10. Boyland, E., Manson, D., and Nery, R., *Ibid.*, 1962, 606.
11. Feigl, F., *J. Chem. Educ.*, 1944, **21**, 479.
12. Emschwiller, G., *Compt. Rend.*, 1954, **238**, 341.
13. Murati, I., and Cupahin, I. O., *Acta Pharm. Jugoslav.*, 1953, **3**, 20.
14. Anger, V., *Mikrochim. Acta*, 1937, **2**, 3.
15. Ohkuma, S., *J. Pharm. Soc. Japan*, 1952, **72**, 1101.
16. ——, *Ibid.*, 1954, **74**, 220.
17. ——, *Ibid.*, 1955, **75**, 1342.
18. Ishidate, M., and Sakaguchi, T., *Ibid.*, 1950, **70**, 308.
19. Asperger, S., Murati, I., and Cupahin, I. O., *J. Chem. Soc.*, 1953, 1041.
20. Cambi, L., and Szego, L., *Atti Accad. Naz. Lincei, Rend. Classe Sci. Fis. Mat. Nat.*, 1927, **5**, 636.
21. Shinra, K., and Masuda, I., *J. Chem. Soc. Japan, Pure Chem. Sect.*, 1954, **75**, 1211.
22. Bamberger, E., *Leibigs Ann.*, 1921, **424**, 233, 297.
23. ——, *Ibid.*, 1925, **441**, 207.
24. Boyland, E., and Nery, R., *J. Chem. Soc.*, 1963, 3141.

NOTE—References 10 and 24 are to Parts I and III of this series respectively.

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A Gas-chromatographic Apparatus for the Analysis of Mine Gases

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A gas chromatograph is described for the precise determination of hydrogen, methane, oxygen and nitrogen in mine gases. A novel feature of the apparatus is the use of a transistorised stabiliser circuit for maintaining the column, sample loop and detector block at a constant temperature. Transistorised voltage and current stabilisers are used for supplying the heater and bridge circuits. A zero-suppression technique, in which a standard cell and an automatic standardiser is used in the potentiometric recorder, is incorporated to improve the precision of oxygen and nitrogen determinations.

The 5 per cent. limits of error of a single determination for the instrument are $\pm 0{\cdot}0014$ at the 0·1 per cent. level and $\pm 0{\cdot}0137$ at the 1·1 per cent. level for hydrogen, $\pm 0{\cdot}026$ for methane, $\pm 0{\cdot}03$ for oxygen and $\pm 0{\cdot}12$ for nitrogen.

Gas-solid chromatography applied to mine gases offers the advantages, over classical chemical-absorption and combustion methods, of improved precision, speed, simplicity of operation and freedom from bias on the part of the operator. Systems for analysing permanent gases have been described by Whatmough,[1,2] Timms, Konrath and Chirnside,[3] and Vizard and Wynne.[4] This paper describes an instrument with novel features that has proved accurate and reliable for determining hydrogen, methane, oxygen and nitrogen in mine gases. The improved precision of both oxygen and direct nitrogen determinations is of special benefit in studying underground explosions and heatings.

Apparatus

A flow diagram of the apparatus is shown in Fig. 1.

General description—

The carrier gas passes through a purification train to remove carbon dioxide and moisture, and the sample is injected *via* a six-way nylon-faced valve and a copper loop into the carrier-gas stream passing to the separating column. A "twin-column" technique is used, because the inherent stability of the method is high if both columns are packed and activated at the same time to ensure that their flow impedances are identical. A hot-wire katharometer is used as a detector, and the signal is attenuated and fed to a potentiometric recorder.

Experience has shown that when a chromatograph is used in conditions of fluctuating ambient temperature, some form of temperature stabilisation for the sample loop, column and katharometer is necessary to maintain operating stability. Constant-temperature baths were considered cumbersome. An electronic temperature controller[5,6] that maintained operating conditions to within $\pm 0{\cdot}1^\circ$ C was adapted for this purpose.

A simple transistorised voltage stabiliser is used to supply the temperature controller, and the bridge circuit is supplied by an independent current stabiliser.

For optimum operating conditions the carrier-gas flow-rate is adjusted to give the maximum katharometer response. Small variations of flow-rate above or below this optimum value produce little variation in sensitivity. The temperature controllers and bridge network are switched on and the bridge current adjusted to 210 mA. When the temperatures of the column and katharometer have reached 30° C, as indicated by the drop in current measured by the ammeter in the control circuit, the instrument is ready for use. Before analysing an unknown sample, the instrument is calibrated with mixtures of known composition, whose levels of concentration are close to the levels in the unknown sample. The response of the katharometer can be taken as linear over limited ranges of oxygen and nitrogen, and the composition of the unknown sample is calculated by comparing the peak heights of the unknown sample with those of the calibration sample. Small changes in gas flow-rate, bridge current and atmospheric pressure may alter the response and regular calibration is necessary.

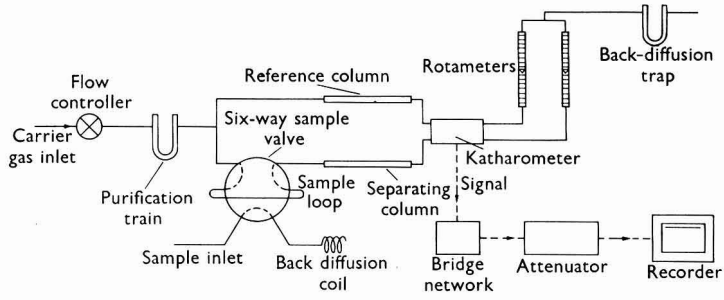

Fig. 1. Flow diagram of gas-chromatographic apparatus

The apparatus is assembled in a case whose dimensions are 30 × 14 × 20 inches. The back is hinged and one side is removable; this allows easy access to the column, katharometer and sampling valve. All tubing is of copper and joints are made by soldering, or by short connecting pieces of poly(vinyl chloride) tubing where soldering is impossible. Controls are conveniently situated on the front of the instrument.

The only external requirements of the apparatus are a 230-volt mains supply and a cylinder of argon fitted with a two-stage reducing valve. The component parts of the chromatograph cost £125 and the potentiometric recorder £200, making a total cost (excluding labour) of £325.

Details of apparatus

Separating columns—

The two columns used in the instrument were 6-foot 2-inch lengths of $\frac{3}{16}$-inch bore annealed-copper tubing packed to within one inch of each end with Union Carbide (formerly known as Linde) Molecular Sieve, Type 13X, and gave 6 feet of active column.

Work with earlier columns showed that, to obtain the precision necessary for the determination of oxygen and nitrogen, some form of temperature control would be essential. A comparison of chromatograms obtained from a mixture of hydrogen and methane in air

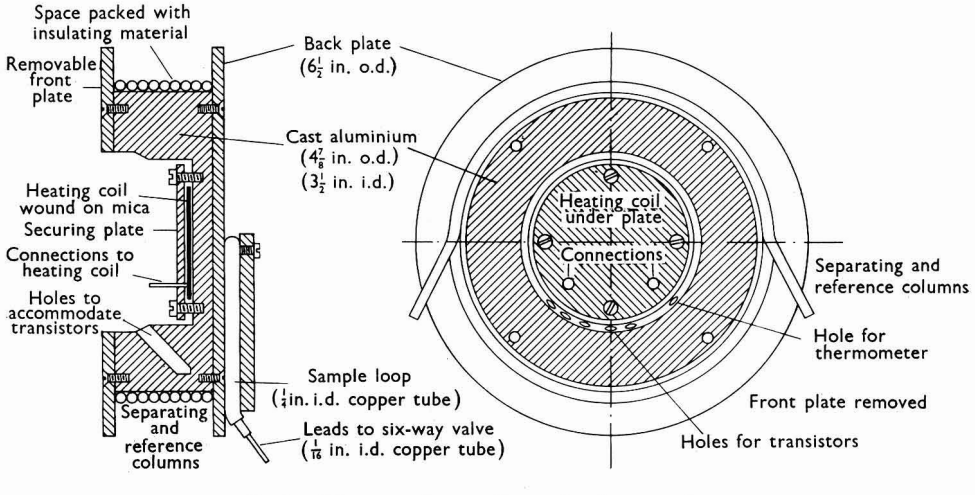

Fig. 2. Diagram of separating and reference columns

showed that peak heights in some instances varied substantially with column temperature. Raising the column temperature decreased the sensitivity of the instrument to hydrogen and increased the sensitivity to oxygen and, particularly, methane. Heating the column to 80° C increased the peak-height sensitivity to methane threefold. It is stressed that only peak heights were affected since the actual amount of constituent emerging from the column, and therefore peak area, was the same. However, the change of the retention time of the constituent altered the peak shape. Peak heights were normally used for evaluating chromatograms as they were easily and accurately measured.

The columns were wound side by side on an aluminium cylinder block, $4\frac{7}{8}$ inches in diameter and 2 inches wide, fitted with end-plates, to form a bobbin, see Fig. 2. Each column required space for four turns, *i.e.*, one inch, so that both columns fitted the available space on the block exactly. The space remaining between the end-plates of the block was filled with insulating material, to reduce the heat loss from the columns themselves. This ensured that the temperature of the columns was the same as that of the block. Provision was made on the side of the block for the sample loop. As the bobbin was a substantial mass of aluminium having high thermal conductivity, its temperature could be accurately controlled.

When the temperature controller (see below, under "Electrical Circuit, (*iv*)," p. 109) was set at 30° C, it would maintain the temperature of the block to within $\pm 0{\cdot}1$° C over a wide range of ambient temperature conditions. The current used was between 0·5 and 0·8 A at 12 V, the initial surge being 1·5 A.

Union Carbide Molecular Sieve, Type 13X, was used as column packing material. The $\frac{1}{8}$-inch pellets were crushed in an agate mortar, wet-sieved through a British Standard sieve, and the 30- to 60-mesh fraction retained. The free water was removed by drying the powder in an air oven overnight at 110° C.

Both columns were plugged at one end with copper gauze and supported vertically over a wooden block. Small amounts of molecular sieve were added, and the columns tamped

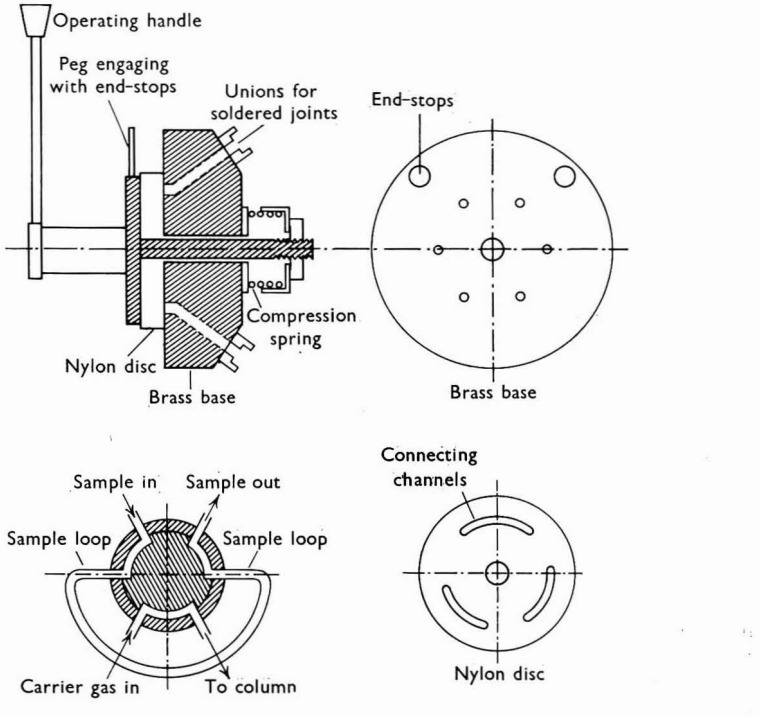

Fig. 3. Diagram of six-way sampling valve

against the wooden block; the filling operation was spread over 10 minutes. Great care was taken to pack both columns identically. The columns were then wound round a 4½-inch diameter former.

The packing material was activated by heating it for 2 hours at 250° C in a muffle furnace to remove water, and then for a further 1½ to 2 hours at 350° C. Argon was passed through the columns during the activation process. The final state of activation of the columns could not be accurately predicted and the preparation required some exploratory work.

SAMPLING VALVE—

The six-way valve is a modification of that described by Timms, Konrath and Chirnside,[3] and is illustrated in Fig. 3. The valve body was made of brass with one face accurately ground flat and polished. A nylon disc was held against the valve body under spring tension. Considerable care was necessary to ensure that both faces were as flat as possible to prevent leaks. A trace of high-vacuum grease was used as lubricant.

Three channels were cut in the nylon face to overlap the six gas-ports drilled in the brass face, so that in one position the carrier gas was carried straight to the column and the sample loop was swept out with the gas sample. The carrier-gas stream was diverted through the sample loop by rotating the nylon disc through 60° C. This swept the plug of sample into the column. End-stops were provided on the valve. Slight score marks appeared on the brass face after some use owing to rubbing by the nylon, and it is suggested that a harder material would be more suitable.

SAMPLE LOOP—

The volume of the sample loop was approximately 5 ml. Since fluctuations in room temperature will affect the amount of sample occupying the loop, it is essential for accurate work that the temperature of the loop should be constant. The constant-temperature block used for the column was convenient for this purpose. The sample loop was bolted to the side of the block under a thick plate of aluminium, to provide good thermal contact. Care was taken to avoid sudden changes of section in the sample-loop assembly, as the ensuing turbulence might have led to the sample plug mixing with the carrier gas. Small-bore copper connecting tubing was used so that the maximum possible volume of the sample loop was in contact with the constant-temperature block. A coil of small-bore tubing was also attached to the sample-outlet port of the six-way valve (see Fig. 3), to prevent air diffusing back into the sample loop.

CARRIER-GAS FLOW-RATE CONTROL—

Control of the argon carrier-gas flow was achieved by using a two-stage reducing valve, set at 5 lb per sq. in., on the supply cylinder in series with an Edwards (Type V.P.C.I.) diaphragm reducing-valve for fine adjustment. The carrier-gas flow-rate was divided equally between the separating and reference columns, and the flow-rate was measured by rotameters at the column outlets. The carrier-gas streams were reunited and passed through a silica-gel drying-train to prevent moisture diffusing back into the columns when they were not in use. The response of the katharometer was dependent on the carrier-gas flow-rate, and there was an optimum flow-rate, at which the response was a maximum. Small variations of flow-rate about this optimum value produced little variation in response. The optimum flow-rate must be determined for each combination of column and detector, by plotting a graph of detector response *versus* flow-rate. It was 60 ml per minute for the column we used.

DETECTOR UNIT—

The katharometer block is shown in Fig. 4. The main features were (*a*) removable sensing-elements, which allowed spares to be used, and (*b*) long tensioning-springs to overcome the thermal expansion of the 0·001-inch diameter platinum sensing-wire, without serious loss of tension. This design also facilitated renewal of the platinum sensing-wires. New elements can be screwed into position and electrically connected while the old elements are being repaired.

Each element consisted of a threaded brass plug which carried a hairpin loop of 14 s.w.g. wire insulated from the element with a Perspex ring. The hairpin loop was varnished with shellac to provide electrical insulation as a precaution against it touching the walls of the

chambers within the block. A length of 0·001-inch diameter platinum wire was soft-soldered to the inside of the support of the hairpin loop and to the end of the tension spring in such a way that exactly 2 inches of free wire was left between the solder joints. A 10 g weight was hung from the outer end of the spring before it was fastened in position. A second element was then made having an identical electrical resistance. Two finely drawn glass capillary-tubes were glued with shellac across each hairpin loop to provide intermediate

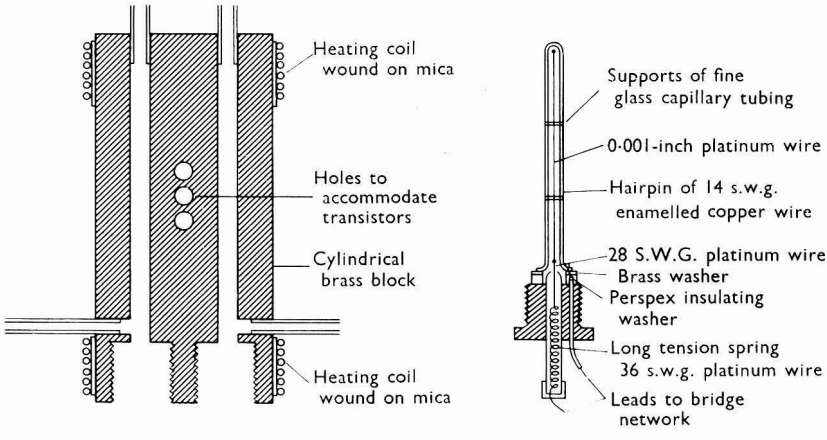

Fig. 4. Diagram of the katharometer

supports for the 0·001-inch diameter wire. This was found to improve the stability of the detector against mechanical vibration. All holes drilled through the brass plug were sealed with Apiezon wax, and a neoprene washer was used to prevent leaks through the plug threads The katharometer block was provided with a temperature control (see "Electric Circuit (*iv*)," p. 109).

ELECTRICAL CIRCUIT—

The electrical circuit is shown in Fig. 5 and can be conveniently divided into five parts—

(*i*) bridge network and attenuator,
(*ii*) current-stabilised bridge supply,
(*iii*) zero-suppression circuit,
(*iv*) temperature-controller circuit and
(*v*) voltage-stabilised temperature controller.

(*i*) *Bridge network*—The detecting and reference elements formed two adjacent arms of a Wheatstone-bridge circuit, and the other two arms consisted of fixed resistors wound from silk-covered manganin wire. The elements were connected in parallel so that each carried half the total bridge current. The fixed resistors were of relatively high value (89 ohms), compared with the hot resistance of the elements (14 ohms). This minimised current changes in the bridge as the peaks emerged. The zero control was a 1-ohm 10-turn helical potentiometer (Colvern type CLR/25/01) that incorporated a straight (not coiled) resistance wire allowing infinite adjustment to be made. The bridge current was measured with a 0- to 500-mA $3\frac{3}{4}$-inch full-scale deflection milliammeter and the bridge voltage with a similar 0- to 10-V voltmeter with a total bridge current of 210 mA. A typical output was 38 mV when a sample containing 20 per cent. oxygen was analysed.

The signal was attenuated by a chain of resistors, whose resistance was high compared with that of the bridge circuit to minimise signal loss. The resistors are shown in Fig. 5 as R_{17} to R_{25}. Nine sensitivity steps were provided, and these were selected by a silver-contact rotary switch mounted in the front panel of the instrument. All resistors were wound with silk-covered manganin wire to a close tolerance.

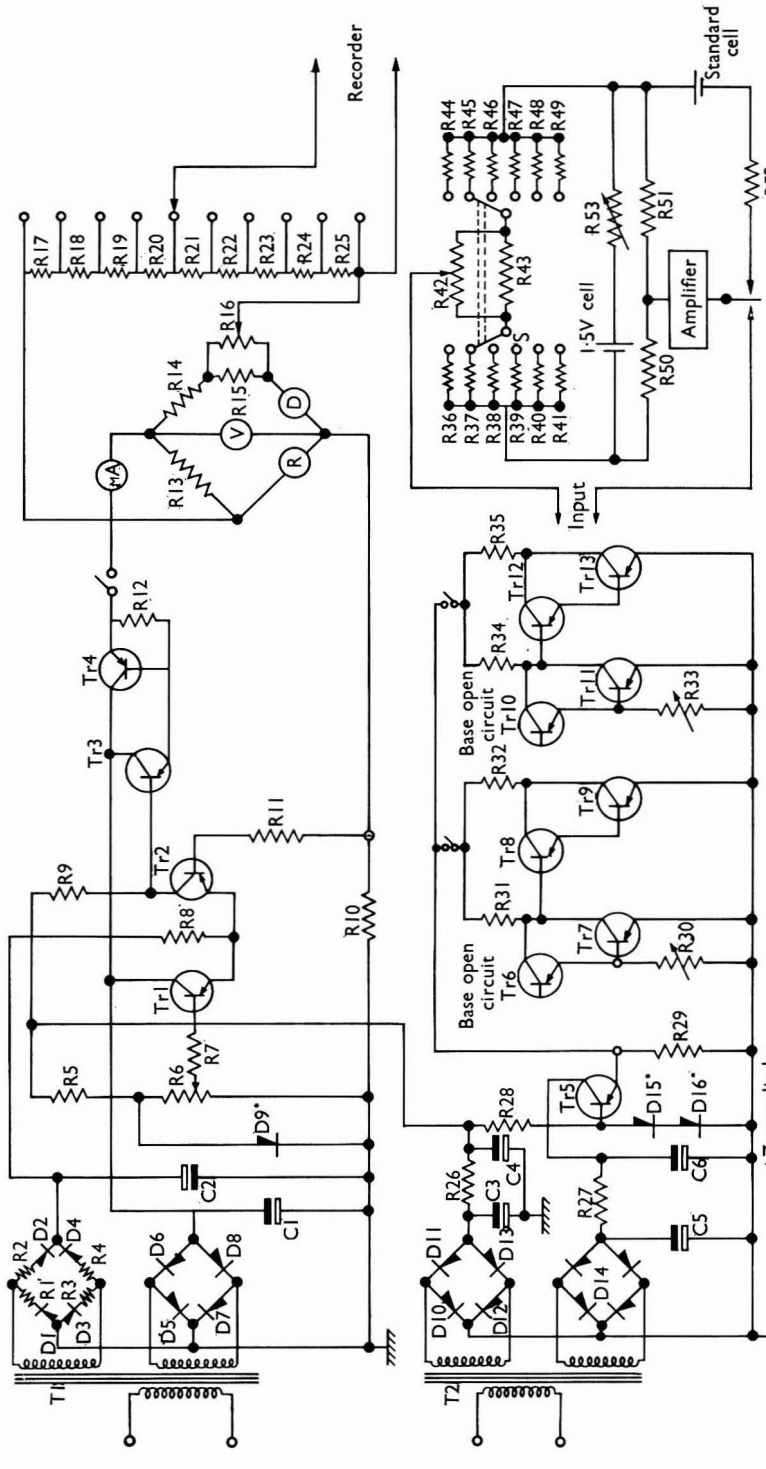

Fig. 5. Diagram of electrical and zero-suppression circuits (for values of components, see Appendix, p. 113)

(*ii*) *Current-stabilised bridge supply*—The reference wire in the katharometer was kept in the carrier gas and the detecting wire in the column-effluent stream. The temperature of the detecting wire changed as a function of the thermal conductivity of the environmental gas. Since the electrical resistance of the wires was a function of their temperature, the electrical bridge circuit was unbalanced proportionately to the change of thermal conductivity of the column effluent. It was therefore important to maintain the sensing wires at a constant temperature. One necessary condition was to supply the katharometer with a stable current supply, since the heating effect in the sensing wires is proportional to the square of the current, and that the resistance of the sensing wire is a function of its temperature. The transistorised current-stabilised supply used was based on the pattern of the voltage stabiliser described by Brown and Stephenson.[7] This was a mains operated d.c. power supply, stabilised by d.c. feedback and incorporating a 6-V Zener diode as a voltage reference. The stabiliser consisted of a two-transistor emitter-follower, Tr_3 and Tr_4 (see Fig. 5), and a phase-reversing amplifier, Tr_1 and Tr_2, providing a feedback that reduced the d.c. output resistance by a factor of several hundred. The feedback was obtained by passing the output current through the resistor, R_{10}, and comparing the voltage developed with that across the Zener diode, D_9. The resistor, R_6, provided adjustment of the reference source and therefore served as bridge-current adjustment. Any variation of voltage across R_{10} was then amplified by Tr_2 and applied to the emitter-follower stages, which stabilised the output.

Variation of output voltage caused by temperature effects was minimised by using a "long-tailed pair" configuration as an amplifier, since the temperature variations related to Tr_1 and Tr_2 were equal. In addition, these two transistors were mounted in holes drilled in the aluminium column block to maintain them at a constant temperature.

The effect of variation in the unstabilised supply on the output was reduced considerably by supplying the feedback amplifier from a separate line.

The transistor, Tr_4, must be mounted on a heat sink. The output of the stabiliser was 200 to 220 mA at 12 V.

(*iii*) *Zero-suppression circuit*—One of the factors limiting the accuracy of the determination of oxygen and nitrogen, the main constituents of mine gases, was the practical difficulty of measuring the peaks on the recorder chart. Vizard and Wynne[4] pointed out that if a 20 per cent. oxygen content was represented by a peak of 10 inches, the peak height would have to be measured to $\pm 0{\cdot}01$ inch for the accuracy to be similar to that obtained by an experienced operator using a Haldane apparatus. They used a zero-suppression technique as a separate circuit to overcome the difficulty of measuring peak height. By using this concept, an improved circuit was devised, making full use of the Leeds and Northrup Speedomax potentiometric recorder (1 mV span). In the present instrument a backing-off potential was applied to the output of the katharometer so that only the output in excess of this was recorded on the chart. It was therefore possible to represent a narrow range of oxygen concentration on the full width of the chart, *e.g.*, 19 to 21 per cent. of oxygen, with a corresponding increase in the accuracy with which these readings could be made. The circuit shown in Fig. 5 involves the use of the existing standard cell and automatic standardiser supplied with the potentiometric recorder. The resistors were hand-wound with silk-covered manganin wire to pre-calculated values. Manganin was used because of its negligible temperature coefficient of resistance. The circuit was simple and had the advantage of being fully automatic. Five ranges of backing-off potential were used, *viz.*, 9 to 13 mV, and each range covered a band of 1·8 per cent. of oxygen and 7·3 per cent. of nitrogen.

(*iv*) *Temperature-controller circuits*—Independent temperature-controller circuits were used for the column and katharometer.

The circuit made use of the fact that the leakage current of a transistor is greatly influenced by temperature. The value rises exponentially, approximately doubling for every 8° C rise in temperature. Transistor, Tr_6, which acted as the sensing element of the column stabiliser, and Tr_{10} controlled the katharometer stabiliser. Tr_7 and Tr_8, with their counterparts, Tr_{11} and Tr_{12}, were signal-amplifying transistors, feeding their respective power transistors, Tr_9 and Tr_{13}. The power transistor, Tr_9, controlled the current passing through the heater coil, R_{32}, in such a way that, as the columns reached a pre-set temperature, the heating current was reduced in an infinitely variable manner. The power transistor, Tr_{13}, controlled the katharometer heater coil, R_{35}, in a similar manner.

The column transistors, Tr_5, Tr_6 and Tr_7 were mounted close together within the bottom of the rim of the aluminium block, as shown in Fig. 2. The heating coil, R_{32}, was wound on mica in the manner of a domestic electric-iron element, and mounted centrally within the block under a securing plate. The transistor, Tr_9, liberated a moderate amount of heat and was therefore kept above and away from the block. The resistor, R_{30}, was a pre-set control that provided a means of setting the temperature. A thermometer was fitted for checking the temperature.

The katharometer temperature control was similar to that of the column. The sensing transistor, Tr_{10}, and the amplifying transistors, Tr_{11} and Tr_{12}, were mounted close together in three separate holes drilled along a radius near the centre of the block (as in the diagram). The power transistor, Tr_{13}, was mounted separately as it generated an appreciable amount of heat. The heating coil of flat nichrome wire was wound on micanite in two portions and in series at each end of the block, to provide a symmetrical arrangement about the transistors. As with the column, the temperature control was to within $\pm 0{\cdot}1^\circ$ C at 30° C. This was measured by a thermometer mounted vertically in a hole drilled in the block. The initial current consumption of the circuit was 1·5 A, rapidly dropping at 30° C to between 0·2 and 0·8 A, depending on the room temperature. The time taken to reach equilibrium was 8 to 10 minutes. The total current consumed by both column- and katharometer-control circuits was measured with an ammeter on the front panel of the instrument.

(*v*) *Voltage-stabilised temperature-controller supply*—The temperature controllers were supplied from a simple 12-V transistorised voltage stabiliser. The single transistor was arranged as an emitter-follower with the base connected to a voltage reference source, consisting of two Zener diodes in series. In this type of circuit the emitter voltage tends to follow the base voltage, which was held constant by the voltage reference source. The difference between these voltages, V_{be}, is of the order of 0·5 V. Owing to the high collector-resistance, the variation of V_{be} with current was negligible, but at a fixed current, V_{be} varied with temperature by 2·5 mV per °C. Hence, even with a constant load, the output voltage varied with the transistor working temperature. The stabiliser has, however, proved adequate. The output was 12 V at 3A.

TABLE I

RESULTS OF HYDROGEN AND METHANE DETERMINATIONS ON GAS CHROMATOGRAPH

Sample number	Hydrogen content, per cent. v/v. Determination A	B	C	D	E	F	Range of differences	Methane content, per cent. v/v. Determination A	B	C	D	E	F	Range of differences
1	0·111	0·113	0·113	0·112	0·113	0·113	0·002	3·70	3·68	3·69	3·70	3·70	3·69	0·03
	0·111	0·113	0·113	0·113	0·112	0·113		3·71	3·68	3·69	3·70	3·70	3·69	
2	0·113	0·112	0·112	0·113	0·113	0·112	0·001	1·10	1·11	1·11	1·09	1·12	1·11	0·03
	0·113	0·112	0·112	0·112	0·113	0·112		1·10	1·10	1·11	1·09	1·12	1·11	
3	1·060	1·060	1·050	1·050	1·050	1·060	0·010	3·73	3·71	3·73	3·74	3·73	3·72	0·04
	1·060	1·060	1·060	1·050	1·050	1·060		3·73	3·70	3·72	3·73	3·73	3·72	
4	1·130	1·120	1·120	1·130	1·120	1·120	0·010	0·99	1·01	0·99	0·99	0·98	1·00	0·03
	1·130	1·120	1·130	1·130	1·120	1·130		1·00	1·01	0·99	0·99	0·98	1·00	
5	0·113	0·110	0·113	0·113	0·112	0·113	0·003	3·16	3·12	3·13	3·12	3·14	3·13	0·04
	0·113	0·111	0·113	0·113	0·113	0·113		3·16	3·12	3·12	3·12	3·14	3·13	
6	0·116	0·115	0·115	0·115	0·116	0·115	0·001	1·05	1·04	1·08	1·05	1·05	1·06	0·04
	0·116	0·115	0·115	0·116	0·116	0·115		1·05	1·04	1·07	1·05	1·05	1·06	
7	1·060	1·060	1·030	1·060	1·050	1·060	0·030	3·48	3·45	3·46	3·44	3·46	3·45	0·05
	1·060	1·060	1·030	1·060	1·050	1·060		3·49	3·45	3·45	3·44	3·46	3·45	
8	1·060	1·060	1·060	1·050	1·060	1·060	0·010	1·08	1·08	1·04	1·06	1·05	1·06	0·04
	1·060	1·060	1·060	1·050	1·060	1·060		1·08	1·08	1·05	1·06	1·05	1·05	
9	0·114	0·114	0·113	0·114	0·114	0·114	0·001	3·68	3·68	3·63	3·65	3·66	3·66	0·07
	0·113	0·114	0·113	0·114	0·114	0·114		3·68	3·68	3·61	3·65	3·66	3·65	
10	0·111	0·109	0·110	0·111	0·111	0·110	0·002	1·18	1·20	1·19	1·19	1·20	1·19	0·02
	0·111	0·109	0·110	0·111	0·110	0·110		1·19	1·20	1·19	1·18	1·20	1·20	
11	1·190	1·190	1·180	1·190	1·200	1·190	0·020	3·45	3·44	3·42	3·43	3·45	3·45	0·03
	1·190	1·190	1·180	1·190	1·200	1·190		3·45	3·44	3·42	3·44	3·45	3·45	
12	1·250	1·250	1·260	1·250	1·250	1·260	0·010	1·39	1·41	1·39	1·38	1·39	1·40	0·03
	1·250	1·250	1·260	1·260	1·250	1·260		1·39	1·41	1·40	1·38	1·39	1·40	
	Minimum						0·001	Minimum						0·02
	Maximum						0·030	Maximum						0·07
	Average						0·008	Average						0·04

Analysis of synthetic mixtures

Twelve argon-free gas mixtures were prepared, covering two levels of hydrogen (0·1 and 1·1 per cent.), two of methane (1·1 and 3·5 per cent.) and ranges of 17·5 to 20·5 per cent. of oxygen and 73·5 to 77·5 per cent. of nitrogen. Carbon dioxide was used to "make up" to 100 per cent. where necessary. The mixtures were prepared in a high-vacuum gas-mixture apparatus and pumped to storage tubes to await analysis. Analyses of all 12 mixtures in random batches were carried out on the chromatograph 6 times over a period of 3 weeks. Each determination was made in duplicate, first without and then with, zero suppression for oxygen and nitrogen (*i.e.*, 144 determinations in all).

The results are presented in Tables I, II and III.

Table II

Results of oxygen determinations, with and without zero suppression

Sample number	Oxygen content, per cent. v/v, without zero suppression. Determination A	B	C	D	E	F	Range of differences	Oxygen content, per cent. v/v, with zero suppression Determination A	B	C	D	E	F	Range of differences
1	18·79	18·68	18·80	18·75	18·81	18·82	0·14	18·76	18·75	18·77	18·74	18·77	18·77	0·03
2	18·50	18·48	18·45	18·40	18·41	18·42	0·10	18·46	18·47	18·47	18·41	18·44	18·44	0·06
3	18·80	18·82	18·71	18·76	18·75	18·81	0·11	18·77	18·78	18·74	18·77	18·77	18·76	0·04
4	18·25	18·17	18·16	18·20	18·19	18·18	0·09	18·18	18·19	18·18	18·18	18·16	18·16	0·03
5	20·29	20·30	20·21	20·24	20·22	20·26	0·09	20·24	20·20	20·20	20·21	20·19	20·20	0·05
6	19·35	19·38	19·29	19·31	19·36	19·35	0·09	19·36	19·38	19·33	19·36	19·36	19·33	0·05
7	19·86	19·84	19·81	19·90	19·83	19·85	0·09	19·82	19·84	19·79	19·82	19·82	19·83	0·05
8	17·69	17·73	17·71	17·70	17·74	17·74	0·05	17·68	17·65	17·66	17·67	17·64	17·66	0·04
9	21·65	21·69	21·68	21·71	21·72	21·71	0·07	21·72	21·70	21·71	21·74	21·70	21·70	0·04
10	20·20	20·14	20·17	20·13	20·15	20·13	0·07	20·14	20·10	20·12	20·12	20·10	20·11	0·04
11	20·47	20·49	20·52	20·48	20·55	20·53	0·08	20·53	20·49	20·50	20·51	20·51	20·50	0·04
12	18·58	18·54	18·56	18·51	18·53	18·49	0·09	18·60	18·56	18·58	18·57	18·59	18·57	0·04
	Minimum						0·05	Minimum						0·03
	Maximum						0·14	Maximum						0·06
	Average						0·09	Average						0·04

Table III

Results of nitrogen determinations, with and without zero suppression

Sample number	Nitrogen content, per cent. v/v, without zero suppression. Determination A	B	C	D	E	F	Range of differences	Nitrogen content, per cent. (v/v), with zero suppression Determination A	B	C	D	E	F	Range of differences
1	77·42	77·31	77·49	77·60	77·29	77·35	0·31	77·38	77·31	77·32	77·28	77·28	77·40	0·12
2	77·19	77·38	77·27	77·09	77·35	77·30	0·29	77·41	77·36	77·33	77·29	77·35	77·31	0·12
3	76·41	76·20	76·21	76·04	76·10	76·24	0·37	76·32	76·22	76·28	76·22	76·20	76·31	0·12
4	76·80	76·70	76·44	76·47	76·51	76·59	0·36	76·63	76·54	76·57	76·61	76·53	76·54	0·05
5	75·85	75·99	76·20	76·18	76·00	76·01	0·35	76·06	76·19	76·11	76·13	76·04	76·06	0·15
6	75·98	75·88	75·87	75·99	76·10	76·10	0·23	76·13	75·96	76·04	75·99	75·96	75·89	0·24
7	75·60	75·83	75·91	75·90	75·63	75·76	0·31	75·83	75·81	75·90	75·95	75·89	75·86	0·14
8	75·73	75·92	75·87	75·68	75·89	75·71	0·24	75·76	75·81	76·86	75·79	75·74	75·74	0·12
9	74·64	74·43	74·71	74·73	74·39	74·76	0·37	74·50	74·48	74·61	74·40	74·42	74·45	0·21
10	76·30	76·01	76·27	75·98	75·87	76·12	0·43	76·01	75·90	75·92	75·89	75·99	75·90	0·12
11	74·63	74·82	74·92	74·60	74·76	74·83	0·29	74·89	74·60	74·80	74·82	74·78	74·77	0·29
12	73·58	73·59	73·47	73·47	73·86	73·72	0·25	73·73	73·68	73·68	73·60	73·77	73·75	0·15
	Minimum						0·23	Minimum						0·05
	Maximum						0·43	Maximum						0·29
	Average						0·32	Average						0·15

The composition of the mixtures (*a*) calculated from making up in the mixture apparatus, (*b*) mean gas-chromatograph results and (*c*) mean of duplicate determinations by one operator on the Haldane apparatus are presented in Table IV.

TABLE IV

COMPARISON OF COMPOSITION OF MIXTURES

(*a*) Gas-mixture apparatus
(*b*) Gas chromatograph
(*c*) Haldane apparatus

Sample number	Apparatus	Composition, per cent. v/v				
		Hydrogen	Oxygen*	Nitrogen*	Methane	Carbon dioxide
1	(*a*)	0·113	18·68	77·20	3·79	0·22
	(*b*)	0·113	18·76	77·32	3·69	—
	(*c*)	0·06	18·59	77·77	3·18	0·40
2	(*a*)	0·113	18·48	77·31	1·06	3·04
	(*b*)	0·112	18·45	77·34	1·11	—
	(*c*)	0·11	17·30	78·42	1·04	3·13
3	(*a*)	1·115	18·64	76·53	3·66	0·05
	(*b*)	1·06	18·77	76·26	3·72	—
	(*c*)	1·05	18·69	76·33	3·83	0·10
4	(*a*)	1·115	18·06	76·46	1·02	3·34
	(*b*)	1·13	18·18	76·59	0·99	—
	(*c*)	1·10	18·19	76·44	1·07	3·20
5	(*a*)	0·114	20·31	76·25	3·21	0·12
	(*b*)	0·113	20·21	76·10	3·13	—
	(*c*)	0·12	20·10	76·53	3·09	0·16
6	(*a*)	0·114	19·44	75·73	1·02	3·70
	(*b*)	0·115	19·35	76·00	1·05	—
	(*c*)	0·05	19·38	78·04	0·96	3·57
7	(*a*)	1·077	20·01	75·34	3·52	0·05
	(*b*)	1·05	19·82	75·87	3·46	—
	(*c*)	1·10	19·71	75·84	3·24	0·06
8	(*a*)	1·150	17·51	75·53	0·99	4·82
	(*b*)	1·06	17·66	75·80	1·06	—
	(*c*)	1·05	17·85	75·34	0·81	4·95
9	(*a*)	0·114	21·76	74·38	3·56	0·19
	(*b*)	0·114	21·71	74·48	3·66	—
	(*c*)	0·10	21·76	74·33	3·64	0·17
10	(*a*)	0·112	20·56	75·61	1·10	2·62
	(*b*)	0·109	20·12	75·94	1·19	—
	(*c*)	0·07	20·16	76·30	0·97	2·42
11	(*a*)	1·261	20·62	74·38	3·58	0·16
	(*b*)	1·19	21·51	74·78	3·44	—
	(*c*)	1·23	20·64	74·37	3·50	0·26
12	(*a*)	1·287	18·71	73·90	1·33	4·77
	(*b*)	1·25	18·58	73·70	1·39	—
	(*c*)	1·24	18·69	73·84	1·45	4·78

* With zero suppression for (*b*).

DISCUSSION OF RESULTS

No evidence was found of bias, systematic drift with time, or interference between constituent gases. The application of zero suppression improved the precision for oxygen and nitrogen determinations.

TABLE V

COMPARISON OF 5 PER CENT. LIMITS OF ERROR BETWEEN THE PROPOSED METHOD AND THE HALDANE METHOD

Gas	Concentration	Gas-chromatographic limits	Haldane limits
Hydrogen ..	0·1 per cent. level	±0·0014	±0·10
	1·1 per cent. level	±0·0137	
Methane	All concentrations	±0·026	±0·07
Oxygen	All concentrations—without zero suppression	±0·07	±0·15
	All concentrations—with zero suppression	±0·03	
Nitrogen	All concentrations—without zero suppression	±0·27	±0·30
	All concentrations—with zero suppression	±0·12	

Details are given in Table V of the 5 per cent. limits of error of a single determination in which the chromatograph was used. These details were calculated from the results in Tables I, II and III. The Haldane limits were calculated from the average within the operator repeatability error found in eight gas analysis trials each carried out in several laboratories.

This evidence confirms the conclusion reached from earlier work that the gas chromatograph is a useful instrument for examining the air in mines, and apart from improved precision and speed over that of the classical Haldane method, it has the advantage of determining directly the nitrogen content of the sample. As with other physical methods of analysis, the value of the results is largely dependent upon the accuracy of calibration, and gas mixtures of known concentration must be available for calibration purposes.

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Appendix

List of components used in the circuits

T_1	= Transformer: primary windings 0, 200 to 250 V; secondary windings 30 V, 20 mA; 24 V, 300 mA.
T_2	= Transformer: primary windings 0, 200 to 250 V; secondary windings 30 V, 150 mA; 20 V, 3 A.
Tr_1, Tr_2	= Silicon transistors, OC201.
Tr_3	= Silicon transistor, OC204.
Tr_4	= Silicon transistor, OC25.
Tr_5	= Germanium power transistor, OC29.
Tr_6, Tr_7, Tr_{10}, Tr_{11}	= Germanium transistors, OC71.
Tr_8, Tr_{12}	= Germanium transistors, OC72.
Tr_9, Tr_{13}	= Germanium power transistors, OC35.
D_1, D_2, D_3, D_4	= Germanium diodes, OA81.
D_5, D_6, D_7, D_8	= Diodes, GJ5M.
D_9, D_{15}, D_{16}	= Zener diodes, OAZ223.
D_{10}, D_{11}, D_{12}, D_{13}	= Germanium diodes, OA5.
D_{14}	= 12-V, 4-A metal rectifier.
S	= Low-resistance precision instrument switch, double-pole, 6-way.
R_1, R_2, R_3, R_4, R_{10}, R_{27}	= 21-ohm 5-watt resistors.
R_5	= 720-ohm 1-watt resistor.
R_6	= 500-ohm wire-wound variable resistor (output control).
R_7, R_{11}	= 2700-ohm $\frac{1}{2}$-watt resistors.
R_8, R_{31}, R_{34}	= 10,000-ohm $\frac{1}{2}$-watt resistors.
R_9	= 50,000-ohm 1-watt resistor.
R_{12}	= 1000-ohm 1-watt resistor.
R_{13}	= 89·7-ohm manganin-wound resistor.
R_{14}	= 89·6-ohm manganin-wound resistor.
R_{15}	= 0·5-ohm resistor.
R_{16}	= 1-ohm 10-turn helical potentiometer.
R_{17}, R_{18}	= 700-ohm manganin-wound resistors.
R_{19}	= 300-ohm manganin-wound resistor.
R_{20}, R_{21}	= 100-ohm manganin-wound resistors.
R_{22}	= 40-ohm manganin-wound resistor.
R_{23}, R_{24}, R_{25}	= 20-ohm manganin-wound resistors.
R_{26}	= 100-ohm 5-watt resistor.
R_{28}	= 237-ohm 2-watt resistor.
R_{29}	= 39-ohm 5-watt resistor.
R_{30}, R_{33}	= 1000-ohm wire-wound variable resistors (temperature control).
R_{32}, R_{35}	= 8-ohm nichrome heating coils.
R_{36}	= 1000-ohm wire-wound resistor.
R_{37}	= 4·605-ohm manganin-wound resistor.
R_{38}	= 5·006-ohm manganin-wound resistor.
R_{39}	= 5·406-ohm manganin-wound resistor.
R_{40}	= 5·807-ohm manganin-wound resistor.
R_{41}	= 6·208-ohm manganin-wound resistor.
R_{42}	= 100-ohm slide-wire resistor.
R_{43}	= 0·413-ohm resistor.
R_{44}	= 407·5-ohm resistor.
R_{45}	= 403·9-ohm manganin-wound resistor.
R_{46}	= 403·5-ohm manganin-wound resistor.

R_{47} = 403·1-ohm manganin-wound resistor.
R_{48} = 402·7-ohm manganin-wound resistor.
R_{49} = 402·3-ohm manganin-wound resistor.
R_{50} = 1-ohm resistor.
R_{51} = 408-ohm resistor.
R_{52} = 1·6-megohm resistor.
R_{53} = 134- to 154-ohm variable resistor.
C_1, C_2, C_5, C_6 = 500-μF, 50-V electrolytic condensers.
C_3, C_4 = 250-μF 50-V electrolytic condensers.

References

1. Whatmough, P., *Nature*, 1957, **179**, 911.
2. ——, *Ibid.*, 1957, **182**, 863.
3. Timms, D. G., Konrath, H. J., and Chirnside, R. C., *Analyst*, 1958, **83**, 600.
4. Vizard, G. S., and Wynne, A., *Ibid.*, 1962, **87**, 810.
5. Kemhadjian, H., *Mullard Technical Communications*, 1958, **4**, 186.
6. ——, *Ibid.*, 1961, **6**, 122.
7. Brown, T. H., and Stephenson, W. L., *Electronic Engineering*, 1957, **29**, 425.

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The Determination of Sorbitol in Chocolate for Diabetics

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A method has been developed for determining sorbitol in chocolate for diabetics. It depends on the same principle as the procedure devised by Manzoni and Turi for estimating sorbitol in bakery products, *viz.*, although an aqueous solution of sorbitol is only slightly *laevo*-rotatory, it exhibits a pronounced *dextro*-rotation in the presence of molybdate in acid conditions. In the method described this change in rotation is specific, and can be used quantitatively. This change in rotation is unaffected by the presence of optically active sugars, so that sorbitol can be determined in their presence. Results from a series of experiments gave a standard deviation of 0·34, for a chocolate containing 30·4 per cent. of sorbitol. Recoveries were between 99 and 101 per cent., when chocolates with sorbitol contents ranging from 24 to 33 per cent. were tested. The procedure can also be applied to the assay of sorbitol as such, and to jam and preserves.

Sorbitol has been used as a sweetening agent in chocolate for diabetics for many years, but no satisfactory specific method has been developed for its determination. As far back as 1891 Gernez[1] reported that mannitol, which, in aqueous solution, has a negligible optical rotation, develops a significant rotation in the presence of molybdates, molybdophosphates and tungstates. This work was extended by Tanret,[2] Hannelaitre,[3] Frerejaque[4] and Richtmeyer and Hudson,[5] all with reference to mannitol. The overall conclusions were that (*a*) the specific rotation of mannitol varies with molybdate concentration unless acid conditions prevail, (*b*) the presence of acid enhances the effect of molybdate and (*c*) the rotatory power reaches a maximum when the ratio of 2 moles of MoO_3 to 1 mole of mannitol, $C_6H_{14}O_6$, is satisfied.

The experiments do not appear to have been extended to include sorbitol although the Merck Index[6] quotes without reference, the specific rotation of sorbitol as $-2{\cdot}0°$ in water, and as $+56°$ in the presence of ammonium molybdate. It was decided therefore to find out whether the effect of ammonium molybdate on the optical rotation of sorbitol could be used as the basis for a quantitative method.

During the course of this investigation, Manzoni and Turi[7] published a procedure, based on the same effect, for determining sorbitol when it is used as a humectant in bakery products. Their conclusions, based on the experimental conditions required to obtain a constant, but enhanced, optical rotation are the same as ours, although no details of the effect of variables, such as acidity and molybdate concentration, were given in their paper. These details are necessary for considerations of the limitations of the method. Further, Manzoni and Turi's method cannot be applied directly to chocolate, because of difficulties both in quantitatively extracting the sorbitol and in obtaining a final extract sufficiently clear and colourless for visual measurement of the optical rotation.

Experimental

In our experiments we used chemically pure sorbitol containing 0·57 per cent. moisture, 0·02 per cent. ash and 0·10 per cent. reducing sugars. All concentrations are corrected for these impurities.

Optical rotation of sorbitol—

The effect of sorbitol concentration on the specific rotation is shown in Table I. The range of concentration studied may seem to be restricted, but it was governed by the amount of sorbitol used in chocolate and the most suitable sample weight of chocolate to take, bearing in mind the problem of removing interfering substances.

Table I

The specific rotation of sorbitol in aqueous solution

Sorbitol content, g per 100 ml	0·5	1·0	1·5	2·0	2·5
$[\alpha]_D^{20}$, degrees	−1·30	−1·50	−1·50	−1·70	−1·50

Mean = −1·50°

EFFECT OF AMMONIUM MOLYBDATE—

Initially, the sorbitol concentration was kept constant and the effect of adding increasing amounts of ammonium molybdate on the specific rotation observed (see Table II). Next, the amount of molybdate present was kept constant and the sorbitol concentration increased (see Table III). From the results it was concluded that the specific rotation of sorbitol changes in the presence of molybdate, but is also influenced by the molybdate concentration.

TABLE II

THE EFFECT OF AMMONIUM MOLYBDATE ON THE SPECIFIC ROTATION AT A CONSTANT CONCENTRATION OF SORBITOL

Concentration of sorbitol was 1·0 g per 100 ml

Concentration of ammonium molybdate, g per 100 ml	$[\alpha]_D^{20}$, degrees	Change in $[\alpha]_D^{20}$, per g of ammonium molybdate degrees
0	−1·5	—
1·0	23·0	24·5
2·0	46·0	23·0
3·0	38·0	−8·0
4·0	30·0	−8·0
5·0	28·0	−2·0

TABLE III

THE EFFECT OF AMMONIUM MOLYBDATE ON THE SPECIFIC ROTATION AT VARYING CONCENTRATIONS OF SORBITOL

Concentration of ammonium molybdate was 4·0 g per 100 ml

Concentration of sorbitol, g per 100 ml	$[\alpha]_D^{20}$, degrees	Change in $[\alpha]_D^{20}$, per 0·5 g of sorbitol degrees
0	0	—
0·5	29·0	29·0
1·0	30·0	1·0
1·5	35·3	5·3
2·0	45·0	9·7
2·5	43·8	−1·2

EFFECT OF AMMONIUM MOLYBDATE IN ACID SOLUTION—

The effect of increasing acidity on the specific rotation of sorbitol in the presence of ammonium molybdate when both sorbitol and molybdate concentrations were kept constant at 1·5 g per 100 ml of solution and 4·0 g per 100 ml of solution, respectively, is shown below—

Acidity, calculated as ml of N H_2SO_4 per 100 ml of solution ..	5	10	15	20	25
$[\alpha]_D^{20}$, degrees	72·0	96·7	104·3	105·5	105·4

The rotation is considerably enhanced, and it becomes constant when the acid strength in the final solution exceeds about 0·15 N.

Having established this, the ammonium molybdate and sorbitol concentrations were again varied with respect to each other in acid conditions. When the concentration of sorbitol was fixed at 1 g per 100 ml of solution, the acidity at a strength equivalent to 25 ml of N sulphuric acid per 100 ml of solution and the concentration of ammonium molybdate varied, we obtained the results given below—

Concentration of ammonium molybdate, g per 100 ml of solution ..	1·0	2·0	3·0	4·0	5·0
$[\alpha]_D^{20}$, degrees	53·0	102·0	105·0	105·5	105·5

When the concentration of ammonium molybdate was fixed at 4·0 g per 100 ml of solution, the acidity at a strength equivalent to 25 ml of N sulphuric acid per 100 ml of solution and the concentration of sorbitol varied, we obtained the results given below—

Concentration of sorbitol, g per 100 ml of solution	0·5	1·0	1·5	2·0	2·5
$[\alpha]_D^{20}$, degrees	105·5	105·5	105·3	103·6	91·5

The ammonium molybdate used contained 82 per cent. of molybdenum, calculated as MoO_3, and it is clear that under these conditions the specific rotation becomes constant once the ratio of 2 moles of MoO_3 to 1 mole of sorbitol, $C_6H_{14}O_6$, is satisfied. These results are similar to those that had been obtained for mannitol.

THE SPECIFIC CHANGE IN ROTATION, $[\Delta\alpha]_D^{20}$—

Since the intention was to use the change in rotation induced by the presence of molybdate in acid solution for quantitative work, once the conditions had been found, the specific change in rotation was therefore determined, and the results given in Table IV show that it remains adequately constant, provided the sorbitol concentration does not exceed 2 per cent. w/v. Additional molybdate would be needed above this concentration to satisfy the ratio of 2 moles of MoO_3 to 1 mole of sorbitol. Under the experimental conditions described, therefore, the specific change in rotation can be used for sorbitol, in the same way that the specific rotation is usually used. Thus—

$$[\Delta\alpha] = \frac{100\Delta\alpha}{c \times l}$$

$$\text{and } c = \frac{100\Delta\alpha}{[\Delta\alpha]\, l}$$

where c = concentration of sorbitol in g per 100 ml,
l = length of polarimeter tube, in decimetres,
$\Delta\alpha$ = observed change in rotation, as a result of adding acidic molybdate solution and
$[\Delta\alpha]$ = specific change in rotation, as a result of adding acidic molybdate solution.

From the results given in Table IV, the value of $[\Delta\alpha]_D^{20}$ can be taken as 107, for a sorbitol concentration of less than 2 per cent. w/v, so that if a 4-dm tube is used, the expression is reduced to—

$$c = 0{\cdot}2336\Delta\alpha$$

As a further check on the value of $[\Delta\alpha]$, two separate batches of 98 to 99 per cent. sorbitol were recrystallised from anhydrous alcohol, dried under reduced pressure, and assayed by using this method. A value of 100·0 per cent. of sorbitol was obtained for each batch.

TABLE IV

THE SPECIFIC CHANGE IN ROTATION OF SORBITOL IN AQUEOUS SOLUTION

Solution contained 4 g ammonium molybdate and 25 ml N sulphuric acid per 100 ml

Concentration of sorbitol, g per 100 ml ..	0·5	1·0	1·5	2·0	2·5
$[\Delta]_D^{20}$, degrees	107·0	107·0	106·8	105·3	93·0

EFFECT OF THE FORMATION OF MOLYBDENUM BLUE—

One of the difficulties experienced in the early stages of this work was the immediate formation of molybdenum blue when the acid solution made contact with the metal screw-threads fixed to the ends of the glass polarimeter tubes. This made it difficult to take the polarimeter readings. The separate addition of citrate, oxalate and fluoride depressed the molybdenum blue effect slightly but introduced other less desirable effects on the optical rotation itself. By using a centre-filling glass polarimeter tube, the difficulty was overcome completely.

In the finally developed procedure, it was initially found essential to take the polarimeter readings immediately after adding the acid and adjusting the volume. This was because of the fairly rapid development of molybdenum blue due to the action of trace constituents. There was, therefore, little time for adjusting the temperature to a suitable value, and so the influence of temperature was studied, and the results given in Table V show that, providing the temperature is about 20° C, the effect can be ignored. However, the method was later modified when it was found that the addition of sodium nitrite, as suggested by Kirsten and Nilsson,[8] successfully retarded the formation of molybdenum blue without affecting the change in rotation of sorbitol either alone, or when extracted from chocolate.

TABLE V

THE EFFECT OF TEMPERATURE ON THE OPTICAL ROTATION OF SORBITOL IN ACID SOLUTION

Readings were taken when a 2-dm tube was used

Temperature, °C	Optical rotation, in degrees, of a solution containing— 2·5 g of sorbitol, 4·0 g of ammonium molybdate and 25 ml of N H_2SO_4 per 100 ml	2·5 g of sorbitol per 100 ml
15	4·18, 4·18, 4·19, 4·18, 4·18, 4·18 (Mean = 4·18)	−0·07, −0·05, −0·05, −0·07, −0·06, −0·06 (Mean = −0·06)
20	4·19, 4·19, 4·18, 4·17, 4·18, 4·18 (Mean = 4·18)	−0·04, −0·04, −0·06, −0·06, −0·06, −0·06 (Mean = −0·05)
25	4·20, 4·18, 4·19, 4·20, 4·20, 4·20 (Mean = 4·195)	−0·07, −0·05, −0·07, −0·07, −0·06, −0·06 (Mean = −0·06)

THE EFFECT OF SUGARS—

Cocoa mass contains traces of reducing sugar and, since these might well influence the specific rotation, the effect of the presence of sugar was investigated. From the results given in Table VI, it was concluded that sorbitol could be determined in the presence of lactose, sucrose, dextrose and maltose up to a sugar concentration of 0·25 per cent. in the final solution. Further results showed that up to 0·5 per cent. could be tolerated, but that above this level, the experimental conditions influenced the optical rotation of the sugar to such an extent that the method was affected. However, since a limit of 0·5 per cent. of sugar in the final solution corresponds to 20 per cent. of sugar in the original chocolate, when the proposed method is used, the method is not limited in this respect. Chocolate intended for sale to diabetics will, at the most, contain only traces of natural sugar.

TABLE VI

THE EFFECT OF SOME SUGARS ON THE OPTICAL ROTATION OF SORBITOL SOLUTION

Solutions contained 0·25 g of sugar per 100 ml; readings were made when a 2-dm tube was used

Type of sugar	Concentration of sorbitol, g per 100 ml	$\Delta\alpha^{20}_{D}$, degrees	$\Delta\alpha$ for sorbitol—$\Delta\alpha$ for sorbitol *plus* sugar, degrees
—	0·5	1·05	—
	1·0	2·11	—
Lactose	0·5	1·06	−0·01
	1·0	2·10	0·01
Sucrose	0·5	1·04	0·01
	1·0	2·10	0·01
Dextrose	0·5	1·06	0·01
	1·0	2·11	0·00
Maltose	0·5	1·04	−0·01
	1·0	2·12	0·01

METHOD

Suspensions of the chocolate were cleared with Carrez' reagents, since clearing agents containing lead could not be used because of the presence of molybdate. However, even when these reagents were used, the filtered extracts produced were insufficiently colourless for polarimetry of the accuracy required when a 4-dm tube was used. This difficulty was overcome by passing the solution through a short column of neutral alumina. The pigments were retained on the alumina and the sorbitol passed through unabsorbed. Separate experiments with aqueous sorbitol solutions proved this latter point.

The method in its final form is given below.

REAGENTS—

Ammonium molybdate solution, 20 *per cent. w/v, aqueous.*
Sulphuric acid, N.
Carrez' reagents—
(*i*) Zinc acetate solution, M.
(*ii*) Potassium ferrocyanide solution, 0·25 M.
Alumina, neutral grade—For chromatography.
Sodium nitrite solution, 2·5 *per cent. w/v, aqueous.*

APPARATUS—

Polarimeter—Calibrated in 0·01° intervals.
Polarimeter tubes—Glass, centre-filling, 4 dm long.
Chromatographic column and receiver.

PROCEDURE—

Weigh accurately about 10 g of a representative sample of the chocolate into a 150-ml beaker. Melt the chocolate by heating it in an oven at 100° C for a short time, then disperse it in 50 to 70 ml of hot water, adding the water in small increments, and mixing well after each addition. All lumps must be broken down. Heat on a boiling-water bath for 15 minutes, stirring occasionally. Add 5·0 ml of zinc acetate solution and 5·0 ml of potassium ferrocyanide solution, mixing well after each addition, and filter the solution, while it is still hot, through a 15-cm Whatman No. 1 filter-paper. Collect the filtrate in a 200-ml calibrated flask. Let the residue drain completely on the paper, then rinse it several times with small amounts of hot water that have first been used for washing the beaker. Transfer the residue from the paper back to the original beaker with the aid of a jet of hot water. Convert the residue into a suspension, by heating it with 50 ml of water on a boiling-water bath for 5 to 10 minutes, with occasional stirring. Filter off the suspension on the same paper, and collect the filtrate in the same 200-ml flask. Wash the residue with separate amounts of hot water, again allowing the residue to drain completely between each washing, until the volume of the combined filtrate and washings is almost 200 ml. Cool the filtrate to 20° C, dilute to the mark with water and mix well.

It is important to follow the above procedure exactly to ensure quantitative extraction of the sorbitol from the chocolate.

Remove the residual colour in the extract by passing it through a chromatographic column, 1 cm in diameter, containing 10 to 20 g of neutral alumina. Use suction, if necessary, to obtain a satisfactory percolation rate. Reject the first 10 to 20 ml of eluant from the column, and collect the remainder.

Measure with a pipette 50 ml of the eluant into each of two 100-ml calibrated flasks. To one flask add 25 ml of N sulphuric acid, dilute almost to the mark with water, add 1 ml of sodium nitrite solution and dilute to the mark with water. Mix the solution well, and measure the optical rotation (see Note 1) in a 4-dm tube. Use a sodium lamp as the light source, and ensure that the temperature is between 20° and 25° C. To the remaining flask add 20 ml of ammonium molybdate solution, 25 ml of N sulphuric acid and 1 ml of sodium nitrite solution, mixing after each addition. Dilute to the mark with water, mix well and measure the optical rotation as before (see Note 2).

CALCULATION—

The sorbitol content of the sample is given as a percentage by the expression—

$$\frac{93{\cdot}46 \times \Delta\alpha}{W}$$

where $\Delta\alpha$ = the algebraic difference in rotation between the two final solutions and
W = sample weight in grams.

This calculation expresses the results for dry and ash-free sorbitol that contains no reducing sugar. Commercial sorbitol, as used in chocolate manufacture, contains 98 to 99 per cent. of sorbitol, as such, and allowance should be made for this.

NOTES—

1. At least six polarimeter readings must be taken, three from each side of the match-point. The average value should be used in the calculation.

2. It is advisable to measure the rotation shortly after diluting the solution to the mark, since, although the sodium nitrite retards the formation of molybdenum blue, there is still a gradual darkening in the tint of the solution.

RESULTS AND DISCUSSION OF METHOD

The results given in Tables VII, VIII and IX were obtained by using the proposed method. The amounts of sorbitol found in laboratory-prepared samples of chocolate having somewhat different recipes were satisfactory. The standard deviation of 0·34 quoted in Table VIII corresponds to an angular rotation of 0·03° under the conditions of the method. Since, in another investigation, the standard deviation of the means of six polarimeter readings by each of 40 different chemists, who used the same solution and polarimeter, was 0·014°, the main source of error in the sorbitol determination lies in the polarimeter reading itself. It is preferable, therefore, to use a 4-dm tube as stated in the method.

Table IX refers to a number of chocolates for diabetics available commercially, some of which contained milk solids that obviously did not interfere with the sorbitol determination.

TABLE VII

RECOVERIES OF SORBITOL FROM LABORATORY-PREPARED CHOCOLATES

Sample	Percentage content of sorbitol (99 per cent. pure) in the recipe	found	Recovery, per cent.
1	33·0	33·4	101·2
2	24·8	25·0	100·7
3	30·5	30·4	99·7
4	32·2	32·0	99·3

TABLE VIII

COMPARISON OF THE RESULTS OBTAINED BY DIFFERENT ANALYSTS ON THE SAME SAMPLE OF CHOCOLATE

Sample contained 30·5 per cent. of sorbitol

Analyst	Sorbitol found, per cent.
1	30·9, 30·7
2	30·6, 30·4
3	29·9, 30·1
4	30·2, 30·1
5	30·6, 30·8
Mean	= 30·4
Standard deviation	= 0·34
$\frac{\text{Standard deviation}}{\text{Mean}} \times 100$	= 1·12

TABLE IX

SORBITOL CONTENT OF COMMERCIALLY AVAILABLE CHOCOLATES FOR DIABETICS

Chocolate sample	Sorbitol content, per cent. declared	found*
Plain	32·2	32·0
Plain	29·0	28·5
Milk	32·5	31·6
Milk	32·1	32·0
Nut milk	27·9	27·6
Milk	25·0	24·8

* Calculated as 99 per cent. pure sorbitol.

SORBITOL IN JAMS AND PRESERVES FOR DIABETICS—

Although in the experiments performed to inhibit the formation of molybdenum blue by using citrates and oxalates it was found that the optical rotation was affected, it was thought that when applied to jams, etc., the concentrations of these anions in the final extracts might not be sufficiently great to influence the result.

The values given in Table X were obtained for raspberry jam and marmalade intended for sale to diabetics. It seems reasonable to assume from these results that the method could well be applied to such foodstuffs.

TABLE X

SORBITOL CONTENT OF PRESERVES

	Sorbitol content, per cent	
	declared	found
Raspberry jam	67·7	67·1, 66·8
Marmalade	66·3	64·6, 65·0

CONCLUSIONS

A satisfactory quantitative method has been developed for determining sorbitol in chocolate sweetened with sorbitol and intended for sale to diabetics. The method is not influenced, within the limits specified, by the presence of sugars, and is capable of wider application, provided that other hexahydric alcohols are absent.

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REFERENCES

1. Gernez, M. D., *Compt. Rend.*, 1891, **112**, 1360.
2. Tanret, G., *Ibid.*, 1921, **172**, 1500.
3. Hannelaitre, A., *Ann. Chimica*, 1925, **3** (**10**), 5.
4. Frerejaque, M., *Compt. Rend.*, 1935, **200**, 1410, and 1939, **208**, 1123.
5. Richtmeyer, N. K., and Hudson, C. S., *J. Amer. Chem. Soc.*, 1951, **73**, 2249.
6. "Merck Index," Sixth Edition, Merck & Co. Inc., Rahway, N.J., U.S.A., 1952, p. 895.
7. Manzone, A. M., and Turi, C. J., *Ann. Chim. Roma*, 1962, **51**, 220.
8. Kirsten, W. J., and Nilsson, S. K., *Anal. Chim. Acta*, 1962, **27**, 345.

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A Method for Quantitatively Isolating "Hydrolysate Lipids" from Biological Materials, and Its Use as a Reference Method for Determining "Fats" in Foods

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Biological materials are hydrolysed in boiling hydrochloric acid in the presence of pyrogallol, which inhibits autoxidation, and dodecylbenzenesulphonic acid, which catalyses the hydrolysis of glyceride esters. Lipids are then extracted with chloroform, and separated from lipophilic artifacts such as furfurals by passage, in benzene solution, through a column of silicic acid that has been de-activated with aqueous formic acid. The eluted material is weighed. This material, for which the term "hydrolysate lipids" is proposed, consists essentially of the total fatty acids *plus* the non-saponifiable fraction. The determination of "hydrolysate lipids" is highly selective and precise, even for materials containing little lipid and much carbohydrate. It is therefore recommended as a reference method for food analysis, although it may not be too elaborate for some routine applications.

The lipids of biological materials may be defined as fat-soluble hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids and amines, and other compounds in which any of these occur in covalent linkage. Although no single definition of lipids has yet met with universal acceptance,[1] this definition is attractive from an analytical standpoint. It includes all the known compounds that have been called lipids, from fat-soluble triglycerides and sterols to water-soluble mucolipids and gangliosides.

The best known lipids are water-insoluble, and extractable from biological materials in two fairly distinct portions. The first portion, often called the "free fats" or, simply, the "fats," is extracted by relatively non-polar solvents, such as hexane and diethyl ether. The second portion, which contains lipoprotein lipids, is extracted by alcoholic solvents. The terms "total lipids" and "fats" are often used to signify a combination of both portions. These lipids are thought to interact with the non-lipid structural components of tissues by van der Waals attraction, dipole - dipole attraction and hydrogen bonding. Some less well known, and generally less abundant lipids (*e.g.*, triphosphoinositides[2]) seem to be bound primarily by ionic linkages. They are not extracted by neutral, alcoholic solvents until hydrochloric acid is added, but then they are readily extracted in the cold. Even after extraction with cold, acidified solvents some tissues still retain lipids. These may be bound by covalent bonds to non-lipid macromolecules (as in lipopolysaccharides[3]); they may be lipids such as triglycerides that are mechanically trapped (naturally or as an artifact of extraction), *e.g.*, in a glassy capsule, or they may be insoluble metallic salts of acidic lipids. These "firmly bound" lipids are seldom detectable, except as fatty acids, *etc.*, found after hydrolysis of the tissues in which they occur.

An extract cannot strictly be said to contain the "total lipids" of a tissue unless no ionically bound lipids or "firmly bound" lipids remain, and unless no water-soluble lipids are washed away.

There is need for some general, "absolute" method for determining fat in foods. Such a method might not supplant the accepted routine methods, but would provide a basis for comparison of results. The accepted methods are relied on diversely for different kinds of foods, and seldom do any two methods give precisely the same result for a single test material. This is because tacitly the methods are based upon empirical assumptions.

For ether and light-petroleum extractions, it is assumed that lipoproteins are absent or else that they are inert towards the extractants. But triglycerides and other types of lipids are found among "free fats" and in lipoproteins and thus, from the analyst's point of view, an artificial distinction is made.

When neutral extractants containing alcohols are used, lipoprotein lipids are readily extracted with "free fats," but still ionically bound and "firmly bound" lipids are ignored. The extracts contain non-lipids, some of which (*e.g.*, lipophilic proteins of cereals) are difficult to remove.

Addition of ammonia solution (*e.g.*, as in the Rose - Gottlieb method) disrupts ionic linkages. It may even liberate "firmly bound" lipids, but this is not known. It is likely to cause ammoniolysis and hydrolysis of some carboxylic and phosphoric ester groups, altering the composition and weight of extracted lipids.

Methods involving mild acid treatments (*e.g.*, Werner - Schmid method and official methods[4 to 8]) partly hydrolyse glycolipids, which are abundant in cereals, and phospholipids: in a routine analysis the extent and effect of such hydrolysis are imponderable. Even when vigorous acid hydrolysis is used[9,10] to ensure recovery of "firmly bound" lipids, the extent of ester hydrolysis is uncertain, and then, too, autoxidative degradation may occur. Moreover, acids convert carbohydrates[11,12,13] and amino-acids[14,15] to lipophilic artifacts, which add to the weight of so-called lipids determined. For example, hexoses yield 5-(hydroxymethyl)-furfural and laevulinic acid, and α-amino-acids yield α-oxo-acids and aldehydes. Such artifacts can, in theory, be removed by distillation or chemical treatment,[16] but in practice the results obtained are seldom precise. This situation, as it concerns sweetened foods, has been commented upon by Morgan and Rawlings.[17] Sandberg[18] has published a revealing study of interference by sugars in the determination of lipids in dairy products, and he was actually able to characterise laevulinic acid and 5-(hydroxymethyl)furfural in his "fat" extracts.

Volumetric methods for determining "fat" (*e.g.* the Babcock and Gerber methods) are obviously empirical. However, as with the other methods, their use for certain purposes can be justified on grounds of convenience and adequate reproducibility. Like the other methods, they would benefit from calibration by a reference method.

In this paper we describe a method for determining the true content of lipids. It gives accurate and reproducible results because the sample, lipids included, is completely hydrolysed, and lipophilic artifacts are removed chromatographically.

METHOD

APPARATUS—

All apparatus should be visibly (but not chemically) dry.

Stopcocks on separating funnels and columns must be greaseless; polytetrafluoroethylene may be used.

Rotary film evaporator—There must be no risk of grease contamination from joints. Evaporators of the original Craig type[19] (horizontal axis) are suitable; a modification of this type has recently been described by Coleman.[20] Evaporators with inclined axes may require traps such as that described by Zacharius and Krulick.[21] The evaporator is maintained at a temperature of 50° ± 5° C, and reduced pressure is created by means of a water-pump. Solvents are not recovered.

Pipettes—All transfers of liquids on the small scale are made with non-graduated pipettes, of about 3-ml capacity, fitted with teat tops.

Chromatographic columns—Glass tubes (internal diameter 2 to 2·5 cm, length roughly 30 cm) are constricted at the lower ends and (preferably) fitted with stopcocks. Small plugs of cotton wool are placed in the constrictions.

REAGENTS—

All reagents should be of analytical-reagent grade unless otherwise specified.

Hydrochloric acid, diluted (1 + 3).

Catalyst—Extract commercial "85 per cent." sodium dodecylbenzenesulphonate* with ether in a Soxhlet apparatus for 4 hours, and dry in air the solid that remains in the thimble.

Pyrogallol, resublimed.

Boiling chips, 30-grit carborundum.

Chloroform.

* The domestic detergent "White Tide" (Procter & Gamble, Ltd.) is a convenient source of the sulphonate. Ether extraction removes fluorescent and fatty acid-containing compounds from it, giving a powdery product, which is more easily handled than that obtained from the "85 per cent." material.

Benzene—Redistil the benzene, rejecting the first (wet) fraction of distillate.

Silicic acid—Use Mallinkrodt, analytical-reagent grade. Activate the 100-mesh powder as described below.

Formic acid, 98 to 100 per cent. w/w.

Methanol.

ADSORBENT—

Preparation of activated silicic acid—Place a 2-litre measuring cylinder containing 1·5 litres of methanol in a fume cupboard and slowly add 600 g of silicic acid, while stirring with a thick glass rod. After being set aside for 1 hour, the suspension will settle into three visibly different layers: bottom, dense slurry; middle, less dense slurry; top, cloudy liquid. Pour off as much as possible of the top two layers and then, with the aid of a little fresh methanol, pour the bottom layer into shallow glass or aluminium dishes to a depth of about 1 cm. Heat these, first at 80° C for 1 hour, to evaporate most of the liquid, and then at 100° to 120° C for 6 hours, raking the powder hourly with a spatula. Then quickly transfer the powder to a screw-cap bottle and store this in a desiccator.

Test for the activity of silicic acid—Quickly and accurately weigh about 1 g of the adsorbent into a small test tube, fitted with a rubber bung and containing two glass beads. Add about 0·5 ml of water from a microburette, and shake the tube vigorously for about 2 minutes, until the adsorbent regains its original uniform, free-flowing appearance. Then add more water, to raise the total addition to 0·70 ml of water per g of adsorbent. If the adsorbent again recovers its original appearance after further shaking, the result is satisfactory.

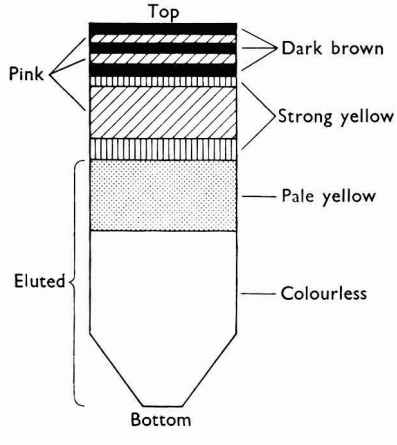

Fig. 1. Appearance of the column immediately after a hydrolysate of 10 g of flour has been run on. (Silicic acid, 10 g)

This is really a determination of pore volume.[22] Low titres indicate that the adsorbent is not fully activated or that its pores are too narrow.[23] Commercially available products labelled "silica gel" are often unsuitable for the latter reason.

PROCEDURE—

Hydrolysis—Coarsely chop or crumble the test material, if necessary, and weigh a suitable sample, ideally containing 200 mg of lipids (*e.g.*, 10 g of flour). Add the sample gradually, with swirling, to 500 ml of 3 N hydrochloric acid in a 1-litre flat-bottomed B24-necked flask that also contains 50 mg of catalyst, 50 mg of pyrogallol and a few boiling chips. Fit a reflux condenser, close it at the top with a tight plug of cotton wool and boil the mixture for 10 hours on a hot-plate.

Extraction—After the hydrolysate has cooled, add 100 ml of chloroform and boil the mixture for 15 minutes. Vigorous refluxing is necessary to wash down lipids that have accumulated in the condenser, from a steam-distillation effect. Pour the mixture after cooling into a 1-litre separating funnel, retaining the boiling chips as far as possible. Run the lower liquid layer into a 1-litre B34-necked flask, but retain the interfacial solid. (Small amounts of this charred solid in the chloroform extract are unimportant.) Wash the hydrolysis flask with a second 100-ml portion of chloroform, pouring it into the separating funnel. Shake the funnel vigorously for 3 minutes, allow the layers to separate and run the second extract into the B34-necked flask. Thoroughly evaporate the chloroform on the rotary evaporator. Extract the residual dark oil with eight 1-ml portions of benzene, transferring the extracts directly to the prepared chromatographic column. (Cloudiness in the extract is unimportant, if it is not caused by water droplets.)

Chromatography—Quickly weigh 10·3 g of silicic acid in a 50 ml beaker. Add 33 ml of benzene, 1·60 ml of water and 0·60 ml of formic acid. Stir the mixture with a small glass rod, and cover the beaker tightly with aluminium foil. Stir the slurry at 5-minute intervals until it appears uniform (usually within 20 minutes). Stir finally, and pour the mixture quickly into the column. A little adsorbent (about 0·3 g) is lost on the walls of the beaker; wash the adsorbent adhering to the walls of the column with two 2-ml portions of benzene. When the adsorbent has settled and begins to run dry at the top, wash it with 10 ml of benzene, run in cautiously to avoid disturbance. When the top dries again, the column is ready for use.

Run in the benzene extract and, when the top runs dry, wash in the last traces of extract with 2 ml of benzene. Then run in 50 to 100 ml of benzene and begin to collect the eluate. Coloured bands appear on the adsorbent (see Fig. 1). Collect the eluate until the lower strong yellow band begins to be eluted. (For test materials that do not give coloured bands collect 100 ml.) Evaporate the eluate in a 500-ml flask, by using the rotary evaporator, wash the residue into a 50-ml tared flask with chloroform and re-evaporate to almost constant weight, that is, until successive weighings agree to within 0·5 mg.

EXPERIMENTAL APPRAISAL OF PROPOSED METHOD

Table I gives the compositions of corn-oil hydrolysates and methanolysates.

HYDROLYSIS—

Hydrolysis medium—Alkaline hydrolysis[24,25] of flour, in which aqueous sodium hydroxide or ammonia solution, sp.gr. 0·88, was used, gave variable yields. When, in preliminary tests, flour was heated at 105° to 110° C with ten times its weight of 6 N hydrochloric acid in sealed tubes, yields of chloroform- or ether-soluble products were roughly 25 times the lipid content. When 6 N acid was used in place of 3 N acid in the proposed method, the yield of lipophilic artifacts rose by about 50 per cent.; much insoluble, charred material was formed, and this hindered extraction, mechanically and by adsorbing fatty acids. For hydrolysing proteins 3 N hydrochloric acid is preferred.[14,15,26]

Ratio of sample to acid—To minimise amino-acid - carbohydrate reactions the ratio of the sample to acid should be low.[14,26] Increased amounts of artifacts were obtained when high ratios were tested, although this was partly a partition effect caused by increased chloroform - acid ratios.

Temperature—For complete hydrolysis of proteins, the temperature should be between 100° and 120° C [14,26]; it is convenient to use the reflux temperature.

Catalyst—Reproducible yields were obtained from flour and egg white before the catalyst was used, but less than half of the glyceride ester bonds were hydrolysed. Thus precision was questionable, since the extent of ester hydrolysis could vary in different samples, and some products of partial hydrolysis, *e.g.*, monoglycerides, were probably removed with the artifacts. Corn oil, present to the extent of 200 mg, was hardly hydrolysed at all (compare spectra A, B and G in Fig. 2), even when 100 mg of cetyl trimethylammonium bromide or 5 g of sodium hexametaphosphate was added, but 50 mg of sodium dodecylbenzene-sulphonate promoted complete hydrolysis.

This cannot be ascribed to emulsification, because dodecylbenzenesulphonic acid (not the salt) must be the active material in the hydrolysis mixture. It seems that hydrolysis is effected inside lipid droplets by dissolved, hydrated molecules of the sulphonic acid. This mechanism is probably also involved in Twitchell's hydrolysis method.[27]

Duration—The yield of lipophilic artifacts from flour increased with the duration of hydrolysis, at least to 24 hours. When corn oil was subjected to a 50-hour hydrolysis (column (*iv*) in Table I) the yield was diminished and the fatty acid composition changed in comparison with the yields and compositions for shorter periods (columns (*i*), (*ii*) and (*iii*) in Table I). The infrared spectrum (F, see Fig. 2) contained a shoulder at 965 cm^{-1} indicating some *trans*-isomerisation of carbon - carbon double bonds. The diminished yield might be explained by hydration of double bonds,[28,29,30] the hydration products being lost on silicic acid.

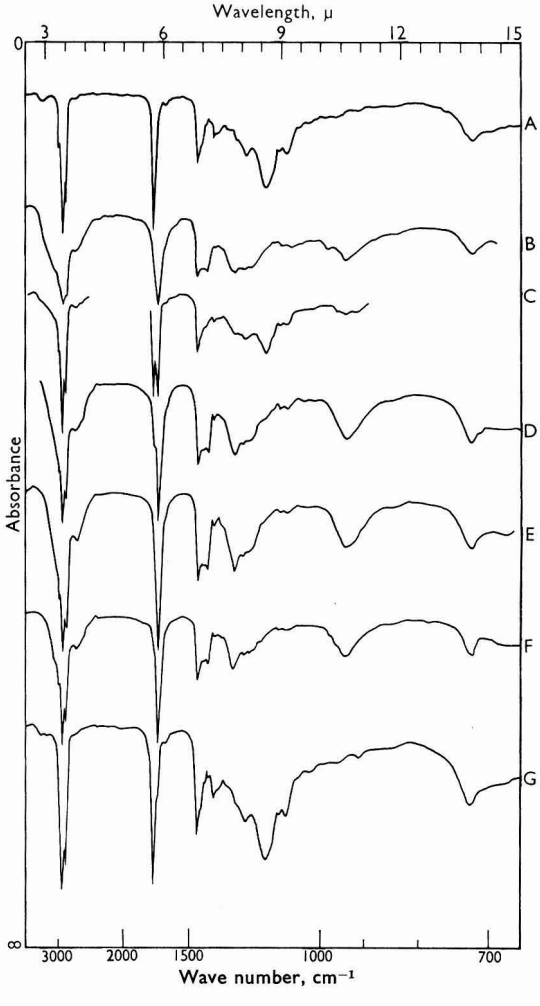

Fig. 2. Infrared spectra of liquid films showing the catalytic effect of dodecylbenzene sulphonic acid on glyceride hydrolysis. Curve A, corn oil ("Mazola"); curve B, corn oil fatty acids, prepared by saponification (with the unsaponifiable fraction removed); curve C, product from corn oil after chromatography (catalyst and pyrogallol included; hydrolysis period, 2 hours); curve D, as for curve C (but hydrolysis period, 6 hours); curve E, as for curve C (but hydrolysis period, 10 hours); curve F, as for curve C (but hydrolysis period, 50 hours); curve G, product from corn oil after chromatography (catalyst, and pyrogallol omitted; hydrolysis period, 10 hours)

Since corn oil is as resistant to hydrolysis as any material likely to be tested, the infrared spectra (see Fig. 2) indicate that the 10-hour hydrolysis period is suitable. Shorter periods and more catalyst are not indicated, because proteins and other macromolecules must be hydrolysed completely, and a larger chromatographic column would be needed to retain more sulphonic acid. Moreover, the fatty acid composition after the 10-hour period (column (*iii*) in Table I) did not differ significantly from that after shorter periods (columns (*i*) and (*ii*) in Table I) or from that of the oil itself, determined directly (columns (*vii*) and (*viii*) in Table I). Thus it can be concluded that lipids undergo little or no undesirable chemical change during the 10-hour period.

TABLE I

PROPORTIONS OF FATTY ACIDS IN CORN-OIL HYDROLYSATES AND METHANOLYSATES
Values are expressed as per cent. total methyl esters, listed in order of emergence from the gas-chromatographic column on which they were separated

	(*i*)*	(*ii*)*	(*iii*)	(*iv*)	(*v*)	(*vi*)	(*vii*)	(*viii*)
Hydrolysis number								
Base-catalysed methanolysis number								
Catalyst added	+	+	+	+	+	+		
Pyrogallol added	+	+	+	+	−	−		
Glass wool added	+	+	+	+	+	−		
Duration, hours of hydrodysis	2	6	10	50	10	10		
Yield (per cent. of corn oil) after chromatography†	88·8	89·4	90·0	81·9	89·9	84·5		
Infrared spectrum (see Fig. 2)	C	D	E	F				
C_{12} saturated	0·5	0·4	0·4	0·5	0·4	0·4	0·3	0·5
C_{14} saturated	0·1	0·1	0·1	0·2	0·1	trace	0·1	0·2
C_{16} saturated	11·9	11·6	12·0	13·7	12·2	11·7	11·3	11·9
C_{18} saturated	2·5	2·5	2·5	2·9	2·8	2·5	2·1	2·3
C_{18} mono-ene	29·8	30·3	30·6	33·5	31·0	29·6	31·0	29·6
Unidentified	0	0	0	5·5‡	0	0	0	0
C_{18} diene	55·2	54·9	54·4	40·0‡	53·5	55·1	55·2	54·3
C_{20} (?)	trace	0·2	trace	2·0	trace	0·7	trace	1·2
Unidentified	0	0	0	1·7	0	0	0	0

* Gas-chromatographic analyses represent only the hydrolysed fatty acids.
† Theoretical yield of linoleic acid from trilinolein = 95·7 per cent.
‡ Incompletely resolved.

Inclusion of pyrogallol—The advantages of adding anti-oxidants to lipids, particularly when they are exposed in the laboratory to heat, light and oxygen, have been emphasised.[31] To be useful in this analysis an anti-oxidant (and any products formed from it) must be retained on the column. 4-Methyl-2,6-di-t-butylphenol and 4-methoxy-2-t-butylphenol are not retained; pyrogallol and hydroquinone are retained, but the latter can be oxidised to *p*-benzoquinone, which is not.

When pyrogallol was omitted in an experiment (column (*v*) in Table I) on corn oil, the linoleate value seemed slightly low. When the glass wool plug was omitted, allowing free access to air, the yield was diminished (column (*vi*) in Table I), but the fatty acid vaiues were not changed significantly; the hydrolysate was unusually colourless.

EXTRACTION—

Two chloroform extractions are sufficient, since when a third extraction was applied in tests on flour and palmitic acid, it recovered only about 1 per cent. of the lipids. A chloroform - methanol mixture was only used once, because it extracted a greatly increased amount of artifacts.

CHROMATOGRAPHY—

Most true lipid fragments expected in hydrolysates are eluted from fully activated silicic acid by chloroform and diethyl ether.[32,33] However, we found that some artifacts were eluted similarly, despite[32,33] their low molecular weight. Several workers have reported that resolution on silicic acid can be improved by de-activating the adsorbent with water and using less polar eluents. Horning, Williams and Horning[34] used benzene for eluting long-chain fatty acids and cholesterol from silicic acid that contained 10 per cent. of water. Because of "tailing," we could not quantitatively recover palmitic acid in a pure form from admixture with glucose

artifacts by elution from silicic acid containing 10, 20, or 30 per cent. of water. We assumed that strong interaction between carboxyl groups and the adsorbent caused the "tailing," and tried incorporating carboxyl groups in the de-activating liquid. "Tailing" was indeed abolished by aqueous formic or acetic acids. The results of numerous tests show that optimum de-activation is obtained with 0·16 ml of water and 0·06 ml of formic acid per g of activated silicic acid. Dodecylbenzene sulphonic acid is not eluted by benzene, but it displaces lipids and artifacts, and so speeds their elution without impairing the resolution.

Fig. 1 shows the typical appearance of a column. Lipid pigments are probably responsible for the pale yellow band. The three bands immediately above are characteristic of artifacts; they are formed by hydrolysates of pure carbohydrates (glucose, starch) and generally by carbohydrate-containing materials, whether or not catalyst and pyrogallol are used. Lipids are eluted quantitatively before the first of these bands. This has been shown (see Table II) by collecting fractions, weighing materials eluted and determining their infrared spectra.

The conditions for concentrating the fractions, and their subsequent chromatography, are considered sufficiently mild to avoid formation of covalent bonds between lipid and artifact molecules.

LIPIDS RECOVERED—

Palmitic acid, caprylic (n-octanoic) acid, cholesterol, ergosterol and β-sitosterol were recovered in yields of over 99 per cent. in the chromatographic procedure. Palmitic acid was isolated in 97·8 per cent. of theoretical yield when the complete analysis was applied to pure tripalmitin. From the results for corn oil (see Table I), it is clear that other long-chain fatty acids, saturated and unsaturated, are recovered likewise. When butyric acid was tested, a part of it was eluted, but the rest was firmly retained (presumably sufficient to saturate the aqueous phase). Batyl alcohol and deoxycholic acid were not recovered.

When the analysis was applied to wheat flour cerebrosides, which on hydrolysis yield a mixture of fatty acids (including α-hydroxystearic) and a mixture of bases of the sphingosine type,[35,36] the former were recovered, but not the latter. Long-chain aldehydes (plasmals) and (probably) Q coenzymes were recovered from heart muscle.

These findings are all to be expected if lipids are eluted in the same order from silicic acid that has been de-activated with aqueous formic acid as from the fully activated adsorbent.[32,33] If this is now assumed, a list of types of lipids recovered in the analysis can be made, and is given below—

Hydrocarbons
Long-chain aldehydes (plasmals)
Long-chain alcohols
Long- and medium-chain fatty acids
Sterols
Q coenzymes (ubiquinones)
Tocopherols (and the synthetic antioxidants, 4-methyl-2,6-di-t-butylphenol and 4-methoxy-2-t-butylphenol)

Notably absent are long-chain bases (sphingosine and similar compounds) and glyceryl ethers (batyl, chimyl, selachyl alcohols). Such compounds could be recovered as aldehydes after periodate oxidation.[37] But generally this would be unnecessary because most lipids that yield these bases and ethers also yield fatty acids, which are recovered.

Recovery from autoxidised materials—Short-chain and highly oxygenated long-chain oxidation products would not be recovered. When lipids undergo autoxidation within biological materials, some become "firmly bound," probably by forming covalent bonds with proteins and other macromolecules.[38] A triglyceride molecule may, for example, undergo autoxidation in one fatty acid chain, and be bound by it; then two fatty acid molecules should still be recoverable by this method.

SPECIFICITY—

Carbohydrate artifacts—5-(Hydroxymethyl)furfural, laevulinic acid, α-angelicalactone and prepared glucose artifacts were all quantitatively retained on the column.

Blank-value determinations—A complete blank-value determination on 500 mg of catalyst (ten times the normal quantity used) yielded only 0·5 mg of residue from 100 ml of eluate.

This is equivalent to only 0·05 mg in a normal determination, or about 0·0005 per cent. of a flour sample. A stringent test of specificity was made by determining the blank value for a mixture of amino-acids and sucrose; this was 0·002 per cent.

TABLE II

COURSE OF ELUTION OF FLOUR HYDROLYSATE

Untreated white wheat flour (10 g): elution with benzene from 10 g of silicic acid that had been de-activated with aqueous formic acid

	Eluate fraction		Non-volatile residue		
Bands (see Fig. 1)	Number	Volume, ml	Weight, mg	Appearance	Constituents, (from infrared spectra)
Colourless + pale yellow	(*i*)	25	54·2	Yellow oil + colourless crystals	Lipids: chiefly fatty acids
Pale yellow	(*ii*)	25	59·3		
	(*iii*)	20	3·9	Yellow gum	
Lower strong yellow	(*iv*)	30	2·9	Orange, pasty solid	Non-lipids, perhaps furan derivatives. Spectra virtually identical with those of corresponding artifact fractions from sucrose + amino-acids
Lowest pink	(*v*)	25	2·1	Pink, pasty solid	
	(*vi*)	25	0·9	Pale pink gum	

REPRODUCIBILITY—

Reproducibility of results is good (see Table III).

TABLE III

RESULTS SHOWING REPRODUCIBILITY

Material	Number	"Hydrolysate lipids" found, per cent. w/w
Dried-sponge mixes that had been extracted with chloroform - methanol mixture	1 (Sample A)	0·69, 0·68
	1 (Sample B)	0·68, 0·70
	2 (Sample A)	0·34, 0·34
	2 (Sample B)	0·32, 0·32
	3	0·24, 0·24
	4	0·30, 0·32
	5	0·31, 0·34
	6	0·30, 0·29
	7	0·31, 0·32
Flours	1	1·17, 1·18
	2	1·27, 1·27
Corn oil		90·0, 89·8
Tripalmitin*		93·3, 93·1

* Theoretical yield of palmitic acid from tripalmitin = 95·3 per cent.

ANCILLARY EXPERIMENTS—

Mixture of amino-acids and sucrose—Five grams of sucrose, 5 g of casein hydrolysate (Difco "Casamino Acids") and 50 ml of water were shaken with 100 ml of chloroform and 50 ml of methanol. The lower layer, which contains any lipid contaminants),[37] was rejected, the upper layer evaporated, and the residue subjected to the complete analysis. The pale yellow band (see Fig. 1) was missing, but the strong yellow and pink bands moved downwards as usual. The eluate, 65 ml, yielded 0·2 mg of residue.

Wheat-flour cerebrosides—The complete analysis was performed on 101 mg of "Early Peak E lipid," resulting from the chromatographic separation of wheat-flour lipids described earlier.[40,41] A 100-ml portion of the eluate yielded 33 mg of an oil (83 per cent. of the theoretical value for α-hydroxystearic acid) that was shown by infrared analysis to contain α-hydroxy fatty acid. A further 20-ml portion of the benzene eluate yielded less than 1 mg

of similar material. A 50-ml portion of diethyl ether eluted 17·2 mg of dodecylbenzenesulphonic acid. Finally, methanol eluted 22 mg of a mixture of formates of long-chain bases (46 per cent. of the theoretical value for phytosphingosine formate).

Beef heart muscle—The complete analysis was performed on 5 g of thinly sliced muscle. The first eluate, whose volume was 40 ml, was yellow. It contained no fatty acids, but its spectrum contained a strong, sharp band at 1735 cm^{-1} (carbonyl band for aldehydes) and a weak, sharp band at 1690 cm^{-1} (suspect carbonyl band for Q coenzymes[42,43]). The second eluate, 60 ml, contained chiefly fatty acids. The combined eluates yielded 89·0 mg of residue.

Tripalmitin—Palmitic acid, of greater than 99 per cent. purity, was esterified with glycerol, and the product purified by elution with benzene from silicic acid that had been impregnated with silver nitrate.[44]

Discussion of method

Broadly, the method can be said to determine the total fatty acids *plus* the non-saponifiable fraction of a sample. More precisely, it determines almost all lipids that survive complete hydrolysis, except bases and glyceryl ethers. For the quantity determined, we suggest the term "hydrolysate lipids." Because the determination is precise, selective, easily carried out and has a reasonable theoretical basis, it seems an excellent reference method for food analysis.

"Hydrolysate lipids" must never be equated mathematically with lipids obtained by other means until the latter have been hydrolysed in the same way. The reason for this can be appreciated by considering the structures of common lipids, such as the digalactosyl diglycerides,[45] which theoretically yield only about 60 per cent. of "hydrolysate lipids."

The method can be used for checking the efficiency of extraction procedures. By avoiding autoxidation, it permits isolation for chemical studies; thus it should facilitate determination of the fatty acid composition of whole tissues, lipopolysaccharides, *etc.* It is also a useful tool for studying the occurrence and composition of "firmly bound" lipids.

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References

1. Lovern, J. A., "The Chemistry of Lipids of Biochemical Significance," Second Edition, Methuen & Co. Ltd., London, 1957, p. ix.
2. Rapport, M. M., and Norton, W. J., *Ann. Rev. Biochem.*, 1962, **31**, 103.
3. Stacey, M., and Barker, S. A., "Carbohydrates of Living Tissues," D. Van Nostrand Co. Inc., New York, Toronto and London, 1962, p. 181.
4. Nicholls, J. R., Colgate, R. T., Fraser, L. S., and Hughes, E. B., *Analyst*, 1943, **68**, 48.
5. British Standard 1743: 1951.
6. Association of Official Agricultural Chemists, "Official Methods of Analysis," Ninth Edition, A.O.A.C., Washington, D.C., 1960.
7. American Association of Cereal Chemists, "Cereal Laboratory Methods," Sixth Edition, American Association of Cereal Chemists, St. Paul, Minnesota, 1957, p. 168.
8. Centre National de Coordination des Études et Recherches sur la Nutrition et l'Alimentation, "Les Méthodes Analytiques des Céréales, Farines et Autres Produits Dérivés," Centre National de la Recherche Scientifique, Paris, 1958.
9. Reichert, R., *Helv. Chim. Acta*, 1944, **27**, 961.
10. Rothe, M., *Getreide u. Mehl*, 1961, **11**, 138.
11. Pigman, W. W., and Goepp, R. M., "Chemistry of the Carbohydrates," Academic Press Inc., New York and London, 1948, p. 69.
12. Newth, F. H., *Adv. Carbohydrate Chem.*, 1951, **6**, 83.
13. Mednick, M. L., *J. Org. Chem.*, 1962, **27**, 398.
14. Dustin, J. P., Czajkowska, C., Moore, S., and Bigwood, E. J., *Anal. Chim. Acta*, 1953, **9**, 256.
15. Hepburn, F. N., Lewis, E. W., and Elvehjem, C. A., *Cereal Chem.*, 1957, **34**, 312.
16. Fuller, C. H. F., and Casson, C. B., *quoted by* Lampitt, L. H., Fuller, C. H. F., and Goldenberg, N., *J. Soc. Chem. Ind.*, 1947, **66**, 117.
17. Morgan, R. H., and Rawlings, H. W., *Analyst*, 1951, **76**, 161.
18. Sandberg, U. H., Proceedings of the XVth International Dairy Congress, London, 1959, Volume III, Section 5, p. 1552.
19. Craig, L. C., Gregory, J. D., and Hausmann, W., *Anal. Chem.*, 1950, **22**, 1462.
20. Coleman, M. H., *Lab. Practice*, 1962, **11**, 543.
21. Zacharius, R. M., and Krulick, S., *Anal. Chem.*, 1959, **31**, 2111.
22. Mottlau, A. Y., and Fisher, N. E., *Ibid.*, 1962, **34**, 714.
23. Klein, P. D., *Ibid.*, 1962, **34**, 733.
24. Mecham, D. K., and Mohammad, A., *Cereal Chem.*, 1955, **32**, 405.

25. Entenman, C., *in* Colowick, S. P., and Kaplan, N. O., *Editors*, "Methods in Enzymology," Academic Press Inc., New York and London, Volume III, 1957, p. 299.
26. Block, R. J., and Weiss, K. W., "Amino Acid Handbook," Charles C. Thomas, Illinois, and Blackwell Scientific Publications, Oxford, 1956, p. 18.
27. Twitchell, E., *J. Amer. Chem. Soc.*, 1906, **28**, 196 and 1907, **29**, 566.
28. Schauenstein, E., and Schatz, G., *Monatsh.*, 1959, **90**, 118.
29. ——, ——, *Angew. Chem.*, 1959, **71**, 681.
30. ——, ——, *Fette, Seif. Anstrichmitt.*, 1959, **61**, 1068.
31. Mattson, F. H., and Volpenhein, R. A., *J. Lipid Res.*, 1962, **3**, 281.
32. Wren, J. J., *J. Chromatography*, 1960, **4**, 173.
33. ——, *Chromatog. Revs.*, 1961, **3**, 111 and 177.
34. Horning, M. G., Williams, E. A., and Horning, E. C., *J. Lipid Res.*, 1960, **1**, 482.
35. Carter, H. E., Hendry, R. A., Nojima, S., and Stanacev, N. Z., *Biochim. Biophys. Acta*, 1960, **45**, 402.
36. Carter, H. E., Hendry, R. A., Nojima, S., Stanacev, N. Z., and Ohno, K., *J. Biol. Chem.*, 1961, **236**, 1912.
37. Sweeley, C. C., and Moscatelli, E. A., *J. Lipid Res.*, 1959, **1**, 40.
38. Schultz, H. W., Day, E. A., and Sinnhuber, R. O., *Editors*, "Lipids and Their Oxidation," Avi, Westport, Conn., U.S.A., 1962.
39. Folch, J., Lees, M., and Sloane-Stanley, G. H., *J. Biol. Chem.*, 1957, **226**, 497.
40. Wren, J. J., and Elliston, S. C., *Chem. & Ind.*, 1961, 80.
41. Wren, J. J., Proceedings of the First International Congress of Food Science and Technology, London, 1962, in the press.
42. Crane, F. L., and Lester, R. L., *Biochem. Preps.*, 1962, **9**, 30.
43. Morton, R. A., Gloor, U., Schindler, O., Wilson, G. M., Chopard-dit-Jean, L. H., Hemming, F. W., Isler, O., Leat, W. M. F., Pennock, J. F., Rüegg, R., Schwieter, U., and Wiss, O., *Helv. Chim. Acta*, 1958, **41**, 2343.
44. Vries, B. de, *Chem. & Ind.*, 1962, 1049.
45. Carter, H. E., Hendry, R. A., and Stanacev, N. Z., *J. Lipid Res.*, 1961, **2**, 223.

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The Determination of Caesium-137 in Irradiated Uranium by using the Ring-oven Technique and Gamma-ray Spectrometry

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A ring-oven method is described for determining caesium-137 in irradiated uranium solutions. A small drop of the solution is placed on a pre-formed ferric hydroxide precipitate situated at the centre of a filter-paper. The paper is positioned on a ring oven, and washed with ammonia solution and water. The caesium-137 migrates to the ring zone, and the other fission products and uranium stay in the centre of the paper. The caesium-137 in the ring zone is determined by using a γ-ray scintillation spectrometer. The method compares well with an ion-exchange procedure.

The determination of caesium-137 in irradiated uranium is used as a measure of the number of atoms in the fuel that have undergone fission. Factors leading to the choice of caesium-137 as a fission monitor have been described elsewhere,[1,2,3] and the methods used for its separation have been reviewed.[4]

In the method described in this paper, the caesium-137 is rapidly separated from a solution of irradiated uranium by using the Weisz ring-oven technique, and is then determined by γ-ray scintillation spectrometry.

General considerations

The ring-oven technique was devised to carry out qualitative analyses on samples of the order of 1 μl. The solution is placed on the centre of a filter-paper situated on an electrically heated ring, and the ions are separated, either individually or into groups, by adding certain reagents. Some ions are thus precipitated on the centre of the filter-paper and others migrate to the edge of the heated ring owing to the capillary action of the filter-paper. Rapid evaporation of the solvent near the heated surface causes the migrating ions to concentrate in a ring, and they are subsequently detected by specific spot tests. The technique has become semi-quantitative by comparing the samples with the standards, and has also been used in conjunction with other analytical techniques, *e.g.*, paper chromatography and solvent extraction.[5]

In radiochemical studies, the ring oven has been used for separating simple mixtures, and the positions of the separated components have been determined by autoradiography.[6] Autoradiography has since been used semi-quantitatively for measuring isotopic concentrations.[7,8,9] γ-Ray scintillation spectrometry permits γ-ray emitters to be quantitatively determined in the presence of one another, provided they have different energies.[10] It should thus be possible to separate complex mixtures of γ-ray emitters into groups by using the ring-oven technique, and to identify the components by their γ-ray energies instead of by spot tests.

The principal γ-ray-emitting fission products in an irradiated uranium solution that has been allowed to cool for 100 days are zirconium-95 (0·72 and 0·75 MeV), niobium-95 (0·76 MeV), ruthenium-106 (0·51 and 0·62 MeV), caesium-137 (0·66 MeV) and cerium-144 0·14 and 0·70 MeV). The γ-ray spectrum of such a solution is so complex that the contribution of caesium-137 cannot be directly determined without prior chemical separation.

The possible methods for separating caesium-137 by using the ring-oven technique were considered to be either (*a*) precipitation of an insoluble caesium salt on the centre of the filter-paper and then washing the other radioactive components to the ring zone or (*b*) fixation of the unwanted radioactive components in the centre of the filter-paper, either by precipitation or adsorption on a surface-active precipitate, *e.g.*, ferric hydroxide, and removal of a soluble caesium salt to the ring zone.

Consideration of the analytical chemistry of the fission products makes it clear that the latter alternative is the more attractive.

The possibilities considered for fixing the unwanted radioactive components in the centre spot were (*i*) precipitation of the zirconium-95 - niobium-95 mixture, cerium-144 and uranium by adding ammonia solution to an irradiated uranium solution that contained added zirconium and cerium carriers, and (*ii*) retaining the carrier-free activities of zirconium-95 - niobium-95 mixture and cerium-144 on a pre-formed ferric hydroxide precipitate. Since the

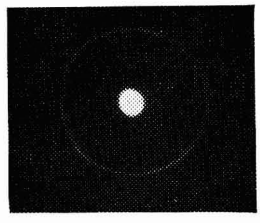

Fig. 1. Autoradiograph showing the separation of caesium-137 to the ring zone

optimum conditions for retaining zirconium-95 - niobium-95 mixture and cerium-144 on ferric hydroxide precipitates are different from those of ruthenium,[11] the behaviour of the ruthenium-106 activity could not be accurately predicted. Similarly, the distribution of the ruthenium activity resulting from the mixed-hydroxide precipitation was uncertain.

These two procedures were tested experimentally.

Experimental

The ring oven used was of similar design to that described by Weisz.[5] Throughout the tests a capillary micropipette of 0·6-μl capacity was used, and the reactions were carried out on Whatman No. 540 filter-papers.

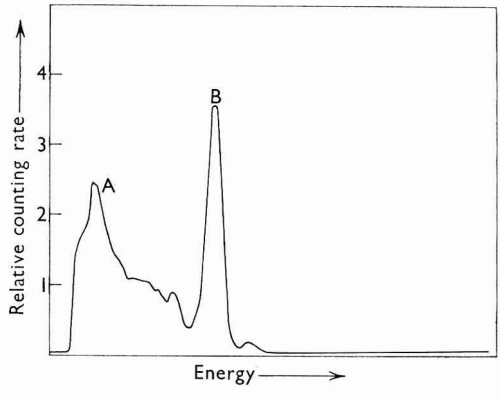

Fig. 2. Gamma-ray spectrum of the separated caesium-137: A, backscatter peak; B, caesium-137 photopeak at 0·66 MeV

Behaviour of caesium-137—

A small drop of caesium-137 solution containing 5 mg of caesium carrier per ml was placed on to a pre-formed ferric hydroxide precipitate and washed to the ring zone. The percentage of caesium-137 removed to the ring zone with each wash is shown below—

Number of washes	1	2	3	4	5
Percentage caesium-137 in outer ring	93·8	97·6	98·9	99·8	100

Complete removal of caesium-137 to the ring zone was also achieved in five washes on a filter-paper containing precipitated ammonium diuranate and zirconium hydroxide.

BEHAVIOUR OF IRRADIATED URANIUM SOLUTIONS—

A sample of irradiated uranium solution, containing added caesium carrier only, was placed on a pre-formed ferric hydroxide precipitate, and washed with ammonia solution and then with water. A typical separation obtained is shown by the autoradiograph reproduced in Fig. 1. The outer ring was then removed and placed on a γ-ray scintillation spectrometer. Fig. 2 shows that the principal photopeak was caused by γ-radiation at 0·66 MeV from the barium-137 daughter of caesium-137, and Fig. 3 shows the γ-ray spectrum of the filter-paper before separation. From these results the loss of zirconium and cerium to the ring zone was calculated to be less than 0·1 per cent., while not more than 2 per cent. of the ruthenium activity had moved to the ring zone.

When it was precipitated with ammonia solution and then washed with water, a sample of uranium solution containing cerium and zirconium carriers, as well as caesium carrier gave a similar separation of caesium. There were in this instance, however, small additional losses of zirconium-95 - niobium-95 mixture, and approximately 20 per cent. of the ruthenium activity was removed to the ring zone.

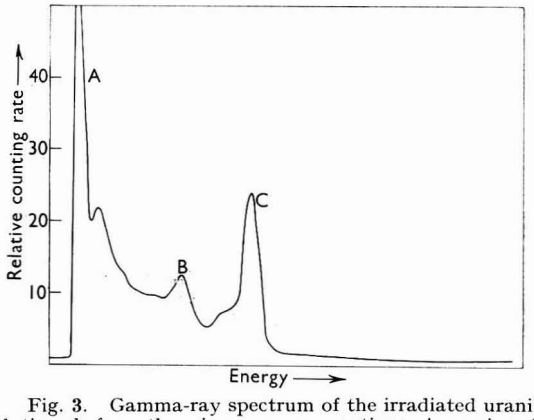

Fig. 3. Gamma-ray spectrum of the irradiated uranium solution before the ring-oven separation: A, cerium-144 photopeak at 0·14 MeV; B, ruthenium-106 photopeak at 0·51 MeV; C, zirconium-95 - niobium-95 photopeak at 0·72 to 0·76 MeV

The separation based on the adsorption of carrier-free radioactive components by a pre-formed ferric hydroxide precipitate was the more efficient procedure. This was mainly owing to the ability of the ferric hydroxide precipitate to retain a higher proportion of the ruthenium activity than ammonium diuranate and the mixed hydroxides of zirconium and cerium.

METHOD

REAGENTS—

Ferric chloride solution—Dissolve 2·4 g of hydrated ferric chloride, $FeCl_3.6H_2O$, in 100 ml of de-ionised water containing 4 ml of concentrated hydrochloric acid, sp.gr. 1·18. This solution contains 5 mg of ferric iron per ml.

Caesium carrier—Dissolve 6·3 g of caesium chloride in 100 ml of de-ionised water. This solution contains 50 mg of caesium per ml.

Ammonia solution, sp.gr. 0·88.

PROCEDURE—

Place a Whatman No. 540 filter-paper on the ring oven, and add sufficient ferric chloride solution to extend to a diameter of 8 mm. Precipitate ferric hydroxide by adding ammonia solution. Add sufficient caesium carrier to the irradiated uranium solution to give an overall concentration of 5 mg of caesium per ml, and adjust the pH to 1. By means of a capillary micropipette of approximately 1-μl capacity, add the irradiated uranium solution to the pre-formed ferric hydroxide precipitate. Add sufficient ammonia solution to reach the edge of the ferric hydroxide precipitate, and then wash the sample five times to the ring zone with

de-ionised water. When the paper is dry, cut away the centre portion containing the ferric hydroxide and mount the ring zone on a suitable tray for counting by γ-ray scintillation spectrometry. Place an aluminium absorber weighing 250 mg per sq. cm on the counting tray, and then determine the caesium-137 photopeak at 0·66 MeV. Repeat the determination with a standardised caesium-137 solution that has undergone the same ring-oven treatment. The proportion of caesium-137 in the unknown solution may then be calculated.

Results

To confirm the method of analysis, a synthetic solution and samples of irradiated uranium having different irradiation and cooling histories, were analysed by the ring-oven method and also by an ion-exchange technique. The ion-exchange technique was based on the method of Woodhead, Fudge and Jenkins.[12] The results obtained are given in Table I and show that agreement between the methods over a range of caesium activities is good, and that the precision of the method is about 2 per cent.

Table I

Analysis of irradiated uranium solutions for caesium-137

Solution	Uranium concentration, mg per ml	Caesium-137 concentration, mC of caesium-137 per g of uranium* by— ion-exchange method	ring-oven method
1	19·8	0·151	0·149 ± 0·0036
2	7·4	2·18	2·16 ± 0·046
3	33·2	2·22	2·21 ± 0·044
4	6·7	7·25	7·26 ± 0·142
5†	14·2	4·26	4·22 ± 0·094

* Errors shown are standard deviations calculated from at least five independent measurements for each solution.

† Synthetic solution.

Conclusions

A microchemical method has been described for determining caesium-137 in irradiated uranium, in which the ring-oven technique was used in conjunction with γ-ray scintillation spectrometry. The advantages of the method include speed (the separation itself takes only a few minutes), simplicity and the negligible use of shielding. Further, the method is precise and does not need any determination of the yield since a calibrated standard is always analysed under the same conditions as the unknown sample.

Further use of the ring-oven technique in conjunction with γ-ray spectrometry should provide an efficient method for determining many radio nuclides in complex mixtures.

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References

1. Crouthamel, C. E., Heinrich, R., and Gatrousis, C., *Talanta*, 1958, **1**, 396.
2. Rider, B. F., Russell, J. L., jun., Harris, D. W., and Peterson, J.P., jun., *U.S. Atomic Energy Commission Report* GEAP-3373, Pleasanton, California, 1960.
3. Sunderman, D. N., *Special Technical Publication No.* 276, American Society for Testing Materials, Philadelphia, 1959, p. 3.
4. Finston, H. L., and Kinsley, M. T., *U.S. Atomic Energy Commission Report* NAS-NS-3035, National Academy of Sciences—National Research Council, Washington, D.C., 1961.
5. Weisz, H., "Micro-analysis by the Ring-oven Technique," Pergamon Press, Oxford, London, New York and Paris, 1961.
6. Weisz, H., and Scott, F., *Mikrochim. Acta*, 1956, 1856.
7. Weisz, H., and Ottendorfer, L. J., *Ibid.*, 1961, 191.
8. Ottendorfer, L. J., and Weisz, H., *Ibid.*, 1962, 725.
9. Weisz, H., and Ottendorfer, L. J., *Ibid.*, 1962, 818.
10. Crouthamel, C. E., *Editor*, "Applied Gamma-ray Spectrometry," Pergamon Press, Oxford, London, New York and Paris, 1960, p. 135.
11. Pushkarev, V. V., *Zhur. Neorg.Khim.*, 1956, **1**, 170, *translated by* Taylor, G. R., *U.K. Atomic Energy Authority Report* IG 1593 (RD/W), Windscale, 1960.
12. Woodhead, J. L., Fudge, A. J., and Jenkins, E. N., *Analyst*, 1956, **81**, 570.

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The Use of Flame-photometry for Determining Phosphate

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Phosphate in rock phosphate can be determined by using a simple and rapid procedure. The sample is dissolved in nitric acid and after dilution is sprayed into an oxy-acetylene - air flame, and calcium is determined from the intensity of its emission. At this temperature, the depressive effect of phosphate ions on calcium emission is virtually eliminated. The ratio of phosphate to calcium is determined by measuring the depression of the intensity of calcium emission when the sample is sprayed into a propane - air flame. With the calcium content known, the phosphate content can be calculated.

THE depression of intensity of emission of calcium, and alkaline-earth elements in general, by phosphate ions has been studied by several workers,[1 to 7] who investigated the factors responsible for this inhibitory effect, and the conditions under which calcium can be determined correctly in the presence of phosphate ions.

It was found that at relatively low phosphate-ion concentration, the intensity of calcium emission decreases linearly with increasing phosphate-ion concentration, until a critical value is reached, beyond which calcium emission is practically independent of phosphate concentration. Values of the molar ratio of phosphate to calcium at this critical point are variously reported in the literature, ranging from 0·33 to 1·0, depending probably upon the apparatus used and on experimental conditions, especially temperature and type of atomiser. Baker and Johnson[1] found the phosphate-to-calcium ratio to be unity, compared with 0·8 found by Mavrodineau and Boiteux,[2] 0·5 found by Leyton[3] and 0·33 reported by Alkemade and Voorhuis.[4]

Alkemade and Voorhuis,[4] Filcek[5] and Schuhknecht and Schinkel[6] reported that the depression of calcium emission by phosphate ions can be virtually eliminated by using higher flame temperatures and an atomiser that gives a good dispersion. In the work described, we used the combination of a high-temperature flame and an atomiser of good dispersion for determining calcium in the presence of phosphate.

Dippel, Bricker and Furman[7] used the inverse relationship between phosphate concentration and intensity of calcium emission as basis for phosphate determination. In their procedure, the sample to be analysed was dissolved, evaporated to dryness, redissolved and passed through a cation-exchange resin. Appropriate amounts of calcium were added to the eluent, and phosphate was determined by a flame-photometric procedure.

The phosphate-to-calcium ratio of calcium orthophosphate, $Ca_3(PO_4)_2$, is 0·66, and since there are compounds of calcium other than the orthophosphate present in rock phosphate, the ratio of phosphate to calcium must always be less than 0·66. Because the critical value of the ratio of phosphate to calcium was 0·7 under the experimental conditions we used, a linear relationship existed between phosphate-ion concentration and intensity of calcium emission. In the proposed method, therefore, phosphate is determined directly in the dissolved sample. The phosphate content was determined in a low-temperature flame, and under these conditions, the calcium emission was inversely proportional to the phosphate concentration. A standard solution, containing a calcium concentration approximately equal to that found in the sample, is sprayed into the flame, and the apparatus is adjusted to give a meter deflection of 100 per cent. The solution to be analysed is then atomised and the percentage emission noted. From a calibration curve of phosphate-to-calcium ratio *versus* percentage emission, the phosphate concentration can be obtained, since the calcium content is now known.

In the flame-photometric determination of calcium, the high-temperature flame is used, since it eliminates depression of the calcium emission by phosphate ions. To measure this depression effect, and hence the phosphate concentration, the low-temperature flame is used as this accentuates the effect.

METHOD

PREPARATION OF THE SAMPLE—

Dissolve 2·5 g of accurately weighed powdered phosphate in 30 ml of nitric acid, sp.gr. 1·42. Gently boil this solution for several minutes in a covered beaker, and set aside on a hot-plate for half an hour; under these conditions all the phosphate dissolves. Transfer the cooled solution to a 250-ml calibrated flask, and make up to the mark with water. Dilute 50 ml of this solution to 1 litre, and use this solution for flame-photometric measurements.

We used an Eppendorf Flame Photometer, fitted with a 620 mμ multi-layer interference-filter, for measuring the intensity of calcium emission.

DETERMINATION OF CALCIUM—

Prepare standard solutions of calcium by dissolving known amounts of calcium carbonate in the minimum volume of concentrated nitric acid. Adjust the nitric acid, sp.gr. 1·42, with water until it is identical to that of the sample to be analysed. Spray these solutions into an oxy-acetylene - air flame (pressures, above atmospheric: acetylene, 500 mm of water; oxygen - air, 0·5 atmospheres) and prepare a calibration curve of calcium concentration *versus* intensity of emission. Spray the rock phosphate solution to be analysed into the oxy-acetylene - air flame, and measure the percentage emission. Read off the calcium content of the sample from the calibration graph.

The relation between calcium content and percentage emission is linear.

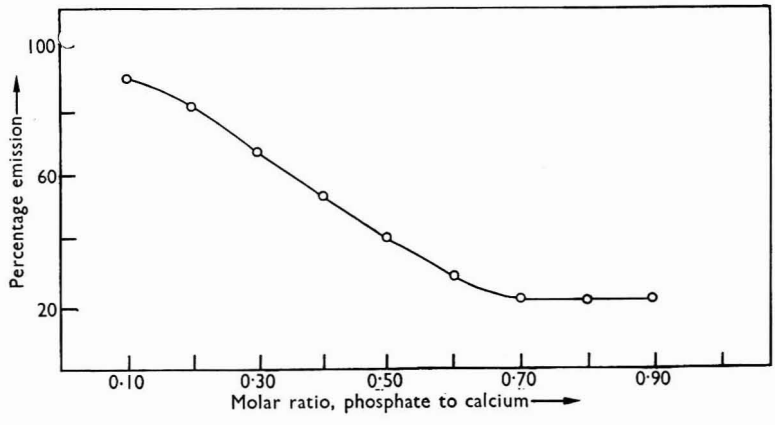

Fig. 1. Calibration curve for phosphate determination

DETERMINATION OF PHOSPHATE—

Prepare separate standard stock solutions of calcium carbonate, dissolved in nitric acid, and potassium phosphate, dissolved in water. Mix these solutions in varying proportions to give a suitable range of phosphate-to-calcium ratios, combined with a suitable range of calcium content. Spray these solutions into a propane - air flame (pressures, above atmospheric: propane, 160 mm of water; air, 0·5 atmospheres), measure the intensity of emission and prepare a calibration graph of phosphate-to-calcium ratio *versus* percentage emission (see Fig. 1). Spray a standard solution of calcium carbonate, whose calcium content is approximately equal to that found in the sample, into the propane - air flame, and adjust the meter deflection to read 100 per cent. Finally spray the sample into the flame, measure the percentage emission and read off the ratio of phosphate to calcium from the calibration graph for an equivalent calcium content, and hence calculate the concentration of phosphate.

The calcium content of samples whose calcium content is within about 10 per cent. of that of the standard used to prepare the calibration curve, can be determined from the same curve. The example quoted on p. 138 shows that small changes in calcium content do not result in false values for the phosphate-ion concentration being obtained. Under the experimental conditions used, the calcium emission obtained with samples of the same phosphate concentration, but different calcium content, will only depend on the calcium content.

EXAMPLE—

Sample No. 10 (see Table I), in which the final concentration of the sample was 0·343 g of calcium phosphate per litre, was found, by spraying in the oxyacetylene - air flame, to contain 4·86 mmoles of calcium per litre. A standard calcium solution, containing 4·5 mmoles of calcium per litre was selected, as it had a calcium content approximately that of the sample. The meter deflection was adjusted to read 100 per cent. for the intensity of calcium emission of this standard solution in the propane - air flame. Sample No. 10 was then sprayed into the flame and gave a 50·5 per cent. emission. By proportion, sample No. 10, sprayed after adjusting a standard solution of calcium containing 4·86 moles per litre to give 100 per cent. deflection would have given a 46·76 per cent. emission. (To check this a standard solution containing 4·86 moles of calcium per litre was specially prepared. Sample No. 10 was sprayed and gave 46·8 per cent. emission, as expected.) If the calcium content of the analysed solution and that of the standard adjusted to 100 per cent. deflection are not identical, the percentage emission of the sample has to be multiplied by a factor equal to—

$$\frac{\text{Calcium content of the standard}}{\text{Calcium content of the sample}}$$

before reading off the ratio of phosphate to calcium from the calibration graph.

From the calibration curve, the phosphate-to-calcium ratio corresponding to 46·8 emission is 0·46, and the phosphate content of the sample can be calculated from the formula—

$$\text{Percentage phosphate content (as } P_2O_5) = 7{\cdot}098 \times \frac{\text{phosphate-to-calcium ratio} \times \text{calcium content (in mmoles per litre)}}{\text{accurate weight of sample}} \times 5$$

In this example, the phosphate content (as P_2O_5), was 31·4 per cent.

INTERFERENCE—

The influence of fluoride, sulphate, silicate and aluminium ions on calcium emission was also investigated, since these ions can remain in the solution of the sample and interfere with the calcium emission. Fluoride, sulphate and silicate ions were added separately to the solution of the sample in amounts up to 5 per cent. of the concentration of the sample, and aluminium ions were added in amounts up to 3 per cent. No interference was observed. To determine the accuracy with which the nitric acid should be added, a number of experiments were carried out with varying amounts of acid. A deviation of 2 ml of the concentrated acid per litre did not affect the results.

RESULTS AND DISCUSSION

Fourteen rock-phosphate samples of different origins, containing from 22 to 25 per cent. of phosphate (as P_2O_5), were dissolved in nitric acid and analysed by the procedure described.

TABLE I

CALCIUM CONTENT IN MMOLE PER LITRE OF 0·5 g OF PHOSPHATE ROCK SAMPLE

Sample No.	Flame-photometric procedure	Titrimetric procedure	Percentage relative difference
1	4·56	4·60	−0·9
2	4·38	4·38	0·0
3	4·50	4·58	−1·7
4	4·92	4·92	0·0
5	4·59	4·56	+0·7
6	4·70	4·62	+1·7
7	4·91	4·96	−1·0
8	4·46	4·48	−0·4
9	4·59	4·62	−0·7
10	4·86	4·84	+0·1
11	4·59	4·56	+0·7
12	4·56	4·54	+0·4
13	4·53	4·54	−0·2
14	4·50	4·50	0·0

Table I compares the calcium content determined by the flame-photometric technique, with that determined by the standard oxalate - permanganate method[8]: the maximum relative difference between the results is 1·74 per cent.

The phosphate content (as P_2O_5) found by the flame-photometric technique is compared with that determined by the standard gravimetric technique[9] in Table II: the maximum relative difference between the results is 3·8 per cent.

TABLE II

PERCENTAGE PHOSPHATE CONTENT (AS P_2O_5)

Sample No.	Flame-photometric procedure	Gravimetric procedure	Percentage relative difference
1	28·4	27·6	+2·9
2	22·0	21·6	+1·9
3	27·8	28·1	−1·1
4	38·5	38·2	+0·8
5	28·4	28·3	+0·4
6	33·8	33·9	−0·3
7	35·4	35·3	+0·3
8	36·2	35·2	+2·8
9	30·0	28·9	+3·8
10	31·4	30·9	+1·6
11	24·9	24·5	+1·6
12	28·0	27·0	+3·7
13	28·0	27·5	+1·8
14	27·3	27·7	−1·4

Standard solutions, used for preparing the phosphate-to-calcium ratio *versus* percentage emission calibration curve, containing 4·0, 4·25, 4·50 and 5·0 moles of calcium, but having the same phosphate-to-calcium ratio, gave exactly the same curve after the apparatus had been adjusted to give a 100 per cent. meter deflection for pure calcium standard solutions of corresponding calcium content. Calibration curves prepared from calcium phosphate and calcium carbonate solutions were identical.

REFERENCES

1. Baker, C. L., and Johnson, L. R., *Anal. Chem.*, 1954, **26**, 465.
2. Mavrodineau, R., and Boiteux, H., "L'Analyse Spectrale Quantitative par la Flamme," Masson et Cie., Paris, 1954, p. 95.
3. Leyton, L., *Analyst*, 1954, **79**, 497.
4. Alkemade, C. T. J., and Voorhuis, M. H., *Z. anal. Chem.*, 1958, **163**, 91.
5. Fileck, M., *Z. PflErnähr. Düng.*, 1959, **85**, 112.
6. Schuhknecht, W., and Schinkel, H., *Z. anal. Chem.*, 1958, **162**, 266.
7. Dippel, W. A., Bricker, C. E., and Furman, N. H., *Anal. Chem.*, 1954, **26**, 553.
8. The Association of Official Agricultural Chemists, "Official Methods of Analysis," Ninth Edition, A.O.A.C., Washington D.C., 1960, p. 21.
9. ——, *op. cit.*, p. 9.

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SHORT PAPERS

The Polarographic Determination of Lead in Antimony Sulphide Flotation Concentrates

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THE accurate determination of low concentrations of lead in antimony sulphide concentrates is important to manufacturers, since smelters impose penalties if certain limits are exceeded. The need for a reliable, rapid method prompted the study of the applicability of polarography to this determination. This problem was also investigated from the atomic-absorption approach, but this paper is confined to the polarographic determination.

The conventional gravimetric method for determining lead as chromate is time consuming, and the results obtained for the samples we encounter are of questionable reliability.

Norwitz, Cohen and Everett[1] have described a gravimetric method for determining lead after liquid - liquid extraction, evaporation of chloroform and precipitation as sulphate. In this procedure the precipitate has to be set aside overnight to settle. The method for dissolving the sample and extracting the lead with dithizone given by these authors has been followed in the proposed method.

Sandell[2] mentions that lead can be stripped from a dithizone extract with 0·02 M hydrochloric acid. This indicated how lead might be obtained in a solution suitable for polarography.

Kolthoff and Lingane[3] reported that a M sodium hydroxide supporting electrolyte is especially useful for the polarographic determination of lead in the presence of tin, antimony and arsenic.

According to Meites[4] it is better to determine lead polarographically in alkaline tartrate solution without the addition of gelatin. This was confirmed in the preliminary experiments.

In the preliminary experimental work it was established that the addition of antimony metal to a lead solution, up to a concentration level of 5 g of antimony per litre of the solution used for the polarographic determination, still produced a well defined wave for lead in the supporting electrolyte used. At higher concentrations antimony was precipitated.

When direct determinations were attempted on samples concentrated by flotation, ill-defined waves were obtained. Precipitation by means of sodium sulphide did not overcome the interference encountered. Extraction with dithizone was tried for separating the lead.

It was verified that a linear relationship exists between concentration and diffusion current.

Experimental

Apparatus—

A Cambridge general-purpose polarograph, capillary stand and galvanometer assembly was used. A suitable recorder was not available.

Extraction solution—

Dithizone solution—Dissolve 0·2 g of dithizone (diphenylthiocarbazone) in 1 litre of chloroform. This solution should be protected from light as far as possible and used within a week after preparation.

Procedure—

Weigh a 1-g sample containing 0·1 to 0·2 per cent. of lead, or 2 g if the sample contains less than 0·1 per cent. of lead, into a 250-ml Pyrex beaker, and add 30 ml of hydrochloric acid, sp.gr. 1·16. Cover, and boil for 1 hour. Add 30 ml of 20 per cent. w/v tartaric acid solution and 20 to 30 ml of hot water. Filter off the insoluble material and wash it with hot diluted hydrochloric acid (1 + 4). Neutralise the combined filtrate and washings to litmus with ammonia solution, sp.gr. 0·91, low in lead, and add 7 drops in excess. Dilute the solution to approximately 200 ml, and transfer it to a clean 500-ml separating funnel.

Extract the lead with two 50-ml portions of extraction solution. Repeat with 25-ml portions until the extract remains green or blue green. Wash the combined extracts with two 25-ml

portions of 0·02 N hydrochloric acid. At this stage, the chloroform solution should be green or blue green.

Reduce the volume of the combined aqueous layers to approximately 30 ml, and add 10 ml of 20 per cent. w/v tartaric acid solution. Neutralise to litmus with 2 N sodium hydroxide, add 4 g of sodium hydroxide pellets and dilute to 100 ml.

Introduce the sample at 25° C into a polarographic cell, remove oxygen with an inert gas and note the galvanometer readings for applied voltages of −0·2 to −0·9 volts, in steps of 0·05 volts, with a sensitivity setting of 1 to 1.

Calculate the concentration of lead from the height of the wave obtained at the half-wave potential (between −0·525 and −0·575 volt) compared to the height of a wave obtained by using a standard.

Discussion of the method

Completeness of dissolution—The acid-insoluble portions of some of the samples were brought into solution by fusion with sodium carbonate, and the proposed procedure applied to the dissolved melt. No significant wave could be detected.

Extraction and washing—The sensitivity of the dithizone extraction for lead is well known, and the colour change is easy to observe. It was established by atomic absorption that the recovery from a standard lead solution was 99·5 per cent.

Concentrations of sodium hydroxide and tartaric acid solutions—Small variations of the sodium hydroxide and tartaric acid solution concentrations about the recommended level did not have a significant effect on the results obtained.

Precision obtained—The standard deviation calculated from 17 determinations was 0·0045 per cent. of lead.

Comparison with other methods—

The results obtained by the proposed method were compared with those obtained by using a classical gravimetric method and by atomic absorption on diluted nitric acid washings. Some typical results are reported in Table I.

Table I

Comparison of methods for determining lead

	Lead found, per cent., by using—		
Sample No.	gravimetric method	atomic-absorption method	polarogragraphic method
1	0·179	0·174	0·185
2	0·106	0·117	0·118
3	0·155	0·157	0·157
4	0·130	0·144	0·137
5	0·168	0·152	0·151
6	0·135	0·142	0·139
7	0·173	—	0·164

Advantages of the proposed method—

The proposed method is more rapid than the conventional gravimetric lead chromate method, and is found to give greater precision. As indicated, the process of extraction and washing with dilute nitric acid can be used for atomic absorption.

If desired the chloroform can be recovered.[5]

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References

1. Norwitz, G., Cohen, J., and Everett, M. E., *Anal. Chem.*, 1960, **32**, 1132.
2. Sandell, E. B., "Colorimetric Determination of Traces of Metals," Third Edition, Interscience Publishers Inc., New York and London, 1959, p. 556.
3. Kolthoff, I. M., and Lingane, J. J., "Polarography," Second Edition, Interscience Publishers Inc., New York and London, 1952, Volume 2, p. 529.
4. Meites, L., "Polarographic Techniques," Interscience Publishers Inc., New York and London, 1955, p. 140.
5. Green, H., *B.C.I.R.A. Journal*, 1961, **9**, 382; *Anal. Abstr.*, 1961, **8**, 5299.

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The Detection of Orange RN and Orange II in Meat

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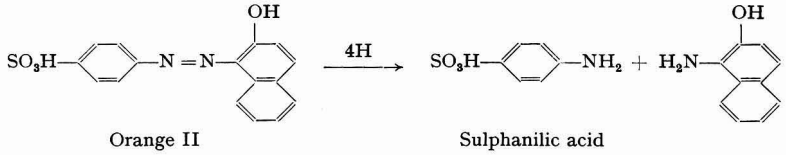
Orange RN (1-phenylazo-2-naphthol-6-sulphonic acid) is a dye permitted in Singapore for use as colouring matter, under the Singapore Food and Drugs Regulations,[1] whereas Orange II (*p*-2-hydroxy-1-napthylazobenzenesulphonic acid) is a dye not permitted for use in foodstuffs. The United Kingdom has similar regulations under "The Colouring Matter in Food Regulations."[2] Orange RN has been used extensively as a colouring matter in a popular Chinese preparation of sweetened roasted pork known as "Char Siew." The meat is made to look more appetising by dipping the seasoned meat in a solution of the dye, and then roasting it. Orange II is occasionally used and it is possible that a mixture of these two dyes may be used. The present investigation was undertaken to differentiate the dyes when they occur separately or together in "Char Siew." The proposed method should be applicable to any such type of meat preparation.

Experimental

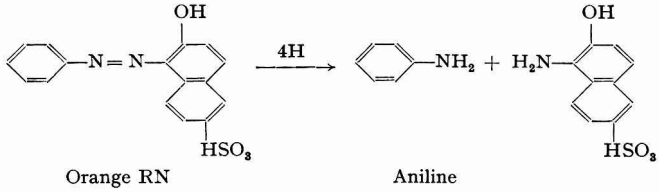
Laboratory investigations on Orange II revealed that it had the same R_F value as Orange RN in all the seven solvents recorded by the Association of Public Analysts.[3] Of the twenty-seven solvents used by Tilden,[4] only 1 per cent. ammonium hydroxide saturated with amyl alcohol gave streaks slightly different in length for Orange RN and Orange II. However, even this solvent failed to resolve a mixture of the two dyes. The method of Yanuka, *et al.*,[5] in which solvents of different pH values were used, gave identical chromatograms for the two dyes. The absorption curves of the dyes in acid and alkaline solutions were found to be similar. Further, when the dyes are present together their absorption spectra would be useless. Some knowledge of the identity of the two dyes, when stained on wool and when not present together, may be obtained by their reaction with 50 per cent. sulphuric acid solution; Orange II becomes purplish, but Orange RN becomes reddish orange. If the dyes are present together, this test would serve no useful purpose.

The need for a solvent system for resolving these two dyes is apparent, especially in routine examination of food samples. Investigations showed that downward elution on Whatman No. 1 filter-paper with a freshly prepared solution of 50 per cent. v/v ammonium hydroxide, sp.gr. 0·88, in water resolved a mixture of the two dyes sufficiently to permit them to be distinghished. However, when the dyes were recovered from meat, it was observed that water-soluble degradation products interfered with the movement of the dyes in the chromatogram. Further work indicated that by adding sodium chloride solution to the solvent the degradation products could be successfully separated from the dye spots, leaving the spots to be eluted smoothly down the paper.

Differentiation of these two dyes may also be achieved by reduction; this technique was recently used by Reed and Heinekey,[6] to differentiate Orange GGN from Sunset yellow. When Orange II is reduced it gives sulphanilic acid and Orange RN gives aniline—



Orange II Sulphanilic acid



Orange RN Aniline

It was found that instead of reducing the dyes in solution with tin and hydrochloric acid,[6] the dyes could be conveniently reduced on the paper with a drop of a solution of stannous chloride in hydrochloric acid. Van Buuren and Hoeke[7] have recently used titanous chloride for the same purpose. The chromatogram with the reduced dye spot was developed by the method of Panchartek, Allan and Huzik.[8] The spot was eluted downward with n-butanol saturated with 2·5 N hydrochloric acid. The spots of aniline and sulphanilic acid were detected by exposing the dried chromatogram to nitrous fumes, then spraying the paper with 2-naphthol-3,6-disulphonic acid and exposing it to ammonia vapour. Orange RN formed a red spot (similar in colour and R_F value to aniline), and also a dark spot. Orange II formed an orange spot (similar in colour and R_F value to sulphanilic acid). It was again observed that degradation products interfered with the dyes extracted from meat. Addition of sodium chloride in the solvent helped the separation between the degradation products and the reduced dyes, but caused the dark spot resulting from Orange RN to overlap the orange spot from Orange II, thus making it difficult to distinguish the dyes. This problem can be overcome by using a Whatman No. 1 filter-paper, impregnated with 2 per cent. sodium chloride solution. The degradation products are separated during elution, and proper resolution of the hydrogenated products of the dyes is achieved.

The colouring matter present in "Char Siew" was found to be about 10 mg of dye per 100 g of meat, the colour being on the surface of the meat. Various proportions of Orange II and Orange RN, with a total colour intensity equivalent to 10 mg of dye per 100 g of meat, were added to undyed fried pork and the dyes extracted, with some modification, by the wool-dyeing technique described by the Association of Public Analysts.[3] The proposed methods were found to be satisfactory for distinguishing about 3 per cent. of Orange II in Orange RN by the ammonium hydroxide - sodium chloride solvent system, and about 2 per cent. of Orange II in Orange RN by the reduction technique. All chromatograms were eluted at room temperature (about 28° C).

Method

Apparatus—

Chromatographic tanks—Suitable for downward elution of paper chromatograms (size approximately 30 × 34 cm).

Chromatographic papers—(*I*) Whatman No. 1 filter-paper, cut into strips (10 × 33 cm): (*II*) Whatman No. 1 filter-paper strips (31 × 33 cm). Dip the strips into 2 per cent. sodium chloride solution and hang them at room temperature to dry. Store in paper envelopes. On each of the papers draw a line parallel to, and 6 cm from, one edge.

Reagents—

Wool—De-greased white wool.

Stannous chloride solution—Dissolve 33 g of analytical-reagent grade stannous chloride, $SnCl_2.2H_2O$, in 20 ml of hydrochloric acid, sp.gr. 1·18, warming if necessary, and dilute to 100 ml with distilled water. Filter through a Whatman No. 42 filter-paper (or equivalent). Mix well, and store it in a dark-coloured bottle.

Aniline containing 2 per cent. sulphanilic acid—Dissolve 0·98 g of twice redistilled aniline and 0·02 g of sulphanilic acid in 15 ml of N hydrochloric acid, dilute to 100 ml with distilled water and mix well.

Orange RN containing 3 per cent. of Orange II—Dissolve 0·97 g of Orange RN and 0·03 g of Orange II in distilled water, dilute to 100 ml and mix well.

Eluting solvents—(*a*) Ammonium hydroxide - sodium chloride system; add 50 ml of ammonia solution, sp.gr. 0·88, to 50 ml of 10 per cent. sodium chloride solution. Use a freshly prepared solution. (*b*) n-Butanol - hydrochloric acid system; saturate n-butanol with 2·5 N hydrochloric acid.

Nitrous fumes—Place about 1 g of sodium nitrite in the bottom of a dessicator. Add about 1 ml of dilute hydrochloric acid, and replace the lid. Prepare freshly before use.

2-Naphthol-3,6-disulphonic acid spray reagent—Dissolve 1 g of the sodium salt in 100 ml of distilled water.

Extraction and recovery of dyes—

Take 50 g of the meat and boil it gently for about one hour with 250 ml of distilled water. Filter the solution while still hot through a Whatman No. 32 filter-paper, and add about 4 g of de-greased wool and 2 ml of glacial acetic acid to the filtrate. Boil gently until the wool appears to be sufficiently dyed. Remove the wool and boil it for about 30 minutes in about 200 ml of distilled water containing a drop of glacial acetic acid. Fat adhering to the wool is dislodged by

this procedure. Repeat the boiling with successive amounts of water, each portion containing a drop of glacial acetic acid, until the washings appear to be clear. Remove the wool and strip the dyes from it with about 100 ml of 1 per cent. ammonium hydroxide solution. Discard the wool when it becomes colourless and evaporate the ammoniacal solution of the dye to dryness. Wash the residue with about 50 ml of warm (50° C) water. Filter off any solid, and evaporate the filtrate to about 0·5 ml.

CHROMATOGRAPHIC DIFFERENTIATION OF THE EXTRACTED DYE—

Place 0·005 ml of standard Orange RN - Orange II solution on the chromatographic paper (*I*). Transfer sufficient of the extracted dye solution to the paper chromatogram to give a spot that has an intensity approximately that of the standard dye spot. Hot air may be used to restrict the size of the spots. Elute the chromatogram downward with the ammonium hydroxide - sodium chloride solvent for about eight hours. The heavier, lower Orange RN spot is separated from the weaker and lighter Orange II spot by about 2 cm. Remove the paper from the tank and mark out the spots immediately, since the spots tend to diffuse on drying, owing to the sodium chloride in the paper.

CHROMATOGRAPHIC DIFFERENTIATION OF THE EXTRACTED DYE AFTER REDUCTION—

Place 0·005 ml of the standard dye solution and 0·005 ml of standard aniline - sulphanilic acid solution on to the chromatographic paper (*II*). Transfer enough of the extracted dye solution to give a spot that has an intensity equal to that of the standard dye spot. Hot air may be used to restrict the size of the dye spots. Dry the aniline - sulphanilic acid spot in air. By means of a capillary tube, add sufficient stannous chloride solution (1 or 2 drops) to wet the dye spots and dry the paper in air. If the spots are not completely de-colourised, add more stannous chloride solution and dry the paper again in air. Elute the spots downwards in the n-butanol - hydrochloric acid system for about six hours. Remove the paper, dry it in air and place it in the dessicator containing nitrous fumes for about a minute. Spray it with the 2-naphthol-3,6-disulphonic acid reagent and while it is still wet expose the paper to ammonia vapour by holding it over the mouth of a bottle containing ammonia solution. Orange RN will form an orange spot of R_F value 0·83 (resembling aniline) and a dark spot of R_F value 0·35, and Orange II will form a single orange spot of R_F value 0·18 (resembling sulphanilic acid).

DISCUSSION OF THE METHOD

The amount of dye used for colouring "Char Siew" is small, since only the outer surface is dyed by the dipping process used for applying the dye. Consequently, a substantial proportion of the extracted dye contains water-soluble degradation products. The detection of small amounts of one dye in the presence of another necessarily involves transferring relatively large amounts of the dye extracted, together with degradation products, on the paper. The proposed method should, therefore, be particularly suitable for meat products containing small amounts of dyes.

The faint dark-coloured spots resulting from the reduction of Orange RN was further investigated. Sunset yellow and Fast red E, both of which have the same component as Orange RN and which would give the same hydrogenation product, (1-amino-2-naphthol-6-sulphonic acid), gave similar faint dark-coloured spots of similar R_F values when subjected to the reduction technique. It is possible that this spot is that of 1-amino-2-naphthol-6-sulphonic acid. The other hydrogenation product of Orange II (1-amino-2-naphthol) begins to show as a faint dark spot, with an R_F value of 0·85, at much higher concentrations of the dye, and is therefore out of the context of this paper.

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REFERENCES

1. The Food and Drugs (Amendment) Regulations, Singapore, 1961, No. S36 of 1961, Government Printer, Government Printing Office, Singapore.
2. Statutory Instrument No. 1066, H.M. Stationery Office, London, 1957.
3. "Separation and Identification of Food Colours Permitted by the Colouring Matters in Food Regulations, 1957," The Association of Public Analysts, London, 1960.
4. Tilden, D. H., *J. Ass. Off. Agric. Chem.*, 1952, **35**, 423.
5. Yanuka, Y., Shalon, Y., Weissenberg, E., and Nir-Grosfeld, I., *Analyst*, 1962, **87**, 791.
6. Reed, R. H., and Heinekey, D. M., *Ibid.*, 1962, **87**, 391.
7. van Buuren, J. H. W., and Hoeke, F., *Chem. Weekbl.*, 1962, **58**, 155.
8. Panchartek, J., Allan, Z. J., and Muzik, F., *Coll. Czech. Chem. Commun.*, 1960, **25**, 2783; abstr. in *J. Chromatography*, 1961, **6**, D12.

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The Determination of Cyanide in Cyanophoric Plants

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Existing procedures for determining cyanide in cyanophoric plants do not have great accuracy. They use either direct distillation or steam-distillation in conjunction with acid or enzyme hydrolysis. A large volume of distillate is produced, causing reduced accuracy of titration. Methods that involve boiling may cause hydrogen cyanide to be converted into other substances, and volatile compounds, such as hydrogen sulphide, may appear in the distillate and interfere with titration.

The preservation and aeration technique of Briese and Couch[1] increases the accuracy of the determination. Three aspects, however, could be improved: the time required to take sub-samples of the minced material for moisture determinations might permit cyanide loss, the process of transferring the stored tissue from preservative jars to distillation flasks is cumbersome and Liebig's turbidimetric titration for cyanide is difficult to perform.

In this paper a method is proposed in which the harvested plant is cut into small pieces rather than minced, and the moisture content is determined on a sub-sample. The remaining material is stored in a distillation flask rather than a preservative jar. The final estimation is by Archer's method.[2] This titration is performed in a water - alcohol mixture to which a little dithizone has been added. The sensitivity of the method is claimed to be about ten times as great as that of the Liebig method. When 0·01 N silver nitrate is used as titrant, the end-points are extremely sharp.

Method

Preservation—

Chop the harvest material into $\frac{1}{8}$- to $\frac{1}{4}$-inch lengths. Take a sample of some material for the moisture determination, and introduce 30 to 40 g of fresh tissue into a weighed 500-ml conical flask containing 200 ml of distilled water and 10 ml of 2 per cent. mercuric chloride solution. Seal the flask and weigh it; hence determine the weight of added material. Specimens can be preserved for six months.

Table I

Recovery of hydrogen cyanide from cyanide-containing samples

System analysed for HCN content	Total HCN content of material analysed	KCN recovered, as HCN, from plant material, %	Standard error of mean of six determinations
KCN	1·75	94·4	±3·5
S. almum	0·86		
S. almum + KCN ..	2·63	95·0	±3·2
S. almum	1·76		
S. almum + KCN ..	3·52	94·3	±2·5
S. almum	3·48		
S. almum + KCN ..	5·27	96·2	±3·4
S. sudanense	1·77		
S. sudanense + KCN	3·54	95·1	±3·7
T. repens	0·06		
T. repens + KCN ..	1·69	90·6	±3·3
C. camphora	0·00		
C. camphora + KCN	1·78	95·8	±3·8

Determination of cyanide—

The technique for liberating cyanide is a modification of Briese and Couch's method[1] and requires apparatus similar to that used by van der Walt.[3] In the proposed method, however, the hydrogen cyanide liberated from the plant material is held as sodium cyanide in two 100-ml collecting flasks, each containing 50 ml of 1 per cent. sodium hydroxide solution in 95 per cent. ethanol. Prepare daily a 0·01 per cent. solution of dithizone (diphenylthiocarbazone) in acetone, and add 2 ml of this solution to each of the collecting flasks. Titrate the contents of the flask, by means of a microburette, with 0·01 N silver nitrate until a deep red-purple colour is formed. Carry out a blank titration exactly as above and deduct the blank value from the sample titre.

1 ml of N silver nitrate ≡ 0·05204 g of cyanide.[4]

At the end-point there should be not more than 20 ml of water present, including the volume of titrant added. If there is more than 20 ml of water present, the end-point is less sharp.

RECOVERY OF LIBERATED HYDROGEN CYANIDE—

To test the efficiency of this method, 0·10 milliequivalents of potassium cyanide were hydrolysed with tartaric acid in the absence of plant material. The amount of hydrogen cyanide liberated was calculated from the titres of 0·01 N silver nitrate for portions of unhydrolysed potassium cyanide. The procedure for hydrolysing potassium cyanide was repeated in the presence of *Sorghum almum* (three different samples), *Sorghum sudanense*, *Trifolium repens* and *Cinnamomum camphora*. The cyanide content of the plant material had been previously determined; this value was subtracted from the value for the total cyanide content. Recovery results are summarised in Table I.

DISCUSSION OF RESULTS

By using former methods of analysis, the recovery of potassium cyanide was found to vary between 85 and 95 per cent. The proposed method appears to improve reproducibility by permitting an accurate assessment over a wide range of total cyanide contents of cyanophoric plants.

REFERENCES

1. Briese, R. R., and Couch, J. F., *J. Agric. Res.*, 1938, **57**, 81.
2. Archer, E. E., *Analyst*, 1958, **83**, 571.
3. van der Walt, S. J., *Onderstepoort J. vet. Sci.*, 1944, **19**, 103.
4. Vogel, A. I., "Quantitative Inorganic Analysis," Second Edition, Longmans Green & Co., London, New York and Toronto, 1955, p. 264.

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The Spectrophotometric Determination of Caffeine

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SINCE the publication of a method for the spectrophotometric determination of caffeine in coffee and in coffee and chicory mixtures,[1] evidence has been obtained that this method can be applied to the determination of caffeine in other caffeine-containing products, provided any 5-hydroxymethylfurfural that may occur in the sample is first removed from the extracts by treatment with aluminium amalgam.[2] This applies to mixtures of coffee and roasted cereals, and to caramelised sugar products sometimes used as coffee substitutes. 5-Hydroxymethylfurfural in such products is formed by the pyrolytic decomposition of sugars.

A sample of a German soluble coffee substitute, consisting only of a dried extract of roasted barley, was examined by the spectrophotometric method. An apparent caffeine content of 0·63 per cent. was found for the sample, from the absorption readings at 273 mμ, before it had been treated with aluminium amalgam. The presence of 5-hydroxymethylfurfural in the clarified water extract was confirmed by a peak of maximum absorption at 282 mμ in the absorption curve. After 5-hydroxymethylfurfural had been removed by treatment with aluminium amalgam, the "caffeine" content was found to be negligible (0·03 per cent.).

The determination of small amounts of residual caffeine in de-caffeinated coffee by any known method is difficult, because interfering substances occur in larger proportions, relative to caffeine, than in normal coffee. Thus Barbera[3] found that a similar photometric method could be used for determining caffeine in raw de-caffeinated coffee, but not in roasted de-caffeinated coffee, because of an impurity in the chloroform extracts of the latter samples. This impurity is now known to consist of derivatives of furfural, whose presence in the clarified extract of a sample of a German de-caffeinated coffee was confirmed by a "shoulder" at 282 mμ in the absorption curve of a clarified extract. Results obtained by using the published method[1] showed the caffeine content of this sample to be 0·07 per cent., compared to a value of 0·61 per cent. obtained without aluminium amalgam reduction.

Base-line corrections cannot be made to the optical-density readings of those extracts of de-caffeinated coffee that contain insufficient caffeine to show an absorption peak at 273 mμ. The value of the optical density at 400 mμ was deducted from the value of the optical density at 273 mμ, to allow for the small background absorption, and the caffeine content was calculated from the corrected optical density value at 273 mμ. A value of 0·510 for the $E_{1\,cm}^{0·001\%}$ value of a pure solution of caffeine was used for calculating caffeine content.

The published method[1] has been found to be suitable for determining the caffeine content of those caffeine-containing carbonated beverages that contain 5-hydroxymethylfurfural derived from the sugar syrup used for sweetening[4] and from the caramel used for colouring. The results obtained for two samples of such beverage, when 50 ml of sample were used for each determination, agreed closely with those obtained from micro-Kjeldahl determinations of nitrogen in chloroform extracts, by the method of Bower, Anderson and Titus.[5] Without the aluminium amalgam treatment, the results obtained were high by about 3 mg of caffeine per 100 ml of sample solution, see Table I. Wilson[4] obtained a blank value of 0·4 mg of caffeine per 100 ml of caffeine-containing beverage, caused by the presence of 5-hydroxymethylfurfural in the invert-sugar syrup used in manufacture.

TABLE I

SUMMARY OF RESULTS

Sample	Caffeine content found by using— micro-Kjeldahl method[5]	spectrophotometric method, without reduction	spectrophotometric method, with reduction
Soluble coffee substitute (roasted cereal) ..	0·09%	0·63%	0·03%
De-caffeinated coffee	0·04%	0·61%	0·07%
Cola beverage 1	13·4 mg per 100 ml	16·5 mg per 100 ml	13·5 mg per 100 ml
Cola beverage 2	7·2 mg per 100 ml	10·3 mg per 100 ml	7·1 mg per 100 ml

REFERENCES

1. Smith, R. F., and Rees, D. I., *Analyst*, 1963, **88**, 310.
2. Smith, R. F., *Café, Cocoa, Thé*, 1963, **7**, 223.
3. Barbera, C. E., *Z. Lebensmitt. Untersuch.*, 1962, **117**, 483.
4. Wilson, J. B., *J. Ass. Off. Agric. Chem.*, 1958, **41**, 617.
5. Bower, R. S., Anderson, A. D., and Titus, R. W., *Anal. Chem.*, 1950, **22**, 1056.

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Determination of Hypobromite and Bromite with Tartrazine as Indicator

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HYPOBROMITE is a strong oxidant in alkaline solution, and has been used for determining nitrogen,[1] sulphur,[2] urea[3] and many other compounds.[4,5,6,7] This necessitates determining the strength of hypobromite and its decomposition products by a simple and accurate method.

Belcher[8] found that tartrazine is a good reversible indicator for the titration of arsenite with sodium hypochlorite in a solution containing sodium hydrogen carbonate and potassium bromide. The presence of bromide is necessary; otherwise the indicator is destroyed at the end-point. In Kjeldahl's method for determining nitrogen in organic compounds, tartrazine was used as an indicator in the final titration with hypochlorite.[9,10]

In the present investigation, a procedure was developed for determining hypobromite and bromite by direct titration with arsenous oxide with tartrazine as an indicator. The method is simple, accurate and rapid, and, with the same indicator, hypobromite and its decomposition products can be determined, since tartrazine is bleached by hypobromite only, whereas bromite and bromate have no effect on its colour.

Method

Reagents—

All reagents were of analytical-reagent grade.

Sodium hypobromite solution—Prepare solutions of various strengths[1,2] and standardise these against ammonium sulphate[11]; by using this method the hypobromite and bromite contents can be determined.

Arsenous oxide, 0·12 N, aqueous—Dissolve the approximate amount of arsenous oxide needed to make 1 litre of solution in 10 per cent. sodium hydroxide solution. Render the solution acid to phenolphthalein, and add 10 g of solid sodium hydrogen carbonate. Dilute the solution to 1 litre, and standardise it against iodine solution.

Sodium thiosulphate, 0·1 N—Prepare an approximately 0·1 N solution and standardise it against a 0·1 N potassium iodate solution prepared from reagent that has been previously dried at 120° C.

Tartrazine solution, 0·05 per cent. w/v aqueous.[8]

Procedure—

Use potassium iodate as the primary standard, and carry out the determination by using the methods described below—

(*a*) Add 1 g of solid sodium hydrogen carbonate and two drops of tartrazine indicator solution to 5 ml of standard arsenous oxide solution. Titrate the contents of the flask with hypobromite solution. The end-point is reached when the yellow colour of the indicator disappears. Since the colour of the indicator fades near the end-point, one or two trial titrations must be made to determine the end-point. For accurate titrations, add the indicator solution only when the end-point is approached. The normality determined from this titre, titre *A*, corresponds to the hypobromite and bromite contents.

(*b*) Add 1 g of solid sodium hydrogen carbonate to 5 ml of a solution containing hypobromite and bromite, and titrate the solution with standard arsenous oxide with tartrazine as an external indicator. The end-point is reached when a drop of the reaction mixture, removed from the flask, does not bleach a drop of tartrazine on a tile. This indicates the approximate end-point range. Repeat the experiment and add a drop of tartrazine solution directly to the titration flask when the end-point is approached, instead of using it as an external indicator. If the end-point has not been reached, the colour of the added indicator disappears; if the end-point has been reached, the indicator imparts a yellow colour to the solution. In the former instance, add a drop of arsenite from a burette and then another drop of tartrazine solution. Repeat the process until tartrazine solution imparts its colour to the solution in the flask. The normality determined from this titre, titre *B*, corresponds to the hypobromite content only.

Calculations—

The hypobromite and bromite contents may be determined from titres *A* and *B* since—

$$\text{Hypobromite content} \equiv \text{titre } B$$
$$\text{and bromite content} \equiv \text{titre } A - \text{titre } B$$

Results

The proposed method was used for determining hypobromite and bromite contents in different samples of hypobromite solution previously standardised by the ammonium sulphate method.[11] The results obtained are shown in Table I and indicate that almost all the hypobromite and bromite present in the solution could be determined accurately.

Discussion of the method

The method has been based on the observation that bromite does not react with arsenous oxide until all the hypobromite present in the solution has been completely removed. With the same indicator, hypobromite and its decomposition products can be determined, since tartrazine indicator solution is bleached only by hypobromite, whereas bromite and bromate have no effect on its colour. The total hypobromite, bromite and bromate contents of the solution can be determined by adding an excess of potassium iodide solution and titrating the liberated iodine with sodium thiosulphate solution. The bromate content of the solution can thus be calculated by subtracting the bromite and hypobromite contents, determined by using method (*a*), from the total contents determined by using the latter titration.[11]

TABLE I

DETERMINATION OF HYPOBROMITE AND BROMITE WITH TARTRAZINE AS INDICATOR

Sample	Hypobromite content, as normality— By ammonium sulphate method[11]	By proposed method	Difference	Bromite content, as normality— By ammonium sulphate method[11]	By proposed method	Difference
A	0·0905	0·0901	−0·0004	0·0096	0·0097	+0·0001
B	0·1285	0·1278	−0·0007	0·0116	0·0120	+0·0004
C	0·0890	0·0886	−0·0004	0·0072	0·0070	−0·0002
D	0·0728	0·0734	+0·0006	0·0049	0·0047	−0·0002
E	0·1904	0·1890	−0·0014	0·0139	0·0144	+0·0005
F	0·1365	0·1367	+0·0002	0·0126	0·0125	−0·0001

It was observed that the most suitable pH value for enhanced sensitivity of tartrazine indicator solution lies between pH 7 and pH 9. Sodium hydrogen carbonate[8] must be added to maintain the pH within this range. This effect may also be achieved by adding sodium tetraborate or small amounts of boric acid.

REFERENCES

1. Hashmi, M. H., Ali, E., and Umar, M., *Anal. Chem.*, 1962, **34**, 988.
2. Hashmi, M. H., Elahi, M., and Ali, E., *Analyst*, 1963, **88**, 140.
3. Margosches, B. M., and Rose, H., *Biochem. Z.*, 1923, **137**, 542.
4. Cullis, C. F., and Hashmi, M. H., *J. Chem. Soc.*, 1957, 3080.
5. Willson, V. A., *Ind. Eng. Chem., Anal. Ed.*, 1935, **7**, 44.
6. Tomick, O., and Jasek, M., *Coll. Czech. Chem. Commun.*, 1938, **10**, 353.
7. Friedman, A. H., and Morgulis, S., *J. Amer. Chem. Soc.*, 1936, **58**, 909.
8. Belcher, R., *Anal. Chim. Acta*, 1950, **4**, 468.
9. Belcher, R., and Bhatty, M. K., *Mikrochim. Acta*, 1956, 1183.
10. Ashraf, M., Bhatty, M. K., and Shah, R. A., *Anal. Chim. Acta*, 1961, **25**, 448.
11. Hashmi, M. H., and Ayaz, A. A., *Anal. Chem.*, 1963, **35**, 908.

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Book Reviews

METHODS IN CARBOHYDRATE CHEMISTRY. Volume I. ANALYSIS AND PREPRATION OF SUGARS. Edited by ROY L. WHISTLER, M. L. WOLFROM, JAMES N. BEMILLER and F. SHAFIZADEH. Pp. xxii + 589. New York and London: Academic Press Inc. 1962. Price 143s.

METHODS IN CARBOHYDRATE CHEMISTRY. Volume II. REACTIONS OF CARBOHYDRATES. Edited by ROY L. WHISTLER, M. L. WOLFROM and JAMES N. BEMILLER. Pp. xvi + 572. New York and London: Academic Press Inc. 1963. Price 139s.

Progress in biological chemistry has been so rapid during the last decade that workers in this field, or even in a specialised section of the field have difficulty in keeping pace with published work. This applies, in particular, to carbohydrate chemistry so that the publication of the first two volumes of "Methods in Carbohydrate Chemistry" will be welcomed by all laboratories engaged on research or routine work involving the preparation, separation or analysis of carbohydrates.

Inevitably personal experiences and preferences of the individual authors will be reflected in the choice of methods. The relegation of the classical methods of sugar analysis, such as those based upon Fehling's solution or modifications thereof, *e.g.*, Lane and Eynon, or Munson-Walker, that are still the official methods of the A.O.A.C. and the International Commission for Uniform Methods of Sugar Analysis to the comparative obscurity of an "honourable mention" in favour of the various modifications of the Somogyi copper-reduction method or of the colorimetric methods in which phenol - sulphuric acid mixture or anthrone is used, may be premature. It is appreciated, however, that these methods have great advantages on the micro scale met in research.

Volume I, "Analysis and Preparation of Sugars," sets out to compile a set of reliable methods for routine or experimental use. Ninety-seven different authors, all of world eminence, have contributed 146 items covering their own particular specialities and the result is the production

of an extremely complete treatise. The main sections of this volume are headed: Laboratory Techniques, Monosaccharides (Trioses to Heptoses, Deoxy Sugars, Amino Sugars, Branched Chain, C-14 labelled sugars, Ionososes), Oligosaccharides (reducing and non-reducing), Analyses, Colour Reactions of Carbohydrates and Physical Measurements.

Volume II, "Reactions of Carbohydrates," is the result of the efforts of 85 contributors. It is concerned with the various reactions of the simple carbohydrates and in addition contains a broader choice of selected methods for successful variants found in other reference lists.

Some sixteen sections (embracing 121 papers) cover the whole field of simple carbohydrate reactions. These extend to 1961 (as far as one can discover from the references, although many contributions only reach up to 1959) and this, in my opinion, is as near to perfection as an editorial panel of even this high calibre can get. The printing presses of every country are so clogged with ephemeral material and eminent men are invariably too fully occupied with their work to be able to undertake authorship, that from "instructions to author" to "publication" may well take two or three years. Thus the references may well be out of date long before the material of the article has ceased to be of interest.

Drs. Roy L. Whistler, M. L. Wolfrom and (in Volume II) James N. BeMiller are to be congratulated not only for the excellence with which they have edited the two volumes but also for their individual contributions and for collecting such a large and high-powered group of authors to obtain practically complete coverage of the two fields with which the volumes deal.

The information in both volumes is adequate for any properly trained chemist; the field covered is as complete as is humanly possible, the material being written by specialists and edited by well known active workers in this field; the bibliographies and indices are accurate and up to date and it is well written, produced, printed and adequately bound. J. A. RADLEY

BRITISH PHARMACEUTICAL CODEX, 1963. Published by direction of the Council of the Pharmaceutical Society of Great Britain. Pp. xxxvi + 1433. London: The Pharmaceutical Press. 1963. Price 105s.

The British Pharmaceutical Codex was intended originally as "a book of reference for those engaged in prescribing and dispensing medicines." The seventh edition has become very much more than that, providing standards and methods of analysis for many important materials used in medicine that are not included in the British Pharmacopoeia. The movement towards complete standardisation, which was well seen in the B.P.C. 1959, has progressed even further with the present edition. Many new specifications have been set, not only for drugs but also for preparations in the formulary, and increasing use has been made of recent techniques for isolating, separating and determining active material and ingredients. New monographs include those for 120 drugs, together with new materials used to produce improved vehicles for administration of drugs. Noteworthy among the latter are the fluorocarbon propellants for pressurised packs, for analysis of which an appendix is given.

The use of spectrophotometric, fluorimetric and chromatographic techniques, and of complexometric, potentiometric and non-aqueous titrations has been extended, in addition to those newer methods of the British Pharmacopoeia to which the Codex refers the analyst. Among methods noted in passing are the use of tetraphenylboron as a precipitant for certain alkaloids, the determination of quinine fluorimetrically, the separation of strychnine chromatographically from Easton's syrup and tablets, and the chromatographic separation of aneurine hydrochloride, nicotinamide and pyridoxine hydrochloride on an alginic acid column from tablets containing them. Chlorides and bromides in mixtures are all to be determined electrometrically while complexometric determination of bismuth, calcium and mangesium is used for the more complete analysis of alkaline powders, mixtures and tablets. A completely new departure is the introduction of infrared spectrophotometric methods to distinguish and identify related compounds with similar chemical structures. For this purpose arrangements are announced for the Pharmaceutical Society to supply authentic specimens of substances to those analysts who require them. The fruits of the labours of the joint Panel of the Pharmaceutical Society of Great Britain and the Society of Public Analysts appear in the assay procedures for determining the reserpine-like alkaloids in rauwolfia and of capsaicin in capsicum and its preparations.

The British Pharmaceutical Codex has partially changed from the Imperial to the Metric system, provision being made for the period of change while it is intended that the next Codex will have changed completely. There has been a change also in the basis for atomic and molecular weights, the carbon-12 scale being used in place of the oxygen-16 scale.

Together with the British Pharmacopoeia, the British Pharmaceutical Codex covers an extremely wide range of pharmaceutical materials and formulated preparations in more frequent use, and possession of the volume is essential to those engaged in their analysis. Arrangements have been made for both volumes to be published and to become effective simultaneously, thus enhancing their value. H. E. BROOKES

WEST-EUROPEAN SYMPOSIA ON CLINICAL CHEMISTRY. VOLUME 2. THE CLINICAL CHEMISTRY OF MONOAMINES. Edited by HAROLD VARLEY, M.Sc., F.R.I.C., and A. H. GOWENLOCK, M.Sc., M.B., Ch.B., Ph.D. Pp. xvi + 242. Amsterdam, London and New York: Elsevier Publishing Company. 1963. Price 60s.

This book contains the proceedings of a two-day symposium on the clinical chemistry of mono-amines arranged by the Association of Clinical Biochemists, and held in Manchester in July, 1962. It consists mainly of short reviews by invited participants, and falls into three parts.

The first part deals with the catecholamines and includes papers on formation and metabolism (Axelrod), the determination of catecholamines (Callingham and Cass) and of metabolites (Peter Smith, Ruthven), together with reviews of clinical and pathological aspects of catecholamine-secreting tumours. The plethora of methods described for assaying the catecholamines and the metabolites in biological material reflects both the great interest shown in these substances and the unsatisfactory state of current analytical procedures. These problems are considered both in papers and in the subsequent discussions.

The second part of the book deals with hydroxyindoles in a similar manner. Formation and metabolism are discussed by Jepson, and determination by Sandler. The clinical features and pathology of carcinoid tumours are also discussed.

The third part of the book consists of a stimulating paper on the toxicology of mono-amine-oxidase inhibitors and tranquillisers by A. S. Curry, and reviews of the rôle played by mono-amines in the central nervous system, and of drug-induced alterations of mono-amine metabolism.

This volume is not bed-time reading for the analyst. Most of the participants have succeeded in the difficult task of reviewing large and technically controversial subjects within the compass of a few pages and as a result the proceedings of this Symposium give an admirably concise and up-to-date picture of the field.

It is in some ways invidious to single out individual contributions when so many are good. One cannot forbear, however, to mention Sandler's feat, in his review of the determination of hydroxyindoles, of referring to 190 papers in nine pages, and yet remaining perfectly intelligible. The programme committee should also be congratulated for including Crossland's review of the rôle of mono-amines in the central nervous system. A. D. MUNRO-FAURE

PARTICLE SIZE: MEASUREMENT, INTERPRETATION AND APPLICATION. By RIYAD R. IRANI and CLAYTON F. CALLIS. Pp. x + 165. New York and London: John Wiley & Sons. 1963. Price 60s.

The arrangement of the contents of this book does not correspond to the title; rather is it the exact opposite. The opening chapter describes applications of the measurements of particle-size distribution and is presumably intended to whet the reader's appetite. It made me somewhat impatient to find out what the authors had to say about the definition and measurement of particle size. I think that the chapter on applications could have been made more meaningful if it had been placed at the end of the book.

The definitions do not seem to me to be very logically ordered; they might have been made clearer if examples had been given. The section on particle shape is brief and rather superficial. Succeeding chapters deal with methods of data presentation and distribution functions. The Gaussian distribution is shown to be derived from the binomial distribution, and the chapter continues with a more extended treatment of the lognormal distribution and its modifications. In fact, the section on limited and multimodal distributions of the lognormal type, with the examples, was, for me, one of the more interesting parts of the book.

Then follow chapters dealing with particle-size measurement grouped under the main headings: Sedimentation Techniques, Microscopy, Sieving, and Miscellaneous Techniques. Together with the last chapter, which gives general guidance, they are intended to permit the reader to choose the appropriate method of particle-size measurement. To some extent this object is fulfilled; comparison of the book with the Classification of Methods published by the Society for Analytical Chemistry Particle Size Analysis Sub-Committee in *The Analyst*, March, 1963, shows that most

of the well known methods are described in the book, but that many of the less well known methods are omitted. This part of the book, therefore, is a critical review of selected methods of measurement and could be of some assistance to those faced with a choice of methods. However, it is an exaggeration to claim that this book fulfils a need for a reference work that gives an analyst a simple choice of the preferred technique; it guides him to the appropriate group or groups of techniques, and that is all. The publication mentioned above gives this sort of guidance and so do other books, such as Herdan's *Small Particle Statistics*.

The amount of detail given varies greatly. Sieving is treated in considerable detail, but the Andreasen pipette and its derivatives, which are probably used more than any other method, receive less than a page. (In fact, the classification in *The Analyst*, March, 1963, gives the pipette method at least as much space, and provides more useful details.) No mention is made of the use of the light-extinction method for the rapid routine control of the specific surface of powders.

The penultimate chapter, which presents the results of various comparisons of methods, is of considerable interest, though it appears from the list of references that American work predominates or may even have a monopoly; have no comparisons been made elsewhere?

The price is high for a book of this size and type. I can give it only a lukewarm commendation.

D. G. BEECH

THE APPLICATION OF MATHEMATICAL STATISTICS TO CHEMICAL ANALYSIS. By V. V. NALIMOV. Translated by PRASENJIT BASU. English Translation Editor: DR. M. WILLIAMS. Pp. x + 294. Oxford, London, Paris and Frankfurt: Pergamon Press. 1963. Price 84s.

Any book that helps to rid chemists of their inhibitions over the use of statistical methods deserves every encouragement from a reviewer. The average chemist is usually dissuaded from the application of statistics and theory of error, either by the lack of mathematical background needed to understand the relatively indigestible fare in books on the mathematics of statistics, or by the enormous number of words required to describe results that can be expressed succinctly in mathematical terms. Fortunately, more chemists are beginning to recognise the importance of the subject and, as Nalimov states, they are finding that journal editors are curtailing the experimental part of original papers so that readers are unable to assess reliability of results from the texts. As a consequence, authors should provide a compact quantitative estimate of error, and this, together with other useful results, can be achieved by use of a statistical treatment.

This book will undoubtedly be of great assistance to chemists who wish to expand their knowledge of statistics at a reasonable pace. It provides a short mathematical treatment for each section (derivations of statistical functions are generally omitted) and uses established methods and formulae on results from simple genuine examples that are critically examined. The explanations and descriptive matter are painstakingly done, almost to the point of verbosity, but the reader is able to obtain a feeling of statistics and error—a state of affairs rarely achieved from the average book on the subject. Much credit must go to the translator for this, in that he has treated the material faithfully and although some examples of Russian construction remain, he has anglicised the text well and made it readable.

The analytical chemist will find the title to be accurate, in that the book gives all the definitions, treatment of variables, distributions, comparisons of results, analysis of variance, analysis of linear functions, design of experiment and most of the tables of statistical data that are likely to be required. The reader with an elementary knowledge of mathematics should have no difficulty in understanding how to use them. The bibliography is good and most of the useful Western texts are quoted, despite the author's not wholly accurate criticism of Anglo-American statisticians who, he claims, find an end in classifying and summarising results rather than in interpreting them.

One question remains: is the book worth the outlay of four guineas? Even bearing in mind the difficult typography and translator's fees the price is high for such a monograph. When it is realised that certain small English and American monographs cover much of the same ground for a small fraction of the cost, the potential buyer may decide that the price is too high. Nevertheless, the subject matter is of a high standard, the book is almost self-contained, and chemists concerned with quantitative measurements should read and use it.

D. A. PANTONY

ORGANIC FUNCTIONAL GROUP ANALYSIS. By F. E. CRITCHFIELD. Pp. viii + 187. Oxford, London, New York and Paris: Pergamon Press. 1963. Price 42s.

The rapid expansion of the organic chemical industry and the increasing need for ever more detailed information about the presence of particular groups in organic molecules has of late led

to considerable development in the area of functional-group analysis. Frequently, analyses have to be performed on compounds that have similar molecular weights, and empirical formulae that differ little from each other, so that even the most reliable of ultimate organic analyses leaves room for doubt about the identity of the molecule. Moreover, such analyses, valuable though they may be, require to be supplemented by information from infrared spectra, and even here the fingerprint of a complex molecule is not always easily identified. In such instances direct chemical analysis for functional groups in the molecule is invaluable in establishing identity and purity. This area of analysis may indeed lack the glamorous chromium-plated, electronic modernity of many instrumental methods, yet despite its atmosphere of staid classical glassware, bunsen burner and burette it is suprisingly quick and economic. Frequently in terms of reliability, speed and cost as well as accuracy it far outstrips those instrumental methods that overlap into its zone of application.

This new book on the topic is a really good one, and within its range is both an admirable exposition of the scientific basis of the chemical reactions involved and an excellent laboratory manual for use at the bench. The chapter headings best reveal the area covered by the text. Following a brief introduction these are: Acids and Bases, Nitrogen, Carbonyl, Hydroxyl, Unsaturation, 1,2-Epoxy Compounds, Esters, Carboxyl Anhydrides, Peroxides and Sulphur Compounds. Many alternative methods are listed under most headings, and the scope and known limitations of most of them are well discussed. The techniques used are mainly titrimetric for macro scale and colorimetric for micro scale or trace amounts. Generally no special apparatus other than glassware is involved. Most of the reactions are carried out at room temperature or at 100° C. In these latter instances the use of heat-resistant pressure bottles immersed in a steam-bath in place of conventional reflux apparatus strikes, for me at least, an unusual and original note. The points made by the author in favour of this procedure, *viz.* convenience and freedom from atmospheric contamination most certainly have appeal. It may well be that this book will set a new trend in laboratory customs in this respect.

The test is well written and the experimental instructions are clearly expressed, though an unusual turn of phrase startles one occasionally, *e.g.*, on page 4 the end of the second paragraph suggests that there are possibly such things as "invisible" spectrophotometers! From a student's view-point, the author has disappointingly not included much background to the general literature on each analytical species, but the reasons for this are indeed expressed at the outset. I would have liked to have seen a short chapter on *qualitative* functional-group analysis at the beginning of the book, but this is largely a personal point and is no reflection on the value of this new book.

I have no reservations whatsoever in recommending this book. T. S. WEST

PRACTICAL MATHEMATICS FOR CHEMISTS. By F. H. C. KELLY, D.Sc., M.Sc., A.Melb.T.C., F.R.A.C.I., A.Aust. I.M.M. Pp. viii + 148. London: Butterworth & Co. (Publishers) Ltd. 1963. Price 20s.

The need for the science undergraduate to specialise in the subject of his choice has a detrimental effect on his knowledge of subsidiary subjects. Thus the graduating physicist knows no advanced chemistry, and the graduating chemist knows little advanced physics or mathematics.

This book is designed to help the newly qualified chemist acquire an understanding of the capabilities, workings and limitations of the mathematics he is most likely to need. This it does: it covers the sort of mathematics he might have been expected to use in his academic training, and considers some topics he may need as a practising chemist. For example, in the first category there are chapters on Reaction Rates and Temperature Dependent Functions, whereas the sections on Statistics, Curve Fitting and (elementary) Alignment Charts are likely to be of more value to the chemist in the laboratory.

It is perhaps fortunate (or unfortunate) that I am one of those people at whom this book is directed, and can criticise it in the light of my deficiencies. I found the book readable (apart from a few split infinitives), concise and logical, but not perfect: improvements could have been made by the inclusion of more graphs and diagrams, and by slightly longer derivations of some of the more complicated equations.

Publishers have, at last, realised that there is an undergraduate market for paper-backed text-books: I wonder, therefore, why this small book has been cloth-bound, with a consequent increase in price. S. D. L. KEATING

HANDBOOK OF LABORATORY DISTILLATION. By ERIK KRELL. Translated from the Second German Edition by C. G. VERVER. Edited by E. C. LUMB. Pp. x + 561. Amsterdam, London and New York: Elsevier Publishing Company. 1963. Price 100s.

"Like it or not, distillation is going out of chemical analysis" (if one dares to paraphrase one of the Masters of the art). The great days are over; instead of spending hours standing by a fractionating column, nowadays we get our answer with our little chromatographic set, often so quickly that we can take action on the results. Even so, distillation is still an important laboratory technique; mixtures still have to be separated on a scale far larger than the chromatographic, the working of a plant has sometimes still to be simulated in the labotarory, and the analyst may wish to separate a small percentage of "light ends" or "heavy residue" from a large bulk of liquid for further examination.

The book under review is not addressed particularly to analysts; it is a thorough discussion of the principles of fractional distillation and their realisation in the laboratory. It is difficult to pick out particular sections for mention, but that on "Physical Fundamentals of the Separation Process" (175 pages) is outstanding in a laboratory hand-book, and includes details of calculations of the requirements of theoretical plates, column dimensions, heat input, etc., both for ideal and non-ideal mixtures. There is a chapter on separation of isotopes by counter-current distillation, 135 pages on the actual processes of separation from micro to large-scale laboratory units, including azeotropic, carrier, and extractive distillation, and also a chapter on short path or "molecular" stills. There are 160 pages on materials of construction and apparatus, a glossary, a brief section on the lay-out of a distillation laboratory, and, in a pocket at the end of the book, a list of symbols and eleven nomograms.

The literature references extend to 1961, but not many are later than 1958; not many important papers have appeared since 1958. The editing and translation are well done, but it is a pity that theoretical plates are sometimes "separating stages" and sometimes "theoretical stages," and the section on random packings for columns is inconveniently separated from "Columns" by 42 pages on other matters. The indexes could be better; the author index, for example, does not include the names of Raschig, Dixon, Prym or Wilson (which Wilson? No initials given), though all devised random packings that are described and named in the text; Dixon is further referred to on page 426, where a reference given to his papers, but there appear to be no references to the original papers of the other three. Plate and other symmetrical packings seem to me to be given too much space compared with random packings, and the difficulties of maintaining spinning band and similar columns in perfect condition could be indicated. More emphasis could have been given to thermocouples or resistance thermometers. These are, however, minor points of editing or of personal opinion, and do not detract from the general value of the work. H. N. WILSON

RESIDUE REVIEWS: RESIDUES OF PESTICIDES AND OTHER FOREIGN CHEMICALS IN FOODS AND FEEDS. Volume 3. Edited by FRANCIS A. GUNTHER. Pp. iv + 170. Berlin, Göttingen and Heidelberg: Springer-Verlag. 1963. Price DM 22.

The third volume of "Residue Reviews" follows the pattern set by the first two except that it consists of three chapters only; one of these occupies over three-quarters of the main text. The main emphasis is again on pesticide residues and while the extent of current interest in these is undeniable, the declared object of the series is to cover other "foreign" chemicals, some of which could properly be considered at the present time. However, it is true that Dr. Lykken's contribution to the new volume, on the collection and preparation of crop samples for residue analysis, says much that is pertinent to food analysis generally. This is a short (16 pages) but valuable chapter, based not only on a lecture presented in 1961 but also on many years of practical experience. The importance of intelligent, realistic sampling is stressed, with special consideration of trial crop experiments designed to assess residue situations. V. H. Freed and M. L. Montgomery (Oregon State University) review the literature on the metabolism of phenoxyacid, dalapon, carbamate and triazine and other heterocyclic herbicides by plant and soil micro-organisms. Dalapon and similar compounds are particularly resistant to metabolic attack; the degradation of other herbicides is briefly indicated, with full supporting references to early 1962 and with particular emphasis on oxidative processes.

The greater part of the volume is devoted to a comprehensive survey by W. Ebling of the basic processes involved in the deposition, degradation and persistence of pesticides. The desirability of increasing the efficiency of the deposition process so that a given degree of control is

achieved with less material is stressed, the current trend being towards specific, non-persistent pesticides. Methods of application and the various types of equipment used are discussed in detail. There is a semi-theoretical treatment of persistence, the factors influencing which are related firstly, to chemical and physical properties, to plant growth-rate, to formulation and to weather and other external factors; and secondly, to the physical and chemical characteristics of the pesticide itself.

Uniformity of format, binding, price (perhaps) and a consistently high editorial standard have their advantages, and there are no doubt other justifications (among which, one hopes, are included speed of publication) for serializing such review contributions as the present ones. When a volume is devoted almost exclusively to a single topic, however, it is somewhat difficult to distinguish the case for an independent monograph. The series is less likely to flourish as a collection of individual monographs than as successive volumes each containing a selection of current topics. H. EGAN

DEVELOPMENTS IN APPLIED SPECTROSCOPY. Volume 2. Edited by J. R. FERRARO and J. S. ZIOMEK. Pp. x + 438. New York: Plenum Press Inc. 1963. Price $16.00.

This book constitutes the Proceedings of the 13th Annual Symposium in Spectroscopy, organised by the Chicago Section of the Society for Applied Spectroscopy, and held in Chicago during April 30th to May 3rd, 1962. It appeared just over a year after the Symposium and contains 41 papers of varying length, covering a wide range of spectroscopic techniques and applications, together with a (non-spectroscopic) section on gas chromatography.

The value of a collection of papers of this sort can be assessed on two grounds. The first is the extent to which it provides a permanent record of the Symposium Proceedings for those people who attended it. On this basis the book can be regarded as a creditable achievement. In contrast to Volume 1 (1962), which recorded the 12th Symposium, all the papers are given in full, with copious high-standard illustrations. From the point of view of the potential reader who did not attend the Symposium, the book must be judged on a different basis, however, and here some of the well known disadvantages that follow the complete publication of symposia proceedings emerge. It has often been argued by reviewers that original work first presented at conferences should then be submitted for publication in an appropriate journal in which, after it has been refereed, it will find its proper and widest audience, and will be properly abstracted. In fact, nine of the papers in this volume are stated to have been published elsewhere or will be submitted to other journals, in either identical or expanded form. In view of the enormous volume of current scientific literature that clamours for attention, duplication of any sort must be deprecated.

The second defect of published volumes of complete conference programmes is that the range of subjects covered is often too broad to appeal to specialist readers. In the volume under consideration the coverage is as follows: Infrared and Raman Spectroscopy (12 papers, comprising two on Information Retrieval and Communication, five on Instrumentation, three on Molecular Structure and two on General Topics); Nuclear Magnetic Resonance (2); Ultraviolet and Vacuum Ultraviolet Spectroscopy (2); Emission Spectroscopy (7); X-ray Spectroscopy (11, comprising six on Absorption and five on Emission Techniques); Gas Chromatography (7, including four on Preparative Techniques and two on Applications). The 41 papers vary considerably in length and character. A few deal with purely scientific uses of the various branches of spectroscopy, whereas the majority emphasise applications to analytical problems of direct industrial interest.

It is in this respect that the book is likely to be of most value to practising analysts because it does give a particularly good impression of the current status of spectroscopic and gas-chromatographic instrumentation, and the way in which contemporary equipment is being used in the analytical field. Two brief papers point to important new developments in interferometric far-infrared spectroscopy, and a third indicates the potential value of the optical maser (laser) as a Raman source. One of the two papers on nuclear magnetic resonance describes the use of this technique for determining fat in cereals, and the paper on ultraviolet absorption spectra of a solution in a vacuum outlines the possible use of this region for steroid studies.

Of the papers on emission spectroscopy, the one on "Time-resolution Spectroscopy" (36 pages with 32 figures) is an outstanding review of instrumentation and application in this field, and includes some results on "nuclear events." In the same section a paper on a direct-reading spectrograph is also copiously illustrated, but mainly with photographs of "consoles," etc., which are impressive but not very informative. The other papers in this section deal with practical analytical

situations, as do most of those in the X-ray-absorption and emission section. But there are also two papers of less routine character, one on unsolved problems in X-ray-absorption spectrometry and another on fine-structure absorption studies at low temperature. Finally, there are four useful papers on gas chromatography that give an excellent idea of the actual equipment assemblies used in this field, especially for preparative-scale work, where increasing sample size may have to be balanced against loss of resolution.

It will be evident from these remarks that despite the diversity of spectroscopic and gas-chromatographic topics covered in the programme of the 13th Chicago Symposium, a proportion of the papers is likely to be of interest to non-specialist readers with general analytical interests.

G. H. BEAVEN

MODERN POLAROGRAPHIC METHODS. By HELMUT SCHMIDT and MARK VON STACKELBERG. Translated by R. E. W. MADDISON. Pp. viii + 99. New York and London: Academic Press Inc. 1963. Price 44s.

The increase in diversity and complexity of polarographic techniques and instrumentation in the last decade has created a need for a book to describe and classify them and to provide details of their basic principles, scope, limitations and applications. This book presumably has this objective, but there is a sense of disappointment that it is too short to do justice to so large a subject. A good account of many techniques is given, but only briefly, and with so few practical details that it must be supplemented by extensive reference to the literature and other works if more than a superficial understanding is required. For example, all the stationary and quasi-stationary methods are covered in the space of eleven pages, while only thirteen pages are devoted to the whole field of cathode-ray polarography. A treatment of each, having a length qeual to the whole book, would not have been inappropriate.

The balance of the book seems to follow the interests of the authors rather than the relative importance of the techniques described. Alternating-current methods, square-wave, pulse and radio-frequency polarography are excellently described, but the coverage of cathode-ray methods is less satisfactory. In this latter instance the description of theoretical principles is adequate, but practical details are lacking and sometimes not in accordance with practice. Some assessments of the value and limitations of these methods are incorrect and misleading. For instance, the single-sweep method tends to be dismissed as of little importance except in a narrow analytical field. In fact it is in increasingly widespread use in both research and chemical analysis. The advantages of oscillo-polarography are probably exaggerated.

The shortness of the book has led to a terseness in style so that it reads like a series of notes. The translation is generally good, but there are some awkward passages, which problably arise from too literal a rendering of the German text. The sense of the last sentence on page 16 appears to have been reversed, while on page 26 the last part of the first paragraph is meaningless. It is a pity more commonly use symbols were not substituted in the English edition, especially in the diagrams. The usefulness of the book would also have been improved if references to English language books had been added to, or substituted for, the German books quoted.

In spite of these and other shortcomings, the book does fill a gap in the polarographic literature and is therefore to be welcomed It is a useful, if brief, guide to principles, and, providing that some of the practical details and opinions are treated with reserve, it will be extremely useful to those wishing to acquire some understanding of the modern techniques of polarography. Most polarographers will wish to have it at least available in their library. G. F. REYNOLDS

Errata

DECEMBER (1963) ISSUE, p. 967, "Method." *After the last reagent* (Tris glycerol buffer), *add the following paragraph—*

"*Combined reagent*—Dissolve 10 mg of glucose oxidase, 1 mg of horseradish peroxidase and 10 mg of *o*-dianisidine hydrochloride in 100 ml of tris glycerol buffer."

IBID., p. 967, 1st line of "Procedure." *Before* "reagent" *insert* "combined."

IBID., p. 967, 4th line of "Procedure." *After* "2 mg" *insert* "per ml."

JANUARY (1964) ISSUE, p. 78, 11th line. *For* "L. S. THEOBOLD" *read* "L. S. THEOBALD."