THE ANALYST

PROCEEDINGS OF THE SOCIETY FOR ANALYTICAL CHEMISTRY

ORDINARY MEETING

AN Ordinary Meeting of the Society was held at 6.30 p.m. on Tuesday, November 5th, 1957, in the Lecture Theatre of The Royal Institution, 21 Albemarle Street, London, W.1. The Chair was taken by the President, Dr. J. H. Hamence, M.Sc., F.R.I.C.

A lecture on "Recent Developments in Chelatometry" was given by Dr. Rudolf Přibil.

NEW MEMBERS

ORDINARY MEMBERS

John Derek Cosgrove, B.Sc. (Dunelm.); Gino Dicastro, Dr. Biochem. (Rome); Cyril Jack Keattch, A.R.I.C.

JUNIOR MEMBER

Raymond Frederick Hall.

NORTH OF ENGLAND SECTION

AN Ordinary Meeting of the Section was held at 2.15 p.m. on Saturday, October 5th, 1957, at the Engineers' Club, Albert Square, Manchester. The Chair was taken by the Chairman of the Section, Mr. A. N. Leather, B.Sc., F.R.I.C.

A discussion on "The Analysis of Trade Effluents" was opened by J. G. Sherratt, B.Sc., F.R.I.C.

MIDLANDS SECTION

AN Ordinary Meeting of the Section was held at 6.30 p.m. on Tuesday, October 8th, 1957, in the Mason Theatre, The University, Edmund Street, Birmingham, 3. The Chair was taken by the Vice-Chairman of the Section, Dr. S. H. Jenkins, F.R.I.C., F.Inst.S.P.

The following paper was presented and discussed: "Analytical Methods in Clinical Biochemistry", by H. Varley, M.Sc., F.R.I.C.

BIOLOGICAL METHODS GROUP

AN Ordinary Meeting of the Group was held at 6.30 p.m. on Wednesday, October 9th, 1957, in "The Feathers", Tudor Street, London, E.C.4. The Chair was taken by the Vice-Chairman of the Group, Dr. J. I. M. Jones, F.R.I.C.

A discussion on "Biological Standards" was opened by J. W. Lightbown, M.Sc., Dip. Bact., F.P.S.

The Determination of 17-Oxo Steroids (17-Keto Steroids) and 17-Oxogenic Steroids (17-Ketogenic Steroids)

A Review

By A. E. KELLIE*

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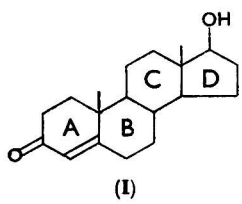
Hydrolysis of steroid conjugates: (*a*) glucuronides and (*b*) sulphates.
Chromatography of 17-oxo steroids.
Identification of individual 17-oxo steroids.
Determination of 17-oxo steroids in blood.
Determination of 17-oxogenic steroids.
Determination of non-ketonic steroid alcohols.

Although the presence of androgens in urine was first demonstrated by biological methods,[1,2] bioassay has now been largely replaced by colorimetric methods, which measure both biologically active and inactive androgen metabolites. Not all urinary androgen metabolites are 17-oxo steroids nor are all 17-oxo steroids androgenic, and it was fortuitous that two of the first crystalline steroids isolated from urine by Butenandt,[3,4] androsterone (3α-hydroxy-5α-androstan-17-one) and dehydro*epi*androsterone (3β-hydroxyandrost-5-en-17-one), were 17-oxo steroids possessing biological activity. Zimmermann[5,6] showed that these and other pure steroids containing an activated methylene group $-CH_2-CO-$ could be quantitatively determined by the colour given with *m*-dinitrobenzene in alkaline solution, and Wu and Chou[7] applied this reaction to measure the concentration of chromogenic androgen metabolites in urine. The Zimmermann reaction for 17-oxo steroids distracted attention from the fundamental importance of biological activity, but without this simple test the rapid expansion that has taken place in the past 20 years would not have been possible. This reaction forms the basis of all reliable methods for the determination of 17-oxo steroids. Recently, chemical methods have been established whereby more complex steroids (the 17-oxogenic steroids[8]) may be converted to 17-oxo steroids for indirect determination.

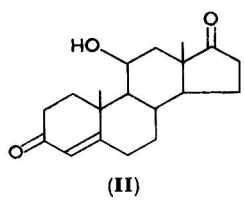
Precursors of 17-oxo steroids and 17-oxogenic steroids

The use of carbon-14 labelling has proved beyond reasonable doubt in man that the synthesis of the biologically important steroids, including cholesterol, can be achieved from acetate.[9] Whereas cholesterol, which can act as a precursor of steroid hormones, is formed by many body tissues, the formation of the steroid hormones is closely associated with the adrenals, the gonads and, during pregnancy, the placenta. Dorfman has proposed that the term biosynthesis should be reserved for those metabolic processes that result in the production of steroid compounds with maximum biological activity and that all subsequent changes should be considered to be strictly catabolic. If this criterion is accepted, then the primary steroid products of the endocrine system (excluding oestrogens) are as follows—



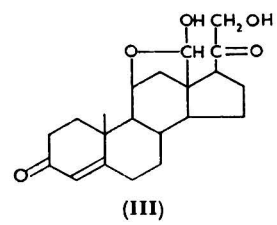
(I)

Testosterone



(II)

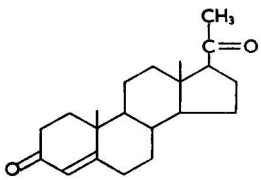
11β-Hydroxyandrost-4-ene-3:17-dione



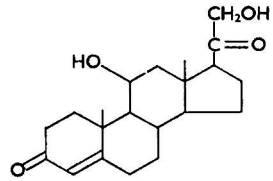
(III)

Aldosterone

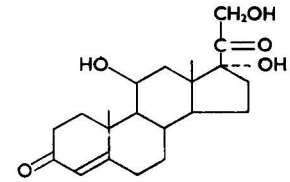
* British Empire Cancer Campaign Research Fellow.



(IV)
Progesterone

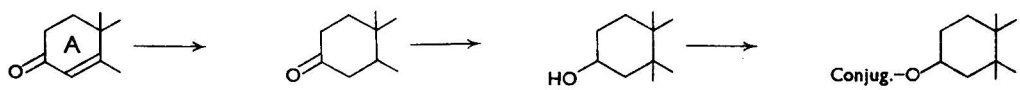


(V)
Corticosterone



(VI)
Cortisol

This is, of course, an over-simplification of the true situation, as many other related steroids of lower biological activity are produced that may have physiological importance; as these compounds are catabolised in a similar way, the concept is both apt and useful. In general, compounds of high biological activity (**I** to **VI**) after intravenous injection disappear rapidly from the blood; for example, the estimated half-life of progesterone (**IV**) in circulation is 5 minutes and that of testosterone (**I**) is 8 minutes. Some of the injected material is excreted in the urine in modified form as water-soluble conjugates. These active compounds have in common the Δ^4-3-ketone structure in ring A of the steroid nucleus and, irrespective of substitution in rings B, C and D, they undergo reduction to the dihydro (**VIII**) and tetrahydro (**IX**) derivatives before excretion, as shown by the following formulae—



(**VII**) Δ^4-3-Ketone

(**VIII**) Dihydro reduction product

(**IX**) Tetrahydro reduction product

(**X**) Conjugated steroid

Although it is uncertain where all the intermediate metabolic changes take place, these compounds are the precursors of the 17-oxo steroids and the 17-oxogenic steroids that appear in blood and urine.

The reactions leading to the reduction of the Δ^4-3-ketone group and the subsequent conjugation with glucuronic acid are catalysed by phosphonucleotides[10,11] and probably take place in the liver, so that peripheral blood contains, in addition to the secreted hormones, conjugates of the tetrahydro and probably also of the dihydro (**VIII**) products. Whether or not all of these circulating metabolites are excreted in the urine depends upon their clearance by the kidney and, because of this complex situation, the dilemma arises as to whether to carry out determinations on blood or urine and what precisely to determine. Although the concentration of steroid metabolites in blood is probably close to that in the intracellular fluid to which cells are exposed, these values refer only to the time of sampling and do not allow for diurnal variations in secretion. On the other hand, determinations carried out on 24-hour urine samples correspond to the average secretion throughout the day, but do not cover the possibility of selective renal clearance producing a urinary steroid composition that does not resemble that of the internal environment.[12] In both blood and urine the amounts of hormone and hormone metabolites in the free form are very low compared with the amount present in the form of conjugates, and most methods recommended for routine use are designed to measure the latter only. The determination of trace amounts of specific free hormones in blood and urine has been described for several hormones,[13,14] but such methods are highly specialised and technically difficult and lie outside the scope of this review, which is limited to the determination of such metabolites of adrenal and gonadal origin as can be measured by the Zimmermann reaction for 17-oxo steroids. This includes, on the one hand, the native 17-oxo steroids and, on the other, those steroid compounds that can be converted by specific reactions into compounds giving a colour in the Zimmermann reaction.

Determination of 17-oxo steroids

In contrast to the uncertain meaning of terms such as "corticosteroids," "11-oxy steroids" and so on, there is no ambiguity about the name 17-oxo steroids, which embraces a group of related steroids, of diverse metabolic origin, that appear in blood and urine and

have as a common structural feature a carbonyl group at the 17-position of the steroid nucleus. The determination of this class of compound is usually carried out by the Zimmermann reaction,[15,16] which, under defined conditions, is reasonably specific. Alternative colour reactions that have been proposed[17,18] have only limited usefulness. A polarographic method for the determination of ketosteroids[19] has also had little application.

The modification of the Zimmermann reaction proposed by Callow, Callow and Emmens[20] has been widely adopted. The reagents (0·2 ml of 2 per cent. w/v ethanolic *m*-dinitrobenzene and 0·2 ml of 2·5 *N* ethanolic potassium hydroxide) are incubated at 25° ± 0·1° C for 1 hour with 0·2 ml of an ethanolic solution containing 0 to 100 μg of 17-oxo steroid and are then diluted with 10 ml of ethanol for absorptiometric measurement. Under these conditions 17-oxo steroids give at 520 mμ strong absorption that obeys Beer's law closely. 3-Oxo steroids give a typical colour after 5 minutes' incubation, but they differ from 17-oxo steroids in that the colour fades rapidly, and at 1 hour there is very little absorption at 520 mμ. 20-Oxo steroids and Δ^4-3-oxo steroids also react slowly with the reagents in the Zimmermann test to give low general absorption. Cholestan-2-one[21] and 2:3:6-trimethylbenzylideneacetone[22] are not 17-oxo steroids, but they do give the typical colour reaction of this group.

The presence of red or purple pigments in urine extracts is sometimes troublesome and colour-correction formulae based on the measurement of background absorption are frequently inadequate.[23] This difficulty can be overcome by preparing a ketonic fraction by means of Girard's[24] reagent T (carbohydrazidomethyltrimethylammonium chloride) or, if it can be shown that the background absorption is linear, by applying the Allen correction.[25] Alternative conditions for the Zimmermann reaction have been described by Holtorff and Koch.[26] This reaction is carried out in aqueous ethanol and the period of incubation is longer. The method is less reliable than that described by Callow *et al.*, because the determination of 17-oxo steroids in impure extracts shows a departure from the linear response required by Beer's law. Under Callow's conditions, and more especially under those of Holtorff and Koch, individual 17-oxo steroids have different molecular extinction coefficients,[27] *e.g.*, 3α-hydroxy-5β-androstane-11:17-dione (11-oxoaetiocholanolone) gives a higher molecular extinction than 3α-hydroxy-5β-androstan-17-one (aetiocholanolone) and for this reason the determination of a mixture of 17-oxo steroids of unknown composition is only approximate. 3β-Acetoxy-14ξ-hydroxyandrost-5-en-17-one[28] and 3β:16α-dihydroxyandrost-5-en-17-one (16α-hydroxydehydro*epi*androsterone), recently isolated from urine,[29] are rare examples of 17-oxo steroids that do not give the Zimmermann reaction. Micro-scale methods have been described[27,30] in which as little as 1 μg of 17-oxo steroid may be determined; such methods depend for their precision on a low reagent blank. Important factors influencing this value include the purity of the *m*-dinitrobenzene and of the ethanol and the stability of the ethanolic potassium hydroxide. The method of Wilson,[31] based on that of Hamburger,[32] gives a satisfactory potassium hydroxide reagent; the solution is prepared in the presence of ascorbic acid and is stored under nitrogen at 4° C.

Determination of 17-oxo steroids in urine

Originally, measurements of urinary 17-oxo steroids involved the preparation of a neutral steroid extract and the determination of a figure for the sum of all neutral 17-oxo steroids. Later, the pathological significance of the appearance of large amounts of dehydro*epi*androsterone in urine was recognised and the original method was extended to permit the ratio of 3α- to 3β-hydroxy-17-oxo steroids to be determined (by precipitation as digitonide[33]) in an attempt to establish a differential diagnosis between adrenal carcinoma and hyperplasia. This period was also associated with many reports of the isolation of individual compounds; these efforts were invariably carried out on a large scale and were not quantitative. In contemporary methods the total 17-oxo steroid fraction is analysed quantitatively in terms of individual compounds, small urine samples being used.

Substantially all the 17-oxo steroids in normal urine are excreted as water-soluble conjugates, and there is good evidence that the total is largely made up of glucuronides and sulphates. Lieberman and Dobriner have pointed out[34] that trace amounts of the C_{19} 3:17-diones that have been isolated cannot be conjugated in this way unless they undergo preliminary enolisation; the suggestion that they may be excreted as thiazolidines has, as yet, little experimental support. Although 17-oxo steroid glucuronides and sulphates can be extracted quantitatively from urine[35] and have been separated from each other,[36] there is no satisfactory method for the quantitative separation of individual conjugates. In the absence of

such methods, it has been customary to hydrolyse the water-soluble conjugates and to extract the liberated steroids into organic solvents. Because of the difficulties associated with this preliminary hydrolysis, most of the methods suggested have been unsatisfactory.

HYDROLYSIS OF 17-OXO STEROID CONJUGATES—

Glucuronides are reasonably stable at room temperatures in mildly acid or alkaline solution; sulphates, on the other hand, although stable under alkaline conditions, undergo progressive hydrolysis at acid pH values at room temperature. Both forms of conjugates are completely hydrolysed on being boiled in acid solution and this method of hydrolysis has been the mainstay of so-called quantitative methods for two decades. This method of hydrolysis, presumably because of its convenience, persists, yet it is for most problems entirely unsatisfactory. It is, perhaps, possible to justify this procedure in quick methods for scanning a large number of urine samples in a preliminary manner,[37] but even in this application it can give rise to misleading results. Hydrolysis with hot acid produces artefacts of dehydration and substitution,[38] and these complicate subsequent analysis of the liberated 17-oxo steroids. Thus, 3-hydroxy- and 11-hydroxy-17-oxo steroids are dehydrated to the corresponding Δ^2 and $\Delta^{9(11)}$ compounds; in addition, the 3β-hydroxy-Δ^5 compound dehydro-*epi*androsterone is converted in the presence of hot hydrochloric acid (or sodium chloride and sulphuric acid) into the corresponding 3β-chloro compound. If these artefacts were equivalent in colour value in the Zimmermann reaction to their precursors, this would not affect the determination of total 17-oxo steroids, but not only is this not so, but there is strong evidence that total destruction of their precursors also occurs.[39] The effect is more marked when isolated sulphate fractions are hydrolysed[30] and, as this fraction contains a high proportion of dehydro*epi*androsterone, it is predominantly this compound that is destroyed.[39] Accurate determination of dehydro*epi*androsterone is not possible after hydrolysis with hot acid, and no method of determining 17-oxo steroids can be considered satisfactory if it fails with this important compound.

Hydrolysis of 17-oxo steroid glucuronides—Hydrolysis by means of enzymes is by far the most satisfactory method for the hydrolysis of 17-oxo steroid glucuronides. β-Glucuronidase from calf spleen,[40] bacteria[41] or molluscs[42] has been used and the hydrolysis can be carried out by adding the enzyme directly to the buffered urine or to urine extracts containing the conjugates. As enzyme inhibitors have been reported in urine,[43] the latter method may be preferable, for, by proceeding in this way, much non-steroid matter is excluded from the enzyme digest and, further, as the dried conjugate extract can be redissolved in small volumes of buffer, much higher concentrations of enzyme can be used. A convenient and prolific source of β-glucuronidase in Britain is found in the visceral hump of the common limpet (*Patella vulgata*)[44] and from this source preparations with an activity of 10^6 Fishmann units per gram can readily be prepared. This enzyme brings about complete hydrolysis of 17-oxo-steroid glucuronides (16 hours at 40° C) without the formation of artefacts.

Hydrolysis of 17-oxo steroid sulphates—According to Bitman and Cohen,[45] 3β-hydroxy-Δ^5-steroid sulphates are hydrolysed when treated with acetate buffer at pH 4·7. This hydrolysis is promoted by limpet preparations because steroid sulphatases are also present. One of these enzymes, studied by Roy,[46] has been shown to bring about the complete hydrolysis of steroid sulphates that have the 3β-hydroxy-Δ^5 or the 3β-hydroxy-5α structure, as in dehydro*epi*androsterone sulphate and 3β-hydroxy-5α-androstan-17-one (*epi*androsterone) sulphate, respectively. Androsterone sulphate (3α-hydroxy-5α) and aetiocholanolone sulphate (3α-hydroxy-5β), both of which are present in urine, are not hydrolysed; nevertheless, for methods exclusively concerned with compounds of the 3β-hydroxy-Δ^5 configuration,[29] this enzyme is an ideal hydrolytic catalyst.

Attempts to bring about the simultaneous hydrolysis of the glucuronide and sulphate conjugates of neutral steroids by limpet powder are unsatisfactory, as the pH optima of the two enzymes are different[47] and, further, owing to the presence of androsterone sulphate and aetiocholanolone sulphate in urine and plasma extracts, hydrolysis is incomplete. By carrying out the hydrolysis with β-glucuronidase at pH 4·0 in the presence of 0·03 *M* potassium dihydrogen phosphate, the sulphatase enzyme is inhibited and only glucuronides are hydrolysed. If the sulphates are subsequently hydrolysed independently, the mode of conjugation of individual 17-oxo steroids can be studied.

Fortunately, alternative methods are available for the hydrolysis of steroid sulphates. Cohen and Oneson[48] demonstrated that dioxan in the presence of trichloroacetic acid will

bring about the hydrolysis of steroid sulphates irrespective of configuration or degree of unsaturation. Hydrolysis takes place at room temperatures overnight and produces few artefacts. Water must be removed from the reagents and excluded from the reaction, a condition that is difficult to ensure with urine extracts. An alternative method[39] of hydrolysing steroid sulphates, which does not split glucuronides, is by continuous extraction of an aqueous solution of conjugates adjusted to pH 1. This method can be readily applied to urine samples and appears to be a mild and quantitative method of hydrolysing the sulphate conjugates. In normal urines, hydrolysis by β-glucuronidase, followed by extraction with ether at pH 1 does not lead to the formation of artefacts and, further, the amount of 17-oxo steroid liberated from conjugation by these combined methods is at least as great as that obtained by hydrolysis with hot acid. Gallagher has reported the analysis of an abnormal urine from a case of adrenal hyperplasia for which this did not appear to be true; this urine, after being hydrolysed with β-glucuronidase and continuously extracted with ether at pH 1, was progressively acidified in stages to 4 *N* and re-extracted with ether. At each stage further amounts of 17-oxo steroid were obtained.[49]

According to Cohen and Oneson, hydrogen chloride in dioxan will hydrolyse sulphate and glucuronide conjugates simultaneously; for this purpose, Buehler[50] recommended continuous extraction with ether of an aqueous solution adjusted to 7·2 *N*. Both of these methods make use of strongly acid conditions, which are best avoided.

When hydrolysis is complete, the liberated steroids are extracted from the aqueous phase by means of an organic solvent. Many solvents have been used for this purpose, *e.g.*, ether, benzene and methylene dichloride, but, as few comparisons have been made of their effectiveness and selectivity, there are no rational grounds for selecting any particular one, especially as 17-oxo steroids are markedly hydrophobic and quantitative extraction is not difficult to accomplish.

CHROMATOGRAPHY OF 17-OXO STEROIDS—

Complete hydrolysis and the absence of artefacts are essential for satisfactory chromatographic separation. Artefacts, when formed, are difficult to separate from each other and frequently interfere with the separation of genuine urinary 17-oxo steroids; thus the dehydration product of 3α:11β-dihydroxy-5α-androstan-17-one (11β-hydroxy-androsterone) formed during hydrolysis with hot acid is 3α-hydroxy-5α-androst-9(11)-en-17-one, which cannot be completely separated from androsterone by any chromatographic system and consequently confuses the separation of androsterone and aetiocholanolone.

After complete hydrolysis of conjugates has been achieved under mild conditions a very large choice of chromatographic systems is available for separation of the mixed 17-oxo steroids. For precise analysis, chromatography on a column is recommended, but several paper-chromatographic systems have also been described. The early work of the Callows[51] and many of the large-scale separations achieved by Dobriner *et al.*[52,53] were carried out by adsorption on alumina; alternative, but less satisfactory, adsorbents include silica[54] and magnesium silicate.[52] Neutral steroids such as the 17-oxo steroids can be recovered quantitatively from alumina, and the potential resolving power of this adsorbent is very high. Dingemanse, Huis in't Veld and de Laat[55] described a method for the quantitative analysis of small samples of 17-oxo steroids, based upon the elution of the individual compounds from alumina by benzene containing increasing concentrations of ethanol. The ethanol concentration was increased stepwise. This method in its original form and in the many modifications to which it gave rise[56,57,58] has been of great value in the study of urinary excretion of 17-oxo steroids. Yet not one of these methods achieved satisfactory resolution of the urinary steroid fraction; the 11-deoxy-17-oxo steroids were poorly separated and the 11-hydroxy compounds, had they survived the hydrolysis with hot acid used in all these methods, would have been more intractable. The Pond method is also open to criticism, because suction, used to increase the rate of flow through the column, may introduce solvent-vapour spaces in the adsorbent column.

A notable improvement of the separation obtainable by the Dingemanse chromatographic procedure was obtained by Lakshmanan and Lieberman,[59] who made use of the principle of gradient elution. In the system alumina/ethanol - benzene, 17-oxo steroids have curved adsorption isotherms, and for this reason the compounds are eluted from alumina with marked "tailing," which prevents the separation of adjacent compounds. The effect of the gradient is to make these compounds behave as though they had linear adsorption

characteristics, so that they are eluted as symmetrical peaks; the movement of the compounds under gradient elution is more rapid than in the absence of a continuous gradient and the development of the chromatogram can be completed with a much smaller volume of eluting solvent. A suitable gradient of ethanol in benzene applied by the simple device shown in Fig. 1 gives excellent separation of the 17-oxo steroids and permits quantitative analysis of the main urinary 17-oxo steroids on small urine samples.[30]

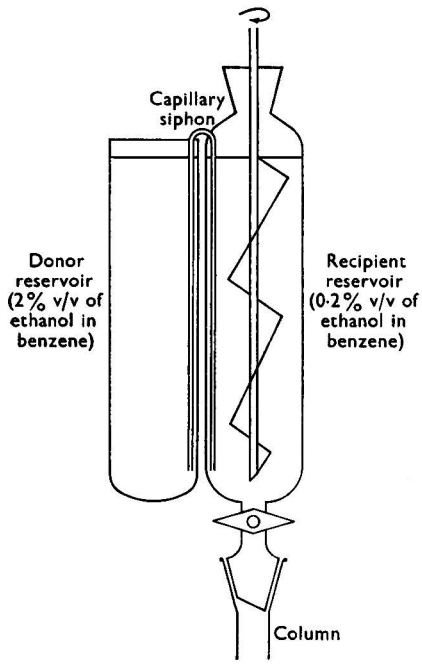


Fig. 1. Apparatus for separation of 17-oxo steroids by gradient elution. Reproduced by permission of the Editors from *Biochem J.*, 1957, 66, 196

It has been known for many years that the behaviour of compounds adsorbed on alumina is greatly influenced by the activity of the alumina, and it has been customary to specify Brockmann grades[60] for the separation of 17-oxo steroids. The activity of alumina for this purpose is greatly influenced by the apparent moisture content (loss of weight on heating at 100° C), which has a pronounced effect on the separation attainable under gradient elution. This has practical importance, as the adjustment of the moisture content of alumina provides the easiest way of reproducing batches of alumina with standard properties. Various complex methods of adjusting the activity of alumina by exposure to aqueous solutions of known vapour pressure are slow and probably unnecessary, as the required activity (corresponding to between 4 and 5 per cent. of water by weight) can be achieved by adding sufficient water dropwise to the alumina exposed in shallow trays and subsequently mixing the alumina to achieve uniform distribution.

Many factors other than the moisture content of the alumina affect the resolution of 17-oxo steroids under gradient elution; these include the dimensions of the column, the rate of flow and the nature of the gradient applied. The theoretical aspects of some of these factors have been studied by Drake.[61]

IDENTIFICATION OF INDIVIDUAL 17-OXO STERIODS—

A further serious disadvantage of methods based on the Dingemanse method is the failure to identify individual compounds other than by the position of elution. Although

it is true that, with identical alumina and solvents, pure compounds behave in a predictable manner, it is unsound to assume for fractions from normal and abnormal urines in the presence of a great excess of non-steroidal matter that the position of elution will be maintained. Infra-red absorption spectroscopy, introduced to the steroid field by Dobriner, Lieberman, Rhoads, Jones, Williams and Barnes[62] offers an excellent method of characterisation when amounts in excess of 100 μg are available. Modern analytical methods, however, do not provide more than a few micrograms of the minor components, and they necessitate alternative methods of identification. When the amount available after determination is in excess of 5 μg, paper chromatography against standard reference compounds provides additional evidence of identity. Bush[63] has described systems suitable for 11-oxy- and 11-deoxy-17-oxo steroids. When smaller amounts are available, the complex formed in the Zimmermann reaction, after being determined absorptiometrically, can be extracted from the solution and used to establish identity.[64]

Many systems of paper chromatography have been suggested for the separation of 17-oxo steroids. Conventional two-phase systems have been described by Axelrod,[65] Bush,[63] Heftman,[66] Neher and Wettstein[67] and Savard,[68] and a reverse-phase system developed by Kritchevsky and Kirk[69] is run on paper impregnated by dipping in stearatochromic chloride. Shüll, Sardinas and Nubel[70] deposited alumina on paper and used this as an adsorbent material. There is little doubt that the labour and time involved in running paper chromatograms is much less than is necessary for column work; on the other hand, without preliminary purification of the material to be chromatographed, the former method is less accurate. The disproportionate amounts of 11-deoxy- and 11-oxy-17-oxo steroids is such that overloading of the paper is necessary to provide sufficient of the minor components for assay, and no single system of paper chromatography satisfactorily separates the wide range of compounds present. When the individual compounds have been located, they are eluted from the paper for determination. Invariably in this procedure unspecific chromogenic material is also eluted from the paper; it may arise from the original extract or from the materials of the paper. This blank value for the paper sets a lower limit on the amount of 17-oxo steroid that can be determined.

A wide range of colour reagents has been suggested for the location of 17-oxo steroids,[66,71] but few compare in specificity with the reagents for the Zimmermann reaction. Papers are treated by immersion in 2 per cent. w/v ethanolic *m*-dinitrobenzene and in 2·5 *N* ethanolic potassium hydroxide and are then dried and developed in a current of warm air.

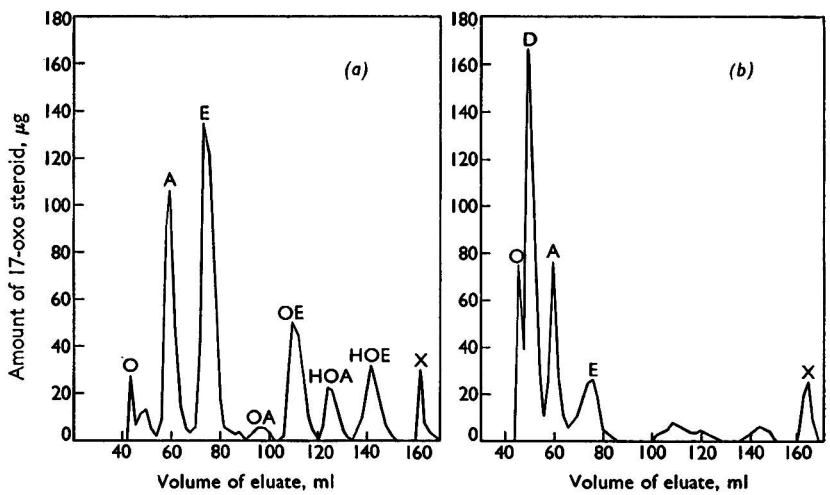


Fig. 2. 17-Oxo steroid excretion patterns of the urine from a normal male subject: (*a*) chromatogram of glucuronide fraction; (*b*) chromatogram of sulphate fraction. D, dehydro*epi*androsterone; A, androsterone; E, aetiocholanolone; OA, 11-oxoandrosterone; OE, 11-oxoaetiocholanolone; HOA, 11β-hydroxyandrosterone; HOE, 11β-hydroxyaetiocholanolone; O, a mixture containing androstane-3:17-dione and aetiocholane-3:17-dione; X, unknown. Reproduced by permission of the Editors from *Biochem. J.*, 1957, **66**, 196

Fig 2 shows typical analyses of the urinary 17-oxo steroid glucuronide and sulphate fractions obtained by gradient elution. In the former fraction the two main components androsterone (A) and aetiocholanolone (E) represent terminal metabolites of C_{19} steroids produced by the testis and adrenal, *e.g.*, testosterone and dehydro*epi*androsterone. In addition, aetiocholanolone may also be formed from C_{21} steroids of the 17-hydroxy-11-deoxy type, *e.g.*, 17-hydroxyprogesterone. 11-Oxoaetiocholanolone and 11β-hydroxyaetiocholanolone arise mainly, by the loss of the side-chain, from the C_{21} steroids cortisone and cortisol formed in the adrenal, whereas the corresponding 11-oxyandrosterone derivatives, although also of adrenal origin, arise from 11β-hydroxyandrost-4-ene-3:17-dione and adrenosterone (androst-4-ene-3:11:20-trione). The absence of dehydro*epi*androsterone from the glucuronide fraction of normal urine is noteworthy.

Dehydro*epi*androsterone is frequently the major component of the sulphate fraction; it is believed to originate exclusively in the adrenal.

Determination of 17-oxo steroids in blood

Very early attempts by Zimmermann[6] to determine levels of 17-oxo steroids in blood gave results that are now known to be too high. Although in principle methods for the analysis of 17-oxo steroids in blood are similar to those outlined for urine, the main practical difference arises because the amounts to be measured are very much lower and the steroids are accompanied by large amounts of protein and fat. The proteins can be removed by hydrolysis with hot acid,[72,73] a procedure that cannot be recommended, or they can be precipitated with ethanol.[74] Methods involving precipitation are open to the criticism that 17-oxo steroids precipitated with the protein, irrespective of whether or not they are bound to the protein in plasma, may result in the loss of some material; the digestion of the protein by proteolytic enzymes appears to offer an alternative procedure. In any event the evidence for protein-bound 17-oxo steroid is largely circumstantial and, even if such compounds exist, the link may be broken by the extraction and hydrolytic procedures normally used. The levels of free steroids that have been established do not exceed their water solubility.

The presence of a large amount of lipid in plasma makes the determination of free 17-oxo steroids difficult. Although free 17-oxo steroids have been identified and determined in adrenal blood,[75,76] such samples are not normally available, and the levels in peripheral blood are too low to be measured by present methods. 17-Oxo steroids present in peripheral blood are predominently in the conjugated form and, because of this, excess of lipid can be eliminated by partition between light petroleum and 70 per cent. ethanol[77] or between benzene and water.[74] The water-soluble conjugates remain in the lower phase and can be hydrolysed and separated by established methods. The very small amounts of 17-oxo steroid liberated necessitate the use of small columns and some modification of the gradient-elution apparatus.

Migeon and Plager[78] and later Migeon[79] showed that, when plasma was subjected to acid hydrolysis, preferably by continuous extraction with ether at pH 1, small amounts of 17-oxo steroids were liberated. The presence of dehydro*epi*androsterone and androsterone in this material was indicated by paper chromatography, and sufficient of the former compound has been isolated to confirm the identification by infra-red spectroscopy.[78] With larger samples of plasma it is possible to detect trace amounts of aetiocholanolone.[77] As this treatment is known to hydrolyse steroid sulphates but not glucuronides, it is probable that these three steroids circulate in peripheral blood as sulphates. Migeon was unable to find free 17-oxo steroids in plasma before or after hydrolysis with β-glucuronidase, although Clayton, Bongiovanni and Papadatos[80] found 12 per cent. of the circulating 17-oxo steroids as glucuronides. With larger samples of plasma than normally available, the presence of small amounts of androsterone, aetiocholanolone, 11-oxoaetiocholanolone and 11β-hydroxyandrosterone in the glucuronide fraction has been demonstrated.[74] In sharp contrast to the large amount of glucuronide conjugates in the urine, in peripheral blood plasma 17-oxo-steroid sulphates preponderate and for all practical purposes they are the only form of circulating 17-oxo steroid that can be determined at the present time.

The levels of 17-oxo steroids in plasma show wide variation in normal subjects, with no obvious relation to age, *e.g.*, males 20 to 40 years, 12 to 136 μg of dehydro*epi*androsterone per 100 ml and 1 to 43 μg of androsterone per 100 ml; females, 20 to 30 years, 5 to 46 μg of dehydro*epi*androsterone per 100 ml and 1 to 27 μg of androsterone per 100 ml. According to Migeon,[79] dehydro*epi*androsterone is detectable in the plasma at 4 years of age and rises steadily to puberty; it is claimed that the concentration of this compound in the blood of

adult females shows some variation during the menstrual cycle. Comparison of the concentration of 17-oxo steroid conjugates in plasma and in urine shows that the glucuronides are excreted at a very much faster rate than the corresponding sulphates.[77]

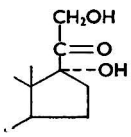
Determination of 17-oxogenic steroids[8]

It has already been shown that C_{21} steroids produced in the adrenal glands can be degraded to 17-oxo steroids, which appear in the urine. In health, only a small proportion of the endogenous and exogenous corticosteroids follow this metabolic pathway and the major part of the material that can be traced also appears in the urine as C_{21} metabolites with side-chain intact, but in various stages of reduction and oxidation. These changes, which are in addition to those in the steroid nucleus, result in the formation and excretion of very many different types of C_{21} metabolites.[81]

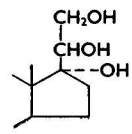
Table I lists the recognised forms of side-chains that may arise from a 17α-hydroxycorticosteroid precursor such as cortisol (**VI**; see p. 723); the list is incomplete and should also contain the corresponding set of compounds lacking the 17α-hydroxyl group, which may be formed from 17-deoxy-C_{21}-steroid precursors.

Table I

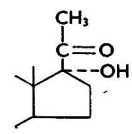
Summary of the reactions of the corticosteroid side-chain structures



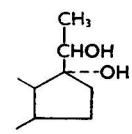
(*a*)



(*b*)



(*c*)



(*d*)

Side-chain type	Molybdophosphate	Triphenyltetrazonium reagents	Formaldehydogenic compounds	Acetaldehydogenic compounds	Porter and Silber chromogens	17-Oxogenic reaction: $NaBiO_3$	$NaBH_4$ $NaBiO_3$	$NaBiO_3$ $NaBH_4$ $NaBiO_3$
(*a*) Dihydroxyacetone	+	+	+	−	+	+	+	−
(*b*) Glycerol	−	−	+	−	−	+	+	−
(*c*) 17:20-Ketol ..	−	−	−	−	−	−	+	+
(*d*) 17:20-Glycol ..	−	−	−	+	−	+	+	−

It is pertinent to emphasise that many of these compounds are thermolabile and unstable in the presence of alkali and that much early work was rendered invalid by the use of hot-acid hydrolysis and the failure to appreciate the need for mild procedures.[82] It seems likely that many compounds of this class are conjugated as glucuronides. The conjugates are hydrolysed by most β-glucuronidase preparations, but there is, as yet, insufficient evidence that the enzymic hydrolysis goes to completion; experimental conditions have been found that give maximum yields of free steroid, but these may coincide with the equilibrium position. The evidence of Norymberski,[83] which suggests a substantial proportion of 17-oxogenic steroid conjugated as sulphate, is not conclusive.

Many novel attempts have been made to determine the several structures of this group of related steroids. The earliest method, that of Heard and Sobel,[84] which depended on the reduction of molybdophosphoric acid, is quite unspecific and has been abandoned. Substituted derivatives of triphenyltetrazonium chloride,[85] prepared for use in solution in sodium hydroxide, depend on the reducing properties of the α-ketol structure –CO–CHOH– and are more specific. These reagents have been of considerable value as spray reagents in paper chromatography for the location of compounds with the α-ketol side-chain structure (Table I, type (*a*)). The Porter and Silber reagent,[86] dinitrophenylhydrazine in sulphuric acid, reacts only with 17α-hydroxy-20:21-ketols and is specific for the side-chain (dihydroxyacetone) found in cortisone and cortisol (Table I, type (*a*)).

Lowenstein, Corcoran and Page[87] in 1946 introduced chromotropic acid into the steroid field as a means of determining small amounts of formaldehyde; this fragment is split off 20:21-glycols (Table I, type (*b*)) and 20:21-ketols (Table I, type (*a*)) by periodic acid or by sodium bismuthate[88] and can be used to determine compounds possessing these structures. The method, although useful with relatively pure compounds,[89] is of less value when applied

to biological materials that normally contain comparatively large amounts of non-steroidal "formaldehydogenic" matter. An analogous method described by Cox and Marrian[90] is based upon the liberation of acetaldehyde by oxidation of steroid glycols with periodic acid; inspection of the possible side-chain structures shows that this fragment is only produced by 17:20-glycols (Table I, type (*d*)), and the method has high potential specificity for 21-methyl-17:20-glycol side-chains.

It is noteworthy that oxidations of this type, in which a fragment containing two carbon atoms is split off from the C_{21} molecule, leave behind a C_{19}-17-oxo steroid that is very much more specific in origin than the acetaldehyde. The new 17-oxo steroid, which, unlike the acetaldehyde, must arise from a steroid progenitor, can be determined by the Zimmerman reaction, and in this way many 17α-hydroxy-C_{21} compounds (the 17-oxo steroids[91]) can be indirectly determined. The oxidising agents used for this type of degradation include periodic acid, lead tetra-acetate and sodium bismuthate, all of which are glycol-splitting agents. The use of chromic oxide in acetic acid for this purpose seems unnecessarily violent, although this reagent will also convert 17-hydroxy-C_{19} steroids to 17-oxo steroids.[92] The claim of Smith and Tompsett[93] that 3:17-dihydroxyandrostanes are oxidised by bismuthate in the presence of chloride to a product that is chromogenic in the Zimmermann reaction has been substantiated,[94] but does not take place when urine is oxidised, because urea is present. No satisfactory method is available for the oxidation of C_{21} steroids that do not have a 17α-hydroxy group.

Of the available oxidants, sodium bismuthate, introduced by Norymberski,[95] offers many practical advantages. With this reagent the oxidation is a surfaced-catalysed reaction that is sensitive to light. Few, if any, soluble by-products are produced and, because of the insolubility of the reagent, oxidation can be stopped by centrifugation or filtration. Traces of bismuthate are removed by the addition of bisulphite. In the original application Norymberski made use of this reaction to determine compounds containing the side-chain structures found in types (*a*), (*b*) and (*d*) (Table I), which are directly oxidised to 17-oxo steroids. For these compounds Norymberski suggested the name 17-oxogenic steroids. When the method is applied to urine, these 17-oxo steroids are formed in addition to those excreted in the urine, and the sum total is referred to as the "total 17-oxo steroids." A separate and independent determination of the preformed urinary 17-oxo steroids is necessary in order to obtain the value of the 17-oxogenic steroids by difference. A further important feature of the oxidation of 17-oxogenic steroids in urine is that, for the purpose of this determination, the hydrolysis of the conjugated forms is no longer necessary. It has been shown that, in addition to the oxidative removal of the side-chain, bismuthate, when present in excess, brings about the oxidative destruction of the glucuronic acid part of the molecule; in this way 17-oxogenic steroid glucuronides are converted to 17-oxo steroid formates (or to the free alcohol), both of which are soluble in organic solvents.[83] It is no longer necessary to hydrolyse in order to extract the steroid residues for determination. This reaction, which appears to be a general one for all classes of steroid glucuronides, has somewhat different consequences when applied to 17-oxogenic steroid sulphates. Compounds of this type, if indeed examples exist, are oxidised to 17-oxo steroid sulphates, which remain, like their precursors, water soluble. In contrast to the straightforward determination of 17-oxogenic steroid glucuronides, some form of mild hydrolysis is necessary before extraction, if these compounds are to be included.

In a subsequent development of the 17-oxogenic steroid method, Norymberski introduced the use of sodium borohydride as a reducing agent to be used in conjunction with sodium bismuthate.[96] Preliminary treatment of compounds of type (*c*) (Table I) with borohydride converts them to type (*d*), so that subsequent oxidation with bismuthate converts all 17α-hydroxy compounds to 17-oxo steroids. During treatment with borohydride, all 17-oxo steroids present in the untreated urine are reduced to the corresponding secondary alcohols, which give no reaction in the Zimmermann test: all 17-oxo steroids present after the oxidation are derived from 17α-hydroxycorticosteroids. This method, which has been of considerable value in following and controlling adrenocorticotrophic hormone and cortisol therapy, determines "total 17α-hydroxycorticosteroids," a term used to distinguish this determination from that of the Porter and Silber chromogens, which are also loosely referred to as 17-hydroxy-corticosteroids.

A further modification of the borohydride - bismuthate method can be used to determine compounds of type (*c*) (Table I) exclusively.[97] When urine or urine extracts are treated with excess of sodium bismuthate, molecules having side-chains of types (*a*), (*b*),

and (*d*) (Table I) are destroyed by oxidation to 17-oxo steroids, whereas molecules of type (*c*) remain unchanged. Subsequent reduction with borohydride reduces all 17-oxo steroids irrespective of origin to the corresponding alcohols and simultaneously converts the unchanged molecules of type (*c*) (Table I) to type (*d*). The latter can then be determined indirectly as 17-oxo steroids by a second bismuthate oxidation. This method has been used to measure the 17α-hydroxy-20-oxo steroids excreted in health, in rheumatoid arthritis and in adrenogenital syndrome.[98]

Table I summarises the reactions that can be used to discriminate between the various types of 17-hydroxycorticoid side-chain. Of the corresponding 17-deoxy compounds, only 20:21-glycols and 20:21-ketols give formaldehyde on oxidation, and all other specific reactions are negative. The use of these methods permits the total amount of any class of corticosteroid to be determined, and this may have clinical value; unfortunately, in the process of determination the precursors are destroyed and their identities are lost. No excretion pattern of corticosteroids, analogous to that of the procedure for 17-oxo steroids, can be obtained by these oxidative methods, as several 17-oxogenic steroids may give rise to the same 17-oxo steroid. In most urines the 17-oxogenic steroids on oxidation give rise to 11-oxy-17-oxo steroids. Recently, methods have been described that give an excretion pattern for corticosteroids based upon the separation of compounds on partition columns of Celite or on silica[99,100]; separation of these compounds on paper chromatograms is also possible.[101]

No attempt has yet been made to determine 17-oxogenic steroids in blood.

Determination of non-ketonic steroid alcohols

The method for the determination of non-ketonic steroid alcohols described below, although not strictly for 17-oxo steroids or 17-oxogenic steroids, is related to these determinations, because it is based upon a reversed Zimmermann reaction.

In contrast to the variety of methods available for the measurement of ketonic alcoholic steroids, very few general methods have been suggested for the determination of steroids that have no functional group other than the alicyclic alcohol group. The Liebermann - Burchardt reaction for cholesterol and the sulphuric acid chromogenic reactions used in the determination of pregnane-3α:20α-diol[102] and pregnane-3α:16α:20α-triol[103] are colour reactions of some specificity, and cannot be applied to a whole class of compounds. It is, however, possible to esterify alcohols quantitatively and in so doing to incorporate a chromogenic group into the ester, which can be used to determine the alcohol indirectly. Engel, Patterson, Wilson and Schinkel[104] showed that it was possible to prepare hemi-3:5-dinitrophthalates of primary and secondary alcohols and to determine the ester quantitatively by means of the red colour formed on treatment with methanolic potassium hydroxide. A more practical method of carrying out this type of determination is to esterify with 3:5-dinitrobenzoyl chloride in the presence of pyridine.[105] By analogy with the Zimmermann reaction, if a steroid ester containing a *m*-dinitrophenyl group is treated with alkali in the presence of excess of a compound containing an activated methylene group, a characteristic Zimmermann-like colour is obtained. Esterifications carried out with 3:5-dinitrobenzoyl chloride are rapid and complete, and the resulting esters when dissolved in acetone (which acts as the compound containing the activated methylene group) give an intense colour when treated with weak ethanolic potassium hydroxide solution. The colour reaches the maximum intensity more rapidly than in a conventional Zimmermann reaction and has a maximum absorption at 560 mμ.

Application of this method to the non-ketonic fraction of urines obtained from patients with adrenal carcinoma and adrenal tumour has shown that in these clinical conditions there is a substantial change in the amount and pattern of excretion.[106] The changes in the non-ketonic fraction are complementary to changes already observed in the ketonic fraction by using the conventional methods for 17-oxo steroids.

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The Determination of Oxygen in Titanium and Titanium Alloys, Based on the Principle of Chlorination

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A chemical procedure for the determination of oxygen in titanium and titanium-base alloys is described. The solid sample is admixed with graphite and chlorinated in an atmosphere of argon and the products of the reaction are subsequently isolated, excess of chlorine being removed by reaction with antimony. The liberated carbon monoxide has been shown to bear a stoicheiometric relationship to the amount of oxygen present in the sample, and in the final stages of the procedure the purified carbon monoxide is oxidised to carbon dioxide and weighed.

The results were reproducible when the procedure was applied to the analysis of titanium - manganese alloys; this potential application is an outstanding advantage over the vacuum fusion procedure.

With a single apparatus, about twelve determinations can be completed in a normal working week of 5 days. This is about the same rate as that at which oxygen can be determined by the macro vacuum fusion procedure, but, whereas a complete vacuum fusion unit costs about £2500 and requires fairly constant attention, apparatus for the recommended procedure costs about £120 and it is estimated that three units could be operated simultaneously.

THE most reliable procedure for the determination of oxygen in titanium and its alloys is that based on the principle of vacuum fusion.[1,2,3,4,5,6,7] In this procedure, the sample is

heated at 1600° C in a vacuum in the presence of an excess of carbon, and oxygen is quantitatively liberated as carbon monoxide. The necessary apparatus is expensive and its operation requires specialised skill. The vacuum fusion procedure can be applied to the determination of oxygen in titanium and most of its common alloys, with the exception of those containing manganese, which readily volatilises, condenses on the cooler parts of the apparatus and combines with some of the oxygen.

The need for a relatively simple chemical method for the determination of oxygen in titanium-base samples, particularly those containing manganese, has been apparent for a long time. A chemical procedure proposed in 1951 by Corbett[8] is similar to the chlorination method of Colbeck, Craven and Murray[9] for the determination of non-metallic inclusions in steels. Corbett's procedure is based on chlorination of the heated sample, whereby titanium is converted into the volatile tetrachloride, and oxygen is indirectly determined in the residue of titania by determining its titanium content. This procedure is very restricted in its applications and involves the assumption that any carbon in the sample is converted into carbon monoxide; a suitable correction for loss of oxygen in this way must be applied. Further, it cannot be used in the presence of metals, such as aluminium, that give residues of uncertain composition.

A similar procedure based on bromination has been proposed by Milner, Hegedus and Dyorsky,[10] but criticisms are equally applicable to both halogenation procedures.

More recently, a procedure has been proposed in which the sample is heated in the presence of excess of carbon in a stream of bromine vapour,[11] whereby titanium and the common alloying elements are converted to their corresponding volatile bromides, and oxygen is evolved as carbon monoxide. After a series of purification stages, carbon monoxide is oxidised to carbon dioxide and weighed, and the weight is used in calculating the oxygen content of the sample. This procedure has met with a mixed reception in the U.S.A., and "further development is necessary before it can be completely relied upon."[12] In addition, our own experience with the method has not been entirely satisfactory.[13]

Bromination of titanium-base materials is slow and, in order to make the procedure reasonably rapid, it is necessary to limit the weight of sample to about 1 g, which in turn necessitates a rigid control on the operating conditions and a very careful evaluation of the blank. Further, the sample for bromination must be in the form of thin slices, and the preparation of such samples increases the risk of atmospheric oxidation.

Other chemical procedures have been either tried or suggested. These include the use of fluorine at elevated temperatures, when oxygen in the sample is liberated as gaseous oxygen[14]; a similar procedure involves the use of hydrofluoric acid, which converts the oxygen into water.[15] Bromine trifluoride has also been suggested as a reagent for this determination,[16] but with all fluorine-containing compounds there is extreme difficulty in obtaining them in an anhydrous condition and specially constructed apparatus, *e.g.*, made of nickel, must be used.

In all these chemical procedures the apparatus must be rendered free from oxygen by the passage of an inert gas, and the need to provide a system free from moisture is of paramount importance.

Unfortunately, the relatively simple expedient of determining oxygen by reduction in either an atmosphere of hydrogen or in the presence of carbon, as has been applied to steel and copper-base materials, cannot be applied in the determination of oxygen in titanium and titanium alloys.[17]

Attempts have been made to determine oxygen in titanium alloys by other methods, including selective solution,[18] radioactivation,[15] by making use of the TiO spectral bands emitted when samples are examined spectrographically in an evacuated system[15,19] and by means of the mass spectrometer.[15,20,21] All these procedures, however, have limitations, and none is entirely satisfactory.

Development of the Method

Consideration of the various factors underlying the possible development of a chemical procedure suggested that a method based on chlorination was worthy of further consideration. Chlorine reacts more readily with titanium and its alloys than bromine does, and consequently a larger sample can be taken with correspondingly less dependence both on sampling and on a careful evaluation of the blank. For the determination of oxygen, chlorine has other advantages over bromine, *e.g.*, it does not condense in narrow-bore tubing or at inaccessible bends

and, more important, it requires no carrier gas during halogenation and so permits a higher concentration of halogen to be present during the reaction.

When chlorine is passed over titanium at 800° C, the following reaction takes place—

$$Ti + 2Cl_2 \longrightarrow TiCl_4.$$

At the same temperature no reaction occurs between titanium dioxide and chlorine, but, when the oxide is in intimate contact with excess of carbon, the reaction under suitable conditions is thought to proceed according to the following equation—

$$TiO_2 + 2C + 2Cl_2 \longrightarrow TiCl_4 + 2CO.$$

Under the conditions used in the development of this procedure, however, this reaction does not occur. Hence, neither titanium tetrachloride nor carbon monoxide could be detected after prolonged chlorination of a heated mixture of graphite and titania. It was also observed that, when reference samples prepared by fusing titanium refined by the iodide process and weighed amounts of titania in an argon-arc furnace were similarly examined, there was a tendency for the recoveries to be low if the oxygen content exceeded about 0·2 per cent., and a corresponding amount of titania was always found in the residue.

It is known that up to about 15 per cent. of oxygen, by weight, can exist in solution in titanium, hence the above-mentioned reaction can be expressed by the following equation—

$$Ti + O + 2Cl_2 + C \longrightarrow TiCl_4 + CO.$$

The contemplated procedure, therefore, was to chlorinate the heated sample in the presence of excess of carbon, use being made of argon as a purge-gas, and to convert the oxygen quantitatively to carbon monoxide, which would subsequently be purified and then oxidised to carbon dioxide and weighed. The final oxidation and weighing would follow conventional lines by using a converter of heated copper oxide and then a weighing tube filled with Carbosorb soda asbestos.

A valid criticism of the use of chlorine under these conditions lies in its ability to react with carbon monoxide to form carbonyl chloride. A tentative procedure was therefore evolved on the assumption that, after chlorination, the ensuing gaseous mixture would contain titanium tetrachloride, the chlorides of the alloying constituents, excess of chlorine, carbon monoxide and some carbonyl chloride.

Titanium tetrachloride has b.p. 136° C and m.p. — 30° C, and therefore condenses as a liquid at room temperature, which affords a ready and simple means of removing almost the whole of the titanium and other volatile metallic chlorides that have very similar properties.

A study of the equilibrium reaction between carbon monoxide, chlorine and carbonyl chloride indicated that a high temperature favours the dissociation of carbonyl chloride, but recombination of the gases at a lower temperature had to be considered. It was essential, therefore, that chlorine from the carbonyl chloride should be removed, not by the conventional method of freezing, but from a stream of hot gas at a temperature as near as possible to that at which the carbonyl chloride was decomposed. Decomposition of carbonyl chloride was achieved by passing the gas leaving the combustion tube through a silica tube maintained at 800° C.

Many attempts were made to remove excess of chlorine from the gas stream. Finely divided tin that was nominally free from carbon was tried at one stage, and this met with some success. The reaction between tin and chlorine proceeds with incandescence, and stannic chloride is the main product of the reaction. Stannic chloride has b.p. 114° C and m.p. — 33° C, and, being a liquid at room temperature, is easily removed from the reaction zone. Unfortunately, the reaction proceeds one stage further and a large amount of stannous chloride is also formed. Stannous chloride is a solid with m.p. 246° C and its presence rapidly chokes the tube. In the next series of experiments, tin was replaced by antimony. The powdered metal was used in order to obtain a large surface area and, although this appeared to be satisfactory chemically, the column soon became choked; the sticks of antimony subsequently used were entirely satisfactory.

Excess of chlorine reacts readily with the antimony and, contrary to expectation, there was no visible sign of chlorine passing beyond the first few pieces of the very loosely packed metal.

Antimony was placed in a vertical column so that liquid antimony pentachloride could be collected in a catch-pot at the base of the column. Passage of gas vertically through the

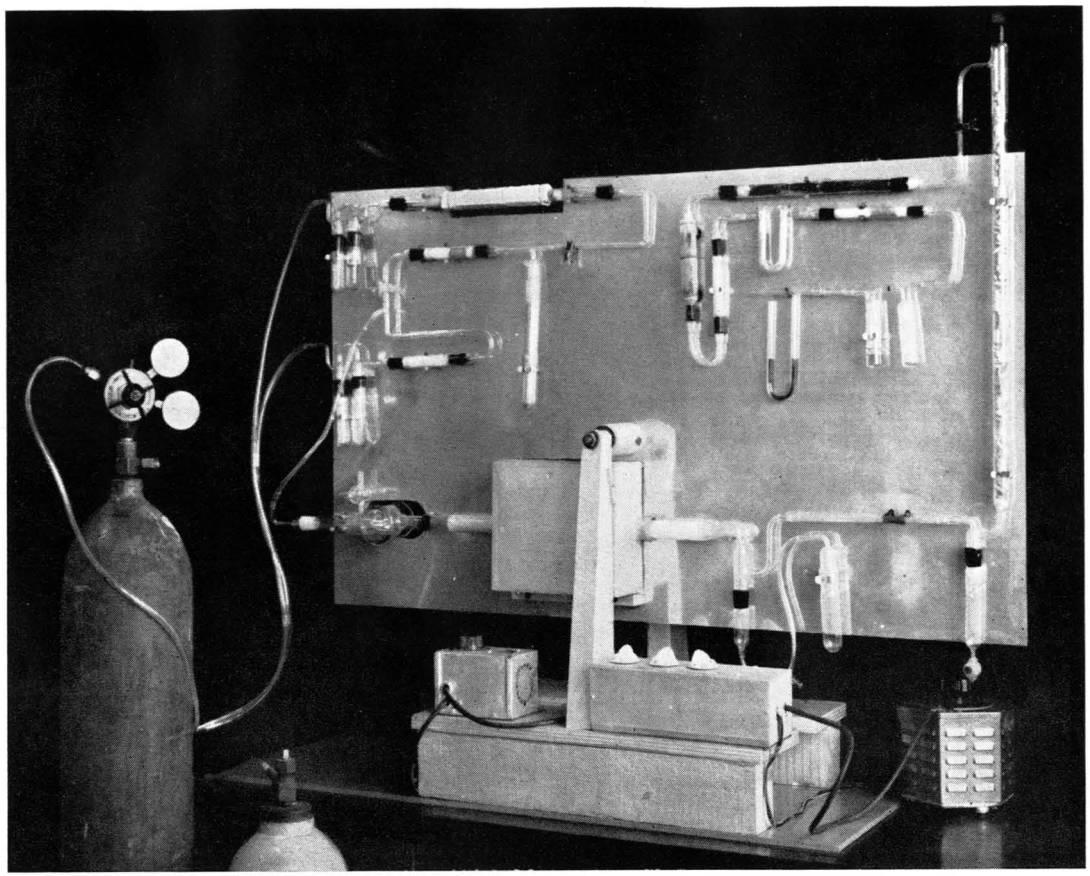

Fig. 1. View of apparatus for determining oxygen in titanium and titanium alloys

[To face p. 736

column was not entirely satisfactory, as a small amount of mist, presumably antimony trichloride, was carried forward by convection currents, and the copper oxide in the converter soon became poisoned. When the gas stream was allowed to enter at the top of the column, solid antimony trichloride was formed and seriously impeded the flow of gas. This idea was soon abandoned in favour of the original idea of passing the gas leaving the combustion tube up through the column, but the problem of the antimony trichloride mist had still to be solved, and several unsuccessful attempts were made to solve it. Plugs of glass-wool, filter-paper and a water trap were separately tried, but none was entirely satisfactory. A scrubber containing Carbosorb soda asbestos admixed with glass beads was tried, and this was reasonably successful over periods of at least 5 days under conditions of daily working, but low erratic results were associated with the incorporation of this scrubber and, finally, a scrubber containing an intimate mixture of manganese dioxide and glass balls was found to be entirely satisfactory.

In each determination it was necessary to place the sample in the cold combustion tube, sweep out the air with dry argon, raise the temperature of the combustion tube and then cool it before the next sample could be examined. This was time-consuming, and, in order to make the procedure more practicable, the apparatus was redesigned so that heating of the combustion tube was uninterrupted and an almost continuous supply of samples could be examined. The way in which this was achieved will be clear from the description of the apparatus.

METHOD

APPARATUS—

The apparatus is shown in Figs. 1 and 2.

The Arnold bubblers, A_1, A_2 and A_3, are each constructed as a single unit and contain sulphuric acid, sp.gr. 1·84; A_1 and A_2 have B24 joints, and B19 cones are attached to their outlet tubes. The purifier for argon, B, is a Pyrex-glass tube, 1 inch in diameter and 18 inches long. The tube is heated electrically to 250° C along 9 inches of its length and is lagged with asbestos; a sheet of asbestos paper covers the tube to prevent any local penetration of the glass by the heating wire. The pressure release valve, C, has a B24 joint and a pressure head of about 7 inches of mercury.

The scrubber for carbon dioxide, D, has a B19 joint at each end; the inlet half of the tube is filled with Carbosorb soda asbestos and the other half with anhydrone. E_1 and E_2 are Quickfit and Quartz S61/2 glass taps. The drying tube, F, has a B19 joint at each end and is filled with phosphorus pentoxide. G is a removable B24 cone and H is a Quickfit and Quartz SC8/6 Pyrex-glass tap having a bore wide enough to allow the silica sample boat and its contents to pass freely into the combustion tube, I. (Tap H was lubricated with Edwards' silicone grease, but the centre part of the tap was not greased so that none could be picked up by the sample boat.) Tap H is joined to the combustion tube by a B24 cone. The combustion tube, I, is a silica tube, 1·25 inches in diameter and 24 inches long, with a B24 joint at each end. This tube is heated electrically to maintain a working temperature of 825° C and the position of the sample boat during a determination is controlled by a silica rod that is locked in position by a polythene collar and tongue. The optimum position of the rod allows the sample boat to be housed in the hottest part of the tube.

The chloride trap, J, has B24 joints and two outlets; one outlet allows the gaseous effluent to pass forward and the other allows the liquid products of the reaction to be transferred, by means of screw clip K, to the chloride reservoir, L, which has a B24 joint, and from which the liquid products are subsequently removed from the system. The silica tube, M, has a diameter of 0·4 inch and is heated to 800° C over 1·5 inches of its length as close to the trap for antimony pentachloride, N, as is practicable. The trap for antimony pentachloride has a B24 joint and is emptied by means of a tap having a core made of Teflon. The cone of the trap and the glass column, O, are constructed as a single unit with trap N.

Column O is made of Pyrex-glass tubing, 1 inch in diameter and 29 inches long. It has a filter-cone made of china at its base and is filled with sticks of antimony of diameter 0·25 inch and about 0·5 inch long. The column has a side arm about 1 inch from the top and a solid stopper in a B24 socket, P, at its upper end. The glass tube, Q, has a B19 cone at each end and is connected to the side arm of column O by a MS5/12 spherical joint. The tube has a diameter of 0·8 inch and is 12 inches long; it is filled with a mixture of manganese dioxide and glass beads with a plug of glass-wool at each end.

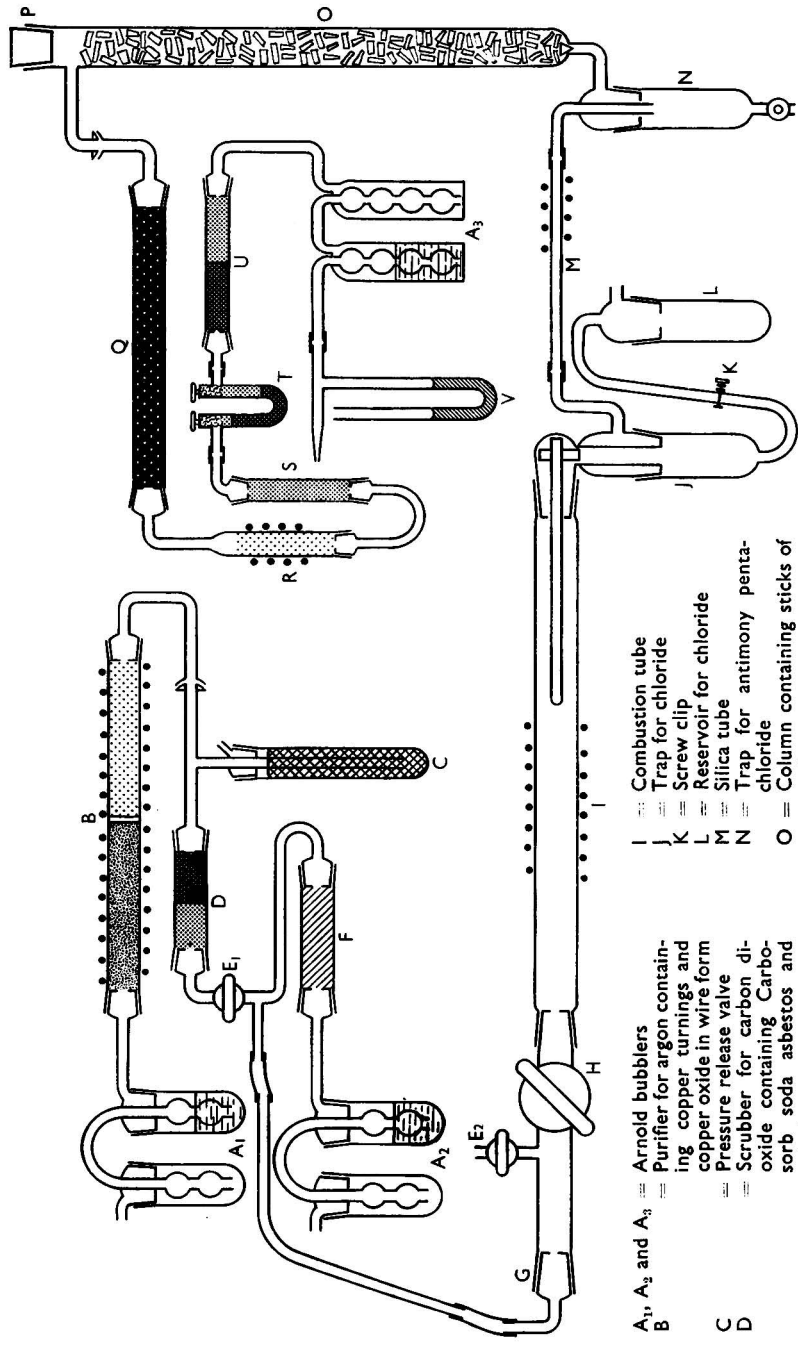

Fig. 2. Apparatus for determining oxygen in titanium and titanium alloys

Tube R and the B19 cone that joins it to tube Q are constructed as a single unit having a B14 joint at the lower end. Tube R is filled with copper oxide, has a glass-wool plug at each end and is heated electrically to 270° C; a layer of asbestos paper covers the tube to prevent any local penetration of the glass by the heating wire. S is a drying tube, 0·6 inch

in diameter and 6 inches long, and is filled with anhydrone with a plug of glass-wool at each end. The weighing tube for carbon dioxide, T, is a U-tube filled with Carbosorb soda asbestos and anhydrone with a small plug of glass-wool between the reagents and a similar plug at each end. The scrubber for carbon dioxide, U, has a B14 joint at each end; the inlet half of the tube is filled with Carbosorb soda asbestos and the other half with anhydrone. V is a flowmeter.

With four exceptions all conical and spherical joints are sealed with black Apiezon vacuum wax. The wax must not penetrate more than half-way down the joints on A_1 and A_2 or more than $\frac{1}{4}$ inch down the joint between H and I. The unwaxed joints are the joint at C, cone G and the joint between I and J; the joints must, however, be lubricated with a silicone grease (Edwards' silicone grease was used). When standard joints are not used and when flexibility is not required, connections are made glass-to-glass by using poly(vinyl chloride) tubing. Lengths of poly(vinyl chloride) tubing are also used to transfer argon and chlorine from the cylinders to the apparatus. The furnaces heating B, I, M and R are controlled by Sunvic switches.

The apparatus as shown in Fig. 1 is mounted on a five-ply board, 54 inches × 29 inches × $\frac{1}{2}$ inch, covered with 16 s.w.g. aluminium sheet to prevent unsightly charring when the waxed joints are heated. Terry spring-clips are used extensively for securing components to the board. Apertures are cut in the board to take the furnaces heating B and I. (The furnace heating I is held in position by wooden supports on the back of the board.) An elongated hole is cut for the larger tap, H, which enables it to be moved from its normal position during assembly and dismantling.

The board is pivoted on an axle, which in turn is supported on a wooden frame. The tilt of the board should be sufficient in both directions to enable the sample boat to slide freely into and out of the furnace. The maximum angle of tilt required is about 30°. In the rest position, there should be a slight clockwise tilt; the apparatus is held in this position by a simple swivel catch mounted on the front support of the wooden frame.

The apparatus must be operated under conditions of good ventilation, preferably in a fume-cupboard.

REAGENTS—

Chlorine—As it is extremely difficult to obtain chlorine free from oxygen and as it is desirable that chlorine with a very low total oxygen content should be used, this must be specified when the material is ordered from the supplier. Freshly supplied cylinders of liquid chlorine tend to have a high oxygen content, but after about one-third of the liquid has been used the oxygen content is considerably reduced.

Argon—Free from oxygen.

Graphite—Rods obtained from Johnson, Matthey & Co., 10 mm in diameter and 30 cm long, were used. The powdered material is prepared by grinding the rods in a pencil sharpener retained exclusively for this work. In order to minimise pick-up of oxygen, the graphite should be powdered immediately before use.

Carbosorb soda asbestos, 10 to 14-mesh B.S.S.
Anhydrone, 10 to 14-mesh B.S.S.
Copper oxide, wire form.
Manganese dioxide—Coarse grade, 20 to 40-mesh B.S.S.
Antimony—The metal in the form of sticks.
Sulphuric acid, sp.gr. 1·84.
Nitric acid, sp.gr. 1·42.
Hydrochloric acid, sp.gr. 1·18.
Hydrofluoric acid, 40 per cent. w/w.
Ethanol.
Ether.

PROCEDURE FOR PREPARING THE SAMPLE—

Cut the sample into pieces of about 1 g and clean them by pickling for about 3 minutes in a mixture of 25 ml of nitric acid and 25 ml of hydrochloric acid, containing about 5 ml of hydrofluoric acid. Wash the pieces free from acid with water, rinse them with 10 ml of ethanol and then 10 ml of ether, and then dry them in a stream of compressed air for about 1 minute; this is usually sufficient to remove the ether completely.

PROCEDURE FOR DETERMINING THE BLANK—

Determine the blank on a weighed sample of titanium (about 5 g), refined by the iodide process and of known oxygen content. Transfer the prepared sample to a previously ignited silica sample boat and fill with freshly ground graphite; level with a spatula and ensure that the sample does not protrude above the graphite. When very pure chlorine is used, it is sufficiently accurate to determine the blank on the graphite only. Place the sample boat in an air-oven at 105° C for 30 minutes and then transfer it, without cooling, to the apparatus. Continue as outlined in the procedure for determining oxygen. The value of the blank B in milligrams is given by—

$$B = W - \frac{X \times Y}{0{\cdot}03636}$$

or, when graphite alone is used, by—

$$B = W$$

where W = increase in weight of weighing tube T in milligrams,
X = weight of titanium taken in grams, and
Y = percentage of oxygen in the titanium.

PROCEDURE FOR DETERMINING OXYGEN—

Assemble the apparatus and heat the furnaces to the appropriate temperatures. Close taps E_1 and E_2, open tap H and sweep out the apparatus with chlorine for 10 minutes. After assembly or partial dismantling and re-assembly of the apparatus, ensure stabilisation of the system by passing argon alone at the recommended rate of flow over heated graphite. The increase in weight of weighing tube T should not exceed 1·5 mg per hour. The preliminary gassing with chlorine is advised if at any time the chlorine supply has been stopped for more than 5 hours. If the column of antimony, O, has been completely renewed, heat the base of the column gently to initiate the reaction. Stop the flow of chlorine, open tap E_1, and then sweep out with argon for 30 minutes. Close tap H, open tap E_2 and remove cone G and carefully remove the silicone grease from the socket. Place the prepared sample, about 5 g, in the glass tube between cone G and tap E_2, lightly grease cone G and replace; ensure that the cone is held firmly in position, *e.g.*, by means of a rubber band. Purge the chamber for 10 minutes with argon, close tap E_2 and stop the flow of argon. Isolate the argon train by closing tap E_1, then open tap H. Weigh the weighing tube T, insert in the train, then open the weighing tube taps. Tilt the apparatus sufficiently to allow the sample to slide into position in the combustion tube, I. Chlorinate the sample for 2 hours at a rate of flow of about 120 ml per minute. At this rate of flow the sample should be completely chlorinated and the trap for antimony pentachloride full. It is important to ensure that, both in determining the blank and determining oxygen in the sample, the volume of antimony pentachloride formed is about equal. Stop the flow of chlorine, open tap E_1 and sweep out the apparatus for 30 minutes with argon at a rate of flow of about 100 ml per minute. During the initial stage of this sweeping-out period, antimony trichloride mist is visible in column O. Stop the flow of argon at this stage for about 2 minutes; this enables the mist to condense and prevents rapid saturation of tube Q. At the end of this period, re-weigh the weighing tube, T, and record the increase in weight in milligrams. Tilt the apparatus to allow the sample boat to slide into the glass tube between G and E_2 and, for a further determination, repeat the procedure from "Close tap H, open tap E_2 and remove cone G. . . ."

The oxygen content of the sample is given by—

$$0{\cdot}03636 \times \frac{(W - B)}{X} \text{ per cent.}$$

where W = increase in weight of weighing tube T in milligrams,
B = value of the blank in milligrams, and
X = weight of sample in grams.

Note that it is an advantage to examine periodically a suitable reference sample of known oxygen content.

RESULTS

Numerous results obtained during the course of the development work are not reported here. In most of the experiments, samples were used whose oxygen content had been determined by the vacuum fusion procedure and invariably, when discrepant results were obtained,

some minor modification to the chemical procedure was made before the determination was repeated. Finally, a bar of commercially pure double-melted titanium of ½ inch diameter was obtained and its precise oxygen content was determined by a vacuum fusion procedure.[7] An independent value for oxygen was supplied by Mr. E. Booth of the United Kingdom Atomic Energy Authority, who used the semi-micro vacuum fusion method of Booth, Bryant and Parker.[22] In this way it was shown that the oxygen content of the bar was uniform throughout at 0·102 ± 0·005 per cent. Replicate determinations of oxygen by the proposed procedure on this material gave the following results—

0·10, 0·11, 0·12, 0·12, 0·10, 0·11, 0·09, 0·09, 0·10, 0·10, 0·09 and 0·09 per cent.

The average value by the vacuum fusion procedure was 0·10 per cent., and it can be seen that the results by the proposed procedure are in good agreement with the established value.

In view of the inability to determine oxygen in manganese-containing alloys by the vacuum fusion technique, the proposed procedure was applied to two rods of double-melted titanium containing 4 per cent. of aluminium and 4 per cent. of manganese. The results of replicate determinations of oxygen by the proposed procedure were as follows—

Rod No. 1 .. 0·13, 0·13, 0·12 and 0·12 per cent.
Rod No. 2 .. 0·18, 0·17, 0·18, 0·18 and 0·16 per cent.

The reproducibility of the results of these tests is satisfactory, and there is no reason to doubt the established oxygen contents, but unfortunately it is not possible to compare them with any results obtained by an alternative reliable procedure.

Two further manganese-bearing materials were examined, but oxygen results ranged from 0·10 to 0·24 per cent. over a total of about twelve determinations. These samples were very difficult to prepare; they were so hard that as many as ten hack-saw blades had to be

TABLE I

DETERMINATION OF OXYGEN IN TITANIUM-BASE MATERIALS

Type of material	Oxygen found by—					
	proposed method, %	vacuum fusion method, %	proposed method, %	vacuum fusion method, %	proposed method, %	vacuum fusion method, %
Titanium refined by the iodide process	0·018	0·012				
Commercially pure titanium	0·34	0·34	0·09	0·10	0·20	0·20
	0·10	0·10	0·13	0·13	0·10	0·10
	0·37	0·38	0·14	0·14	0·11	0·13
	0·09	0·11	0·12	0·13	0·20	0·20
	0·09	0·08	0·11	0·13	0·07	0·08
	0·08	0·10	0·12	0·13	0·20	0·20
	0·14	0·13	0·15	0·14	0·21	0·20
	0·11	0·13	0·49	0·53	0·12	0·13
	0·55	0·53	0·10	0·10	0·07	0·08
	0·11	0·13	0·07	0·08	0·12	0·13
	0·09	0·08	0·08	0·10	0·11	0·11
	0·09	0·10	0·12	0·11	0·10	0·10
	0·11	0·11	0·09	0·11		
Titanium - 12½ per cent. of tin - 2½ per cent. of aluminium alloy	0·12	0·12	0·16	0·17	0·14	0·13
	0·13	0·12				
Titanium - 2½ per cent. of tin - 5 per cent .of aluminium alloy	0·17	0·15				
Titanium refined by the iodide process + 0·2 per cent. of oxygen	0·20	0·20*	0·23	0·20*		

* Theoretical value.

used in the preparation of a 5-g sample. Although every care was taken to minimise the heat evolved during the sawing operation, it is conceivable that the high results are due to atmospheric oxidation. This practical difficulty is associated with the sampling of all titanium-base materials, particularly alloys, and the preliminary pickling treatment incorporated in the recommended method aims at minimising errors introduced in this way.

Table I shows the results of determinations of oxygen in various samples by the recommended method and by the vacuum fusion procedure, together with two theoretical values. It can be seen that the results are all in good agreement.

Application of the recommended procedure to samples of zirconium

Samples of zirconium were examined by the chemical procedure, but there was a practical difficulty to overcome, as zirconium tetrachloride is a solid and chokes the tube leading to the chloride trap. There is little doubt about the successful application of this procedure to the determination of oxygen in zirconium and its alloys, perhaps after some minor modification, but a serious attempt to extend the recommended procedure has not been made.

Conclusions

The method is no more rapid than the existing macro vacuum fusion procedure, but the cost of the equipment represents a considerable financial saving. The installation of a complete vacuum fusion unit costs about £2500, whereas the equipment necessary for the proposed chemical procedure costs only about £120.

The time taken to complete a single determination is about 2½ hours and, allowing for stabilisation of the train and periodic blank evaluations, it is estimated that, with one apparatus, about twelve determinations can be completed in a normal working week of 5 days. Because of the actual working time needed to manipulate a single apparatus, one person should be able to operate three trains simultaneously with a corresponding increase in the number of samples examined.

The outstanding advantage of the chemical procedure is its potential application to the determination of oxygen in alloys containing manganese.

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The Determination of Tellurium in Lead and Lead Alloys

By N. W. FLETCHER and R. WARDLE

A new absorptiometric method for the determination of tellurium in lead and lead alloys is described, in which use is made of the absorption of tellurium bromide in hydrobromic acid. The method is more sensitive than those based on the use of iodides.

The alloy is dissolved in a mixture of bromine and hydrobromic acid and tellurium is precipitated with stannous bromide. After filtration, the tellurium is redissolved in a bromine - hydrobromic acid mixture, most of the bromine is removed by boiling the solution and the remaining traces by addition of ascorbic acid. A standard amount of hydrobromic acid is added and the absorption is measured at 442 mμ.

There is no interference from other alloying elements, as only selenium is precipitated under these conditions and this is volatilised during the removal of free bromine. The range of the method can be altered by selecting a suitable weight of sample, 2 g of sample being required for the range 0·005 to 0·02 per cent.

Nielsch and Böltz have published papers on methods for determining antimony,[1] bismuth,[2] copper,[3] iron[4] and tin[5] by measuring the absorptions of their bromides in concentrated hydrobromic acid. In an attempt to apply their work to the analysis of lead alloys, the absorption spectra, in hydrobromic acid, of the bromides of aluminium, antimony, arsenic, bismuth, cadmium, calcium, copper, iron, lead, magnesium, nickel, selenium, tellurium, tin and zinc were measured.

Tellurium bromide, like the bromides of antimony, bismuth, copper, iron and tin, was found to have a spectrum of analytical interest. The absorptions of the bromides of the remaining nine elements mentioned above were negligible at the concentrations normally found in lead alloys. There was a considerable overlap of the absorption spectra of the bromides of antimony, bismuth, copper, iron, tellurium and tin, and, as the absorption of the other elements could not be suppressed, a separation was required before measurements could be made. Tellurium could easily be precipitated in the elementary form and a method based on this precipitation, followed by re-dissolution of the tellurium in hydrobromic acid, has been developed.

Experimental

Removal of free bromine from solution—

The concentrated hydrobromic acid used, 46 to 48 per cent., contained sufficient free bromine to give a high blank reading, which led to errors when only small amounts of tellurium were present. No reference to a suitable method for removing bromine was encountered in the literature, but, during other work in this laboratory, hydrazine hydrate was found to be effective and was used in some of the preliminary experiments. The reaction, however, was slow, and it was necessary to allow the solutions to stand for at least 1 hour before measurement. At a later stage, ascorbic acid was found to be much superior to hydrazine hydrate for removing bromine, the reaction being instantaneous. The earlier work was repeated with ascorbic acid in place of hydrazine hydrate.

Measurement of absorption spectrum—

The absorption spectrum of a solution of 0·25 mg of tellurium in a mixture of 40 ml of AnalaR hydrobromic acid, 46 to 48 per cent., 5 ml of a 10 per cent. solution of ascorbic acid and 5 ml of distilled water was measured by using a Unicam SP600 spectrophotometer. A blank solution of the reagents was prepared without tellurium and its absorption spectrum was measured in the same way. The absorption spectra are shown in Fig. 1, and all subsequent measurements were made at 442 mμ.

PREPARATION OF STANDARD TELLURIUM SOLUTION—

A stock solution was prepared by dissolving 0·500 g of pure tellurium in hydrobromic acid and diluting to 1 litre with more hydrobromic acid. For use, 10 ml of the stock solution were diluted to 100 ml with hydrobromic acid and aliquots were taken as required.

EFFECT OF HYDROBROMIC ACID CONCENTRATION—

Solutions containing 0·2 mg of tellurium, different amounts of hydrobromic acid and 5 ml of a 10 per cent. solution of ascorbic acid were prepared. The solutions were diluted to 50 ml with distilled water and their optical densities were measured, with the following results—

Hydrobromic acid present, ml ..	40	35	30	25
Optical density	0·421	0·419	0·373	0·132

Although the optical density varies with the hydrobromic acid concentration, this variation can be eliminated when a fixed amount of acid is measured out by pipette for each determination. The fixed amount of concentrated hydrobromic acid chosen was 35 ml.

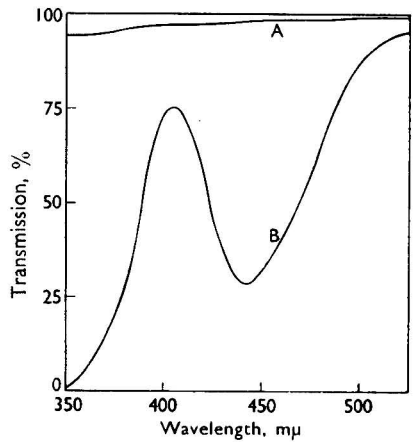

Fig. 1. Absorption spectra: curve A, blank solution; curve B, tellurium bromide in hydrobromic acid

COMPLIANCE WITH BEER'S LAW—

Solutions containing 0 to 0·4 mg of tellurium were measured into 50-ml calibrated flasks and 35 ml of hydrobromic acid and 5 ml of a 10 per cent. solution of ascorbic acid were added. The solutions were diluted to the mark with distilled water and their optical densities were measured at 442 mμ in 4-cm cells. The results are shown in Table I. The figures in the final column represent the optical density per mg of tellurium as measured under these conditions; the almost constant value shows that Beer's law is obeyed.

TABLE I

COMPLIANCE WITH BEER'S LAW

Tellurium taken, mg	Optical density	Optical-density difference	Optical-density difference per mg of tellurium
0·0	0·003	—	—
0·1	0·211	0·208	2·08
0·2	0·411	0·408	2·04
0·3	0·616	0·613	2·04
0·4	0·818	0·815	2·04

CHOICE OF PRECIPITANT—

Sodium hypophosphite was initially used as the precipitant under different conditions, but the recoveries were only about 85 per cent.

Different amounts of tellurium were precipitated from solutions of hydrobromic acid with a 25 per cent. solution of stannous bromide and were allowed to stand for 1 hour to coagulate. The precipitates were collected on No. 4 sintered-glass crucibles and dissolved in hydrobromic acid containing 5 per cent. of bromine. The solutions were evaporated to small bulk and were then diluted to 50 ml by adding 5 ml of a 10 per cent. solution of ascorbic acid, 35 ml of hydrobromic acid and distilled water. Tellurium was determined in each solution by the proposed method, the results being as follows—

Tellurium taken, mg	0·1	0·25	0·3	0·4
Recovery, %	102	103	97	100

Five millilitres of a 25 per cent. solution of stannous bromide were chosen for the precipitation of tellurium.

APPLICATION OF THE METHOD TO SYNTHETIC MIXTURES—

Determinations of tellurium, added as a standard solution, were carried out on 1 g of a lead - antimony alloy containing 12 per cent. of antimony, the results being as follows—

Tellurium added, mg	0·1	0·2	0·3	0·4
Recovery, %	97, 95	96, 92	104, 94	103, 99

The recoveries show that the method is applicable to lead - antimony alloys in the absence of interfering elements.

EFFECT OF OTHER ELEMENTS—

Selenium is the only other element normally present in lead alloys that is precipitated by stannous bromide and so its effect was examined. The effect of arsenic was also investigated in case of possible co-precipitation with tellurium.

Solutions of selenium and arsenic were added to solutions containing 35 ml of hydrobromic acid and 5 ml of a 10 per cent. solution of ascorbic acid and the resulting solutions were diluted to 50 ml. The optical densities of the solutions were measured, the results being as follows—

Arsenic added, mg	0	20	0
Selenium added, mg	0	0	2
Optical density	0·011	0·021	selenium precipitated

Recoveries of tellurium from mixtures of tellurium and selenium were made and it was found that the selenium was volatilised during the evaporation to small bulk without loss of tellurium, provided that dry spots were not allowed to form on the bottom of the beaker. The optimum volume after evaporation was found to be approximately 3 ml.

REPRODUCIBILITY OF RESULTS—

To test the reproducibility of the results by the proposed method, an alloy was prepared having an approximate composition of 85·5 per cent. of lead, 12 per cent. of antimony, 2·5 per cent. of tin and 0·01 per cent. of tellurium, and the tellurium content was separately determined by eight analysts. The results are shown in Table II. It can be seen from Table II that results by the method were reproducible and that the variation between the results obtained by different analysts was negligible.

TABLE II

DETERMINATION OF TELLURIUM BY DIFFERENT ANALYSTS

Analyst No.	Tellurium found, %	Mean, %
1	0·0086, 0·0089, 0·0089, 0·0090	0·0089
2	0·0088, 0·0084, 0·0092, 0·0089	0·0088
3	0·0094, 0·0082, 0·0095, 0·0097	0·0092
4	0·0089, 0·0090, 0·0089, 0·0093	0·0090
5	0·0085, 0·0088, 0·0086, 0·0086	0·0086
6	0·0090, 0·0096, 0·0091	0·0092
7	0·0105, 0·0097, 0·0095, 0·0097	0·0099
8	0·0086, 0·0086, 0·0086, 0·0089	0·0087

METHOD

REAGENTS—

Hydrobromic acid, 46 to 48 per cent. w/v—Analytical-reagent grade.

Bromine - hydrobromic acid mixture—Add 5 ml of analytical-reagent grade bromine to 95 ml of the hydrobromic acid.

Stannous bromide solution, 25 per cent.—Dissolve 10 g of pure tin in 50 ml of the hydrobromic acid, filter if necessary and dilute to 100 ml with distilled water.

Wash solution—Dilute a mixture of 40 ml of the hydrobromic acid and 20 ml of the stannous bromide solution to 200 ml with distilled water.

Ascorbic acid solution, 10 per cent.—Dissolve 10 g of ascorbic acid in distilled water and dilute to 100 ml.

PROCEDURE—

For tellurium contents in the range 0·005 to 0·02 per cent., dissolve 2 g of sample in 20 ml of bromine - hydrobromic acid mixture with gentle heating. (For tellurium contents outside this range, select a suitable weight of sample.) Boil the solution for 5 minutes to remove excess of free bromine and then cool it to about 80° C, add 5 ml of stannous bromide solution, mix well and allow it to stand for 1 hour. Then filter the solution through a No. 4 sintered-glass crucible fitted with a narrow rubber band that does not come into contact with the filtrate. Use the wash solution to transfer and wash the precipitate from the beaker to the sintered-glass crucible and, finally, wash the beaker and precipitate with distilled water. Retain the beaker.

Disconnect the source of suction from the Buchner flask and place a receiver inside it. By means of a pipette, allow 10 ml of bromine - hydrobromic acid mixture to run down the walls of the crucible to dissolve any tellurium not on the sintered disc. After 3 to 4 minutes, re-connect the source of suction and wash the crucible with distilled water, collecting the filtrate and washings in the receiver. Transfer these back to the original beaker and evaporate to about 3 ml. The last part of the evaporation must be done slowly, and on no account must dry spots be allowed to form on the bottom of the beaker.

Allow the beaker to cool and add, by means of a pipette, 35 ml of hydrobromic acid and 5 ml of ascorbic acid solution. Transfer the contents to a 50-ml calibrated flask together with the few millilitres of distilled water used to wash out the beaker. Dilute the solution to the mark with distilled water and measure its optical density at 442 mμ in a 4-cm cell.

Carry out a blank determination on the same amounts of the reagents, but omitting the sample. After subtracting the optical density of the blank solution, read the amount of tellurium present from a calibration curve.

We express our thanks to the Directors of the Chloride Electrical Storage Company Ltd. and to Dr. M. Barak for permission to publish this paper.

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THE CHLORIDE ELECTRICAL STORAGE COMPANY LTD.
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The Determination of Bismuth in Lead and Lead Alloys

BY N. W. FLETCHER AND R. WARDLE

A new spectrophotometric method for the determination of bismuth in lead and lead alloys is described, in which use is made of the absorption of bismuth bromide in hydrobromic acid.

The alloy is dissolved in a mixture of bromine and hydrobromic acid, perchloric acid is added and bromine and volatile bromides are removed by heating. A standard amount of hydrobromic acid and then ascorbic acid, which removes the last traces of free bromine and suppresses the interference caused by copper and iron, are added, and the absorption is measured at 375 mμ.

Tellurium is the only element that interferes and it can be removed by preliminary precipitation with stannous bromide.

The weight of sample is limited to 1 g and the lower limit of the method is 0·001 per cent.

As reported previously,[1] the absorption spectra of a number of elements have been measured in an attempt to apply the work of Nielsch and Böltz[2,3,4,5,6] to the analysis of lead alloys, and a satisfactory method was developed for the determination of tellurium. By applying the principles involved, a method has also been developed for the determination of bismuth in lead alloys.

EXPERIMENTAL

MEASUREMENT OF ABSORPTION SPECTRUM—

The absorption spectrum of a solution of 0·1 mg of bismuth in a mixture of 40 ml of AnalaR hydrobromic acid, 46 to 48 per cent., 5 ml of a 10 per cent. solution of ascorbic acid and 5 ml of distilled water was measured by using a Unicam SP600 spectrophotometer. A blank solution of the reagents was prepared without bismuth and its absorption spectrum was measured in the same way. The absorption spectra are shown in Fig. 1, and it can be seen that the absorption of the bismuth solution was at a maximum at 375 mμ.

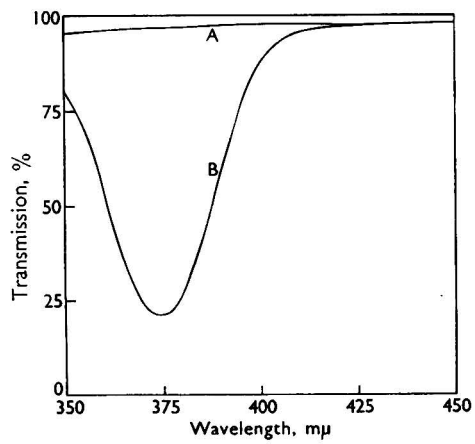

Fig. 1. Absorption spectra: curve A, blank solution; curve B, bismuth bromide in hydrobromic acid

EFFECT OF HYDROBROMIC ACID CONCENTRATION—

Solutions containing 0·1 mg of bismuth, different amounts of hydrobromic acid, water and 5 ml of a 10 per cent. solution of ascorbic acid were prepared. The final volume of each

solution was 50 ml, and optical densities were measured at 375 mμ, the results being as follows—

Hydrobromic acid present, ml ..	40	35	30	25	20	10
Optical density	0·678	0·673	0·670	0·670	0·661	0·648

These results show that the optical density was dependent on the hydrobromic acid concentration, but variations could be avoided by using a fixed amount of acid; 25 ml was chosen, as the interference caused by the bromides of copper and iron decreased rapidly with reduced acid concentration.

REMOVAL OF FREE BROMINE—

The concentrated hydrobromic acid used, 46 to 48 per cent., contained sufficient free bromine to give a high blank reading, which led to errors when only small amounts of bismuth were present.

The addition of ascorbic acid to the solution removed free bromine and also suppressed the interference caused by the bromides of copper and iron.[7]

COMPLIANCE WITH BEER'S LAW—

Solutions were prepared that contained 0 to 0·16 mg of bismuth in a mixture of 25 ml of hydrobromic acid and 5 ml of a 10 per cent. solution of ascorbic acid. The solutions were diluted to 50 ml with distilled water and their optical densities were measured at 375 mμ in 4-cm cells. The results are shown in Table I. The figures in the final column represent the optical density per mg of bismuth as measured under these conditions; the almost constant value shows that Beer's law is obeyed.

TABLE I

COMPLIANCE WITH BEER'S LAW

Bismuth taken, mg	Optical density	Optical-density difference	Optical-density difference per mg of bismuth
0·0	0·004	—	—
0·04	0·266	0·262	6·55
0·08	0·525	0·521	6·51
0·12	0·778	0·774	6·45
0·14	0·910	0·906	6·47
0·16	1·050	1·046	6·54

EFFECT OF PERCHLORIC ACID—

It was known that the bromides of antimony, arsenic and tin in hydrobromic acid absorbed at 375 mμ, and it was proposed to remove these elements by volatilisation as the bromides from perchloric acid.

Various amounts of perchloric acid were added to solutions of 0·1 mg of bismuth in 25 ml of hydrobromic acid. After mixing, 5 ml of a 10 per cent. solution of ascorbic acid were added, the solutions were diluted to 50 ml with water and their optical densities were measured at 375 mμ, the results being as follows—

Perchloric acid added, ml ..	0·0	1·0	2·0	4·0
Optical density	0·655	0·660	0·655	0·657

From these results it can be seen that perchloric acid has little effect on the optical density.

EFFECT OF LEAD AND LEAD ALLOYS—

A large number of determinations of bismuth were made on synthetic solutions and on an alloy prepared from high-purity lead and Specpure antimony. The conditions and amounts of reagents were varied; it was found that the best recoveries were obtained by using the proposed method.

Determinations of bismuth were carried out by the proposed method on 1 g of a lead - antimony - tin alloy containing 12 per cent. of antimony and 2 per cent. of tin, to which 0 to 0·12 mg of bismuth had been added. The results are shown in Table II and it can be seen that lead, antimony and tin did not interfere.

EFFECT OF OTHER ELEMENTS—

No interference was caused by 60 mg of tin, 15 mg of silver, 10 mg of aluminium, 10 mg of calcium, 10 mg of nickel, 4 mg of arsenic, 4 mg of cadmium, 1 mg of selenium, 1 mg of magnesium and 1 mg of zinc when added to 1-g samples of a lead - antimony alloy when the bismuth

in the mixtures was determined by the proposed method. The weights of the elements given are not necessarily the maximum tolerable, but are the maximum amounts likely to be encountered in lead alloys of interest.

TABLE II

RECOVERY OF BISMUTH FROM A LEAD - ANTIMONY - TIN ALLOY

Bismuth added, mg	Optical density	Optical-density difference	Bismuth recovered, mg	Bismuth recovered, %
0·0	0·233	—	—	—
0·04	0·492	0·259	0·040	100
0·083	0·752	0·519	0·080	96
0·117	0·985	0·752	0·116	100

Small amounts of copper, iron and tellurium interfere. Tellurium can be removed by precipitation with stannous bromide before the volatilisation in the presence of perchloric acid. It is essential to use tin free from bismuth and low in copper and iron, *e.g.*, AnalaR or Specpure, for the preparation of stannous bromide.

The completeness of the removal of tellurium was shown when added bismuth was determined on 1 g of a lead - antimony alloy containing 12 per cent. of antimony both with and without the addition of 1 mg of tellurium, the results being 0·0034 and 0·0031 per cent., respectively.

Much work was done to eliminate interference caused by copper and iron by varying the conditions and by the addition of other reagents, but the work was unsuccessful until ascorbic acid was added to the solutions.

Two solutions, each containing 1 mg of selenium, nickel, zinc, copper, iron and magnesium, 4 mg of arsenic and cadmium and 10 mg of calcium and aluminium in an excess of a bromine - hydrobromic acid mixture, were heated to the fuming-point with perchloric acid and then diluted to 50 ml with 25 ml of hydrobromic acid, 5 ml of a 10 per cent. solution of ascorbic acid and water, and their optical densities were measured at 375 mμ, the results being as follows—

Solution No.	.. ..	Blank	1	2
Optical density	.. ..	0·037	0·049	0·048

The addition of 5 ml of a 10 per cent. solution of ascorbic acid eliminated the interference caused by 5 mg of copper and 5 mg of iron present in solution together.

REPRODUCIBILITY OF RESULTS—

An alloy containing 12 per cent. of antimony, 2 per cent. of tin, 86 per cent. of lead and approximately 0·01 per cent. of bismuth was prepared and the bismuth was determined separately by eight analysts. The results are shown in Table III.

TABLE III

DETERMINATION OF BISMUTH BY DIFFERENT ANALYSTS

Analyst No.	Bismuth found, %	Mean, %
1	0·0129, 0·0132, 0·0131, 0·0130	0·0131
2	0·0125, 0·0132, 0·0129, 0·0128	0·0128
3	0·0123, 0·0124, 0·0122, 0·0122	0·0123
4	0·0125, 0·0124, 0·0125	0·0125
5	0·0130, 0·0138, 0·0127, 0·0130	0·0131
6	0·0122, 0·0122, 0·0122, 0·0123	0·0122
7	0·0133, 0·0128, 0·0128, 0·0124	0·0128
8	0·0127, 0·0127, 0·0126, 0·0127	0·0127

It can be seen from Table III that variations between results obtained by different analysts are negligible and that reproducibility is satisfactory.

METHOD

REAGENTS—

Hydrobromic acid, 46 to 48 per cent. w/v—Analytical-reagent grade.

Bromine - hydrobromic acid mixture—Add 5 ml of analytical-reagent grade bromine to 95 ml of the hydrobromic acid.

Perchloric acid, 60 per cent. w/v—Analytical-reagent grade.

Ascorbic acid solution, 10 per cent.—Dissolve 10 g of ascorbic acid in distilled water and dilute to 100 ml.

Stannous bromide solution, 25 per cent.—Dissolve 10 g of analytical-reagent grade tin in 50 ml of the hydrobromic acid and dilute to 100 ml with distilled water.

Wash solution—Dilute a mixture of 40 ml of hydrobromic acid and 10 ml of the stannous bromide solution to 200 ml with distilled water.

PROCEDURE IN THE ABSENCE OF TELLURIUM—

Dissolve 1 g of sample in 20 ml of bromine - hydrobromic acid mixture with gentle heating if necessary. When dissolution is complete, add 10 ml of perchloric acid and boil the solution on a hot-plate until lead bromide begins to crystallise. Remove the beaker from the hot-plate immediately and continue the heating over the flame of a bunsen burner, swirling the beaker vigorously to prevent the contents from bumping and spitting. Continue to heat until the solution is clear and the volume has been reduced to 2 to 3 ml. If a white residue is present in the hot solution, a further 5 ml of hydrobromic acid and 5 ml of perchloric acid should be added and the volatilisation repeated.

Then cool and transfer the solution to a 50-ml calibrated flask containing 25 ml of hydrobromic acid, using distilled water to assist the transfer and to wash the beaker. Add 5 ml of ascorbic acid solution, dilute to the mark with distilled water and adjust the temperature to 20° ± 2° C.

Measure the optical density with a Unicam SP600 spectrophotometer at 375 mμ in a 4-cm cell with the violet filter in position, and the instrument adjusted against water. Carry out a blank determination on the reagents alone in an identical manner.

Read the bismuth content from a calibration curve.

PROCEDURE IN THE PRESENCE OF TELLURIUM—

Dissolve 1 g of sample in 20 ml of bromine - hydrobromic acid mixture and boil to remove most of the bromine. Add 1 ml of stannous bromide solution and set aside for 1 hour, filter and collect the precipitated tellurium in a No. 4 sintered-glass crucible and the filtrate in a suitable receiver. Use not more than 20 ml of the wash solution in small volumes to transfer the precipitate to the sintered-glass crucible and wash the precipitate and beaker. Then wash the precipitate and beaker with distilled water. Transfer the combined filtrate and washings to the original beaker, add 10 ml of perchloric acid and continue from this point as for the procedure in the absence of tellurium.

We express our thanks to the Directors of The Chloride Electrical Storage Company Ltd. and to Dr. M. Barak for permission to publish this paper.

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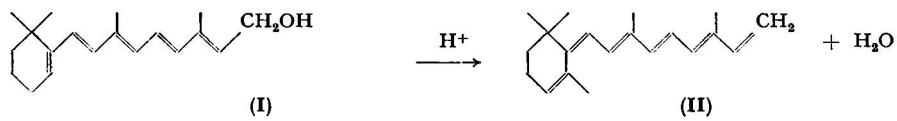
Determination of Vitamin A by Conversion to Anhydrovitamin A*

By P. BUDOWSKI and A. BONDI

Vitamin A may be converted to anhydrovitamin A in benzene solution at room temperature, in the presence of toluene-*p*-sulphonic acid as a catalyst. The increase in extinction at 399 mμ, which results from the dehydration, is proportional to the amount of vitamin A present and can be used for the determination of this vitamin in unsaponifiable extracts. The method exhibits a high degree of specificity and has been applied to a variety of products, including vitamin-A concentrates, fish oils, margarine, butter and poultry mashes, without further purification of the unsaponifiable extracts.

The routine determination of vitamin A by ultra-violet absorption or by the antimony trichloride reaction presents many difficulties with low-potency materials, particularly fortified feeds and foods. Results are often unreliable, in spite of cumbersome chromatographic purification procedures. In the work described here, an attempt has been made to arrive at a simple and accurate assay procedure by making use of a reaction that is more specific for vitamin A and less subject to interference than other non-biological tests. Such a reaction is found in the conversion of vitamin A (I) to anhydrovitamin A (II), which is known to occur in anhydrous solvents in the presence of traces of mineral acids[1,2,3,4,5]—



(I) Vitamin A (II) Anhydrovitamin A

The appearance of the retro structure, together with the lengthening of the double-bond system, results in a considerable displacement of the absorption maximum towards the visible region, an increase in extinction and the formation of a fine structure. By measuring the change in extinction at appropriate wavelengths, it has been found possible, under certain experimental conditions, to determine the vitamin-A content of unsaponifiable extracts from a variety of materials without further purification.

Dehydration procedures involving ethanolic hydrogen chloride have been used occasionally for the detection of vitamin A in biological materials when doubts existed as to its presence.[3,6,7] In one of them an attempt at quantitative measurements has been made.[6] Besides ethanolic hydrogen chloride, the use of chloroformic hydrogen chloride has also been described.[8] For quantitative measurements, however, it was found that greater rapidity and better control of the reaction were achieved by adaptation of a procedure described by Shantz[9] for the preparation of anhydrovitamin A and which involves the use of benzene as a solvent and of toluene-*p*-sulphonic acid as a catalyst.

Experimental

Preliminary tests had shown that the formation of anhydrovitamin A from vitamin A in benzene solution could be carried out at room temperature in the presence of suitable amounts of toluene-*p*-sulphonic acid, provided that the vitamin was in the alcohol form. Routine saponification and extraction procedures[10] were followed for the preparation of the unsaponifiable matter, since no special study of these methods was deemed necessary in connection with the present problem.

Absorption spectra of vitamin A and anhydrovitamin A: difference spectrum—

Fig. 1 shows the absorption spectra in benzene of the unsaponifiable matter from a United States Pharmacopoeia reference standard, before and after application of the dehydration procedure described below. The U.S.P. reference standard consists of gelatin capsules

* Communication from the Agricultural Research Station, Rehovot, 1957 Series, No. 209.

containing 250 mg of a solution of crystalline vitamin-A acetate in cotton-seed oil, at a level of 10,000 i.u. per g.

The spectrum of vitamin A has a maximum at 331 mμ, and anhydrovitamin A exhibits maxima at 358, 377 and 399 mμ, minima at 364 and 389 mμ, and an inflexion from 340 to 345 mμ. It should be noted that the use of benzene as a solvent results in a bathochromic shift of absorption by about 7 mμ in relation to ethanol. The purity of the anhydrovitamin A obtained by dehydration can be judged from the relative extinction values at the three peaks. The ratios E(399)/E(377) and E(358)/E(377) are found to be equal to 0·868 and 0·692, respectively, while the corresponding ratios calculated from Shantz's data[9] for crystalline anhydrovitamin A in ethanol (maxima at 351, 371 and 392 mμ) are 0·870 and 0·690, respectively.

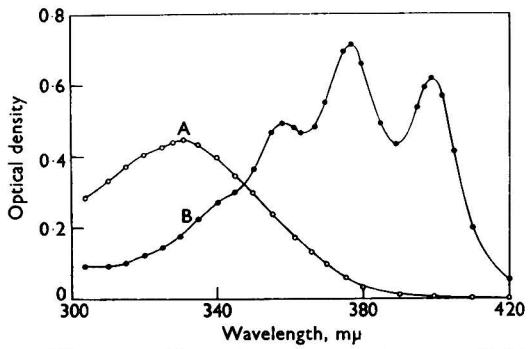


Fig. 1. Absorption spectra of unsaponifiable matter from U.S.P. reference standard: curve A, before dehydration; curve B, after dehydration

Fig. 1 also illustrates the yield from the dehydration reaction, as given by the relative heights of the absorption bands of anhydrovitamin A at 377 mμ and vitamin A at 331 mμ. This ratio is found to be 1·59, while, for crystalline anhydrovitamin A and vitamin A in ethanol, the corresponding ratio of the molecular extinction coefficients is 1·89. This figure is based on extinction coefficients of 1835 for vitamin A[11] and 3860 for anhydrovitamin A.[9] If it is assumed that comparison of such ratios is valid for different solvents, the yield of the dehydration reaction is calculated as 1·59/1·89, or 84 per cent. Robeson and Baxter[12] found a yield of about 60 per cent. for dehydration in ethanolic hydrogen chloride.

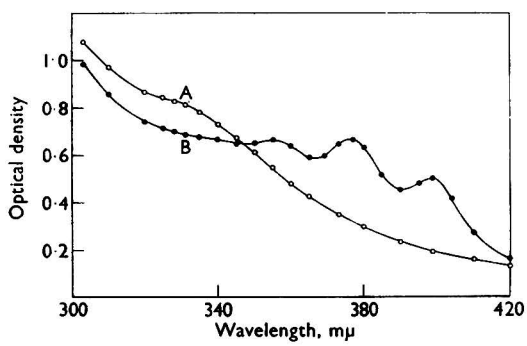


Fig. 2. Absorption spectra of unsaponifiable matter from a poultry mash: curve A, before dehydration; curve B, after dehydration

Fig. 2 shows the absorption curves of the unsaponifiable matter from a fortified poultry mash, before and after dehydration. This is a typical example of a highly impure vitamin-A extract, in which the presence of the vitamin is indicated by a slight hump on the absorption spectrum in the region of the maximum absorption of vitamin A. Similarly, the spectrum of the anhydrovitamin A obtained by dehydration is superimposed upon a strong background

absorption. Absorption curves very similar to those shown in Fig. 2 were obtained for the unsaponifiable matter of other fortified feeds and foods.

Although it is clear that, with such extracts, neither the absorption spectrum of vitamin A nor the curve for anhydrovitamin A can be used for any reasonably accurate determination of vitamin A, because of excessive background absorption, it appears that the "difference spectrum" is relatively independent of irrelevant absorption and can be used for such a purpose. The difference spectrum is obtained by subtracting the optical density of the untreated solution, E_U, from that of the treated (dehydrated) extract, E_T, and plotting the difference, $E_D = E_T - E_U$, against wavelength. It is necessary to point out that such a difference spectrum differs from the absorption spectra of both vitamin A and anhydrovitamin A, and, in fact, corresponds to no specific compound. It should not be confused with the difference spectrum obtained by destructive irradiation[13] or selective adsorption of vitamin A,[14] which is expected to be identical with the absorption spectrum of pure vitamin A.

Fig. 3 shows the difference spectra of the U.S.P. reference standard and the poultry mash referred to on p. 752.

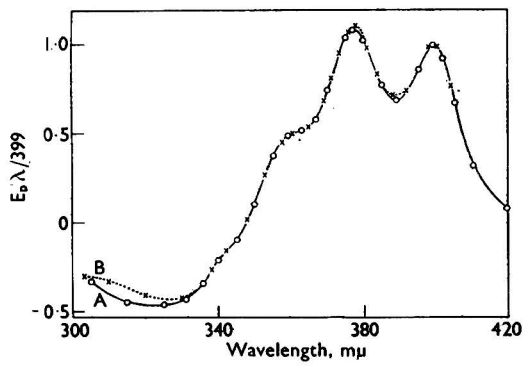


Fig. 3. Difference spectra: curve A, U.S.P. reference standard; curve B, poultry mash

In order to compare the spectra at different concentrations of vitamin A, the curves have been made to coincide at the 399-mμ absorption peak, by using extinction ratios, $E_D\lambda/399$, rather than extinctions, E_D, in accordance with the principle put forth by Oser, Melnick and Pader.[15] It can be seen that, in spite of the very different natures and purities of the unsaponifiable extracts, the difference spectra are nearly identical. Very similar difference spectra have been obtained for a variety of products, including concentrates, fish-liver oils, premixes, poultry mashes, margarine and butter.

TABLE I

EXTINCTION RATIO $(E_D\lambda/399)/(E_D\lambda/377)$ OF UNSAPONIFIABLE FRACTIONS FROM VARIOUS MATERIALS

Designation	Number of samples examined	$\frac{E_D\lambda/399}{E_D\lambda/377}$ Range	Average
U.S.P. reference standard ..	3	0·909 to 0·915	0·913
Fish-liver oils	4	0·903 to 0·912	0·908
Concentrates	2	0·900 to 0·909	0·905
Premixes	4	0·890 to 0·920	0·905
Margarine	2	0·864 to 0·866	0·865
Butter	2	0·815 to 0·825	0·820

Near the visible region, where the absorption due to vitamin A becomes small, the difference spectra resemble the absorption spectrum of anhydrovitamin A: maxima are found at 399 and 377 mμ, and a minimum at 389 mμ. At lower wavelengths, however, the shape becomes different. An inflexion occurs at 360 to 365 mμ, and below 347 and 348 mμ E_D becomes negative, since in this region the curve for anhydrovitamin A lies below the spectrum

of vitamin A. Here, the shape of the difference spectrum becomes more variable, especially for extracts from foods and feeds and for oxidised samples.

The constancy of shape of the difference spectrum in the region of absorption due to anhydrovitamin A is illustrated by the results given in Table I for the extinction ratios $(E_D\lambda/399)/(E_D\lambda/377)$ obtained from different products.

Except for a somewhat lower ratio found for butter, the values are fairly constant and lie within the range 0·86 to 0·92. The constant shape of the difference spectra obtained for such widely different samples constitutes proof of the high degree of specificity of the dehydration reaction. The increase in extinction at 377 or 399 mμ brought about by the dehydration may therefore be expected to correlate with the vitamin-A content of the unsaponifiable extracts.

OPTIMUM CONDITIONS OF DEHYDRATION—

When dehydration is carried out in benzene under the influence of suitable amounts of toluene-*p*-sulphonic acid, maximum formation of anhydrovitamin A occurs almost instantaneously. Further changes, however, result in a subsequent decrease in the amount of this compound. Similar observations have been made when dehydration has occurred in ethanolic hydrogen chloride.[3] It was found convenient to stop the reaction by neutralisation of the catalyst with alkali, whereupon spectrophotometric readings would remain stable for at least 1 hour.

The effect of time of catalysis and concentration of catalyst was tested by using, as before, the unsaponifiable matter from the U.S.P. reference standard and the poultry mash. Some 1-ml portions of a benzene solution of the unsaponifiable matter, which contained 50 i.u. in the case of the U.S.P. reference standard, and 17 i.u. in the case of the poultry mash, were mixed with 4-ml portions of benzene containing different amounts of toluene-*p*-sulphonic acid. The reactions were stopped after various time intervals by thorough shaking with 5 ml of 0·5 *N* sodium hydroxide, and the optical densities of the benzene solutions were read at 399 mμ against the corresponding blanks, which consisted of 1 ml of unsaponifiable extract plus 4 ml of benzene. The results are given in Table II.

TABLE II

EFFECT OF CONCENTRATION OF CATALYST AND TIME OF CATALYSIS ON THE INCREASE IN EXTINCTION, E_D, AT 399 mμ

Designation	Concentration of catalyst in reaction mixture, μg per ml	E_D at 399 mμ, after—		
		0 minute	1 minute	3 minutes
U.S.P. reference standard ..	100	0·565	0·609	0·610
	150	0·585	0·611	0·608
	200	0·602	0·585	—
Poultry mash	100	0·298	0·402	0·399
	150	0·396	0·402	0·395
	200	0·390	0·402	0·374

It is seen that, for all concentrations tested, the readings obtained after a reaction time of 1 minute represent the maximum value, or are very close to it, except for the U.S.P. reference standard, with which the highest concentration tested gave values decreasing from the start. For the analytical procedure, a dehydration time of 1 minute is recommended, together with a concentration of catalyst of 120 μg per ml. Such a concentration results if 1 ml of unsaponifiable extract is mixed with 4 ml of benzene containing 150 μg of toluene-*p*-sulphonic acid per ml. These conditions, however, are not critical, and the dehydration time and concentration of catalyst can be varied by as much as 10 per cent. with negligible effects on the readings.

The effect of temperature was examined by immersing the vessel containing the reaction mixture in a water bath at different temperatures during the 1-minute reaction period. With the U.S.P. reference standard, optical-density readings of 0·610, 0·604, 0·599 and 0·571 were obtained at 18°, 28°, 38° and 48° C, respectively. Small fluctuations in room temperature will therefore have negligible effects on the readings.

The recommended catalyst solution is super-saturated, and crystals will separate at room temperature on standing. Furthermore, toluene-*p*-sulphonic acid tends to form less

soluble hydrates on exposure to moisture. This is accompanied by a loss of catalytic activity. It was found that full catalytic activity could be restored by distilling or removing by boiling 5 to 10 per cent. of the solvent, thereby dissolving separated crystals and simultaneously expelling traces of moisture. Upon cooling, the solution remains in a supersaturated state for 30 minutes or more, depending on the room temperature.

PROPORTIONALITY BETWEEN VITAMIN-A CONTENT AND INCREASE IN EXTINCTION AT 399 mμ—

In order to test the relation between level of vitamin A and increase in extinction at 399 mμ, the dehydration procedure described below was applied to a U.S.P. reference standard and to a poultry mash (containing 6·5 i.u. per g), graded amounts of unsaponifiable matter being used. The results are represented graphically in Fig. 4.

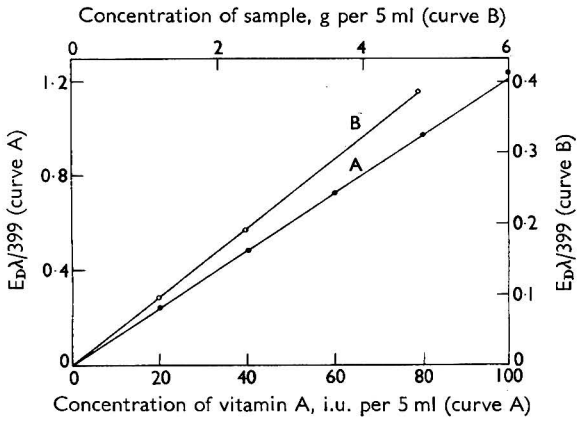


Fig. 4. Relationship between increase in extinction at 399 mμ and concentration of vitamin A: curve A, U.S.P. reference standard; curve B, poultry mash

It is seen that in both cases the readings are proportional to the level of vitamin A. The straight line obtained from the U.S.P. reference standard permits the calculation of the increase in extinction at 399 mμ per i.u. of vitamin A per 5 ml of final solution. The value obtained is 0·0122. This figure can be used for the calculation of the vitamin-A content in unknown extracts in much the same way as the extinction coefficient of vitamin A is used in the ultra-violet absorption method. Indeed, it may be expressed as an extinction coefficient by means of the following equation—

$$(E^{1\%}_{1cm})_D \text{ at } 399\ m\mu = \frac{0{\cdot}0122}{20 \times 0{\cdot}3 \times 10^{-6}} = 2030,$$

where the denominator represents the concentration of vitamin A (1 i.u. per 5 ml) expressed as a percentage.

METHOD

Saponification and extraction of the unsaponifiable matter are carried out by standard procedures.[10] The final extract is made up in dry benzene, so that 1 ml contains between 5 and 50 i.u. of vitamin A.

REAGENT—

Catalyst solution—Heat under reflux 15 mg of toluene-*p*-sulphonic acid monohydrate with 100 ml of redistilled benzene until dissolved. Distil or boil off 10 ml of solvent to drive out moisture, allow the solution to cool, protected from moisture, and re-adjust the volume to 100 ml with dry benzene. Activate before use by repeating the distillation procedure. Glassware used in preparing the catalyst solution should be dry.

PROCEDURE FOR DEHYDRATION—

Mix 1 ml of a benzene solution of unsaponifiable matter with 4 ml of catalyst solution. After 1 minute, neutralise the catalyst by shaking the solution with 5 ml of 0·5 *N* sodium

hydroxide for 1 minute. Alternatively, 1 g of sodium carbonate may be used. Clarify the solution by centrifugation or settling.

Measure the optical density of the clear solution in a Beckman DU spectrophotometer at 399 mμ, against a mixture of 1 volume of unsaponifiable matter in benzene and 4 volumes of benzene, set at 100 per cent. transmission.

Table III

Recovery of vitamin A added to unsaponifiable extracts

Designation	Vitamin A present, i.u. per g	Vitamin A added, i.u. per g	Total vitamin A found, i.u. per g	Vitamin A recovered	
				i.u. per g	%
Fish oils—					
A	24,550	22,660	47,500	22,950	101
B	9160	6600	15,720	6560	99
Foods—					
Margarine ..	19·0	32·3	52·7	33·7	104
Butter A	22·1	32·3	55·9	33·8	105
Butter	14·2	16·7	31·4	17·2	103
Poultry mashes—					
A	6·5	10·5	16·8	10·3	98
B	11·2	28·1	40·1	28·9	103
C	2·9	2·5	5·3	2·4	96
D (unfortified) ..	0·6	8·0	8·7	8·1	101

Table IV

Comparison of results obtained by the antimony trichloride method and dehydration procedure

Designation	Vitamin A found by the antimony trichloride method, i.u. per g	Vitamin A found by the dehydration procedure, i.u. per g
Concentrates—		
A*	1,135,000	1,130,000
B†	663,000	554,000
Fish oils—		
A	26,250	24,550
B	9300	9160
C	8100	7700
Feed premix ..	2400	2350
Foods—		
Margarine A	27·2	19·0
Margarine B	26·0	21·0
Margarine B‡ ..	22·2	—
Chocolate spread ..	20·6	18·5
Butter A	26·1	22·1
Butter B	19·4	14·2
Butter B‡	16·0	—
Poultry mashes—		
A	8·1	6·5
A‡	4·7	—
B	27·2	17·0
B‡	17·6	—
C	20·2	7·0
C‡	7·0	—
D	3·8	2·9
D‡	3·2	—

* Vitamin-A palmitate (obtained from Merck & Co. Inc.) having a potency of 1,000,000 i.u. per g.

† Commercial concentrate, showing an abnormal ultra-violet spectrum.

‡ Chromatographed on a magnesium oxide - Celite mixture (1 + 1), with a light petroleum - acetone mixture (9 + 1) as eluting agent.

CALCULATION—The reading thus obtained represents the increase in optical density, E_D, caused by dehydration, and can be converted to amount of vitamin A in i.u. per aliquot taken for dehydration (1 ml) by the following equation—

$$\text{Amount of vitamin A, i.u.} = \frac{E_D}{0{\cdot}0122},$$

where 0·0122 is the optical density increase corresponding to 1 i.u. of vitamin A. This figure is obtained when the U.S.P. reference standard is treated by the proposed procedure.

APPLICATIONS OF THE METHOD

RECOVERY TESTS—

The results of recovery tests carried out with a number of different samples are shown in Table III.

It should be pointed out that these tests refer to the recovery of vitamin A carried through the dehydration procedure only and do not take into account possible losses caused by saponification and extraction.

The satisfactory results shown in Table III are proof of the absence of significant interference by substances normally present in unsaponifiable extracts.

COMPARISON OF RESULTS BY THE ANTIMONY TRICHLORIDE METHOD WITH THOSE BY THE DEHYDRATION PROCEDURE—

The results obtained by application of the dehydration procedure to a number of samples are compared in Table IV with those given by the Carr - Price method.[10] Aliquots of the same saponified extracts were used in each test.

It is seen that with high-potency materials, such as concentrates, fish oils and premixes, the agreement is generally good. Dehydration values are 0 to 6·5 per cent. lower than Carr - Price values. A large discrepancy was observed for concentrate B. This sample, upon closer inspection, revealed an abnormal absorption spectrum, with a maximum at 330 mμ (in hexane) and marked inflexions at 348 and 368 mμ. After chromatography on alumina, a fraction was obtained that exhibited maxima at 333, 348 and 368 mμ (in hexane), which indicated the probable presence of retrovitamin A or some derivative possessing the same chromophoric structure.[9,16,17,18,19] Retro or rehydrovitamin A, which has only about 8 per cent. of the biological activity of vitamin A,[9] is chromogenic toward antimony trichloride[9] (absorption maximum at 612 mμ), but yields only a small amount of anhydrovitamin A under dehydrating conditions.[20]

Table IV also shows that Carr - Price values obtained from unsaponifiable extracts of foods and feeds are considerably higher than the corresponding dehydration values. Frequently, the antimony trichloride colours are abnormal. This is not surprising, in view of the presence in such extracts of irrelevant chromogenic material, such as sterols, carotenoids, vitamin-A oxidation products and so on. Purification by means of chromatography on magnesium oxide - Celite mixture tends to bring the Carr - Price values closer to the observed dehydration figures. The possibility of applying the dehydration procedure to the unsaponifiable fraction of foods and feeds without further purification gives it a distinct advantage.

APPLICATION OF THE DEHYDRATION PROCEDURE TO AUTOXIDISED VITAMIN-A PALMITATE—

Difficulties are often experienced and abnormal colours observed when the Carr - Price method is applied to products that have suffered losses of potency through atmospheric oxidation. The dehydration procedure was applied to vitamin-A palmitate (obtained from Merck & Co. Inc., having a potency of 1,000,000 i.u. per g, purified by chromatography on alumina), dissolved in liquid paraffin at the level of 12,000 i.u. per g and allowed to undergo autoxidation at 37° C in the dark.

Figs. 5 and 6 show the changes observed in the absorption curve and difference spectrum, respectively, during autoxidation. As in Fig. 3, extinction ratios have been plotted, rather than extinctions.

The absorption spectrum of vitamin-A palmitate, measured in benzene after saponification, is seen to undergo marked changes, which completely obliterate the characteristic peak originally present at 331 mμ. On the other hand, the difference spectrum maintains its characteristic shape, even after 98 per cent. destruction has taken place. Only during the

advanced stages of autoxidation is any appreciable distortion of the difference spectrum observed at wavelengths below 370 mμ.

Table V gives a comparison between results obtained by the antimony trichloride method and the dehydration procedure with vitamin-A palmitate during autoxidation. The two procedures were applied to aliquots of the same unsaponifiable extracts.

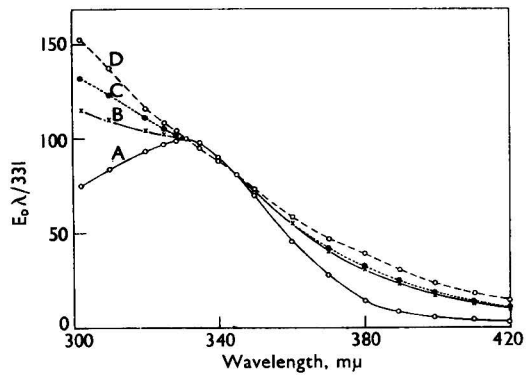


Fig. 5. Changes in the absorption spectrum of vitamin A during autoxidation of vitamin-A palmitate, the extent of oxidation being: curve A, 0·0 per cent.; curve B, 69·1 per cent.; curve C, 92·4 per cent.; curve D, 98·0 per cent.

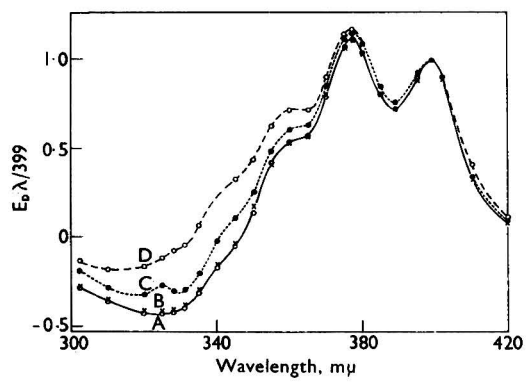


Fig. 6. Changes in the difference spectrum of vitamin-A palmitate during autoxidation, the extent of oxidation being: curve A, 0·0 per cent.; curve B, 69·1 per cent.; curve C, 92·4 per cent.; curve D, 98·0 per cent.

It is seen that the Carr - Price values are consistently higher than the dehydration results. This would be expected from the presence of colour-producing oxidation products. That such substances were actually contributing to the values obtained was seen from the greyish brown colours produced by antimony trichloride with the more highly oxidised samples. With them the readings were quite uncertain, because they increased rapidly from the start. In Table V, the lowest values read, *i.e.*, those read immediately after mixing, have been recorded, and therefore the results obtained with antimony trichloride represent minimum values. It should be noted that saponification does not eliminate the interference caused by oxidation products of vitamin-A palmitate in the Carr - Price test.

TABLE V

COMPARISON OF RESULTS GIVEN BY CARR - PRICE METHOD AND DEHYDRATION PROCEDURE FOR VITAMIN-A PALMITATE UNDERGOING AUTOXIDATION IN PARAFFIN SOLUTION AT 37° C

Time, hours	Vitamin A found by— antimony trichloride method, i.u. per g	Vitamin A found by— dehydration method, i.u. per g	Vitamin A remaining— antimony trichloride method, %	Vitamin A remaining— dehydration method, %
0	12,000	11,500	100	100
23·5	5690	4590	47	40
28·8	4010	3560	33	31
47·5	1250	880	10·4	8·0
71·5	470	226	3·9	2·0

CONCLUSION

The limited results presented here do not permit a rigorous evaluation of the dehydration method. However, a number of observations may be made about its usefulness compared with that of any other non-biological assay procedure.

In the dehydration method, use is made of a chemical reaction, in combination with spectrophotometric absorption measurements. This method may therefore be expected to display features pertaining to both the Carr - Price test and the ultra-violet absorption procedure.

Indeed, dehydration is similar to the development of the blue colour with antimony trichloride, not only in the experimental technique followed, but possibly also in the chemical mechanism involved.[2,3] Further, the products of both reactions are unstable. But, whereas the blue colour must be read rapidly, because of its instability, in the dehydration reaction the product can be stabilised by neutralisation of the catalyst. As a result, the dehydration procedure exhibits the good reproducibility and accuracy of readings (as exemplified by the accurate proportionality between E_D and level of vitamin A) of the ultra-violet absorption method.

The sensitivity of the dehydration method, as measured by the extinction coefficient, $(E^{1\%}_{1\,cm})_D$ at 399 mμ, is slightly superior to that of the ultra-violet absorption method, but inferior to that of the Carr - Price procedure.

Results obtained by the dehydration method are, in general, lower than those obtained by the antimony trichloride procedure, even with materials rich in vitamin A, such as fish oils and concentrates. This should be noted in connection with the higher specificity of the dehydration procedure and the observation[21,22] that other non-biological tests tend to yield higher values than biological assay.

This high degree of specificity, which is evident from the relatively constant shape of the difference spectra obtained from widely different materials, no doubt constitutes the main advantage of the dehydration method. It permits the application of the dehydration procedure to the unsaponifiable fraction of such materials as foods and feeds without further purification. Because chromatography is not needed, time is gained and a potential source of errors is eliminated.

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AGRICULTURAL RESEARCH STATION
REHOVOT, ISRAEL

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o-Dithiols in Analysis

Part V.* The Zinc Complex of Toluene-3:4-dithiol as a Reagent for Arsenic and Germanium

BY ROBERT E. D. CLARK

When the zinc complex of toluene-3:4-dithiol is added to hot acid solutions, toluene-3:4-dithiol is immediately liberated. Oxidising agents, including copper^{II}, uranium^{VI}, and vanadium^{V}, oxidise this to the dithioldisulphide, which separates as a milky suspension. Toluene-3:4-dithiol may also react with cations, and the coloured products formed in strongly acid solution coagulate rapidly. In strongly acid solution only arsenic^{III} and germanium form colourless or nearly colourless complexes with excess of toluene-3:4-dithiol and both form milky suspensions, the turbidities being visible at concentrations of 10^{-5} and 10^{-4} M, respectively. When an excess of the zinc complex of toluene-3:4-dithiol is added to 3 to 4 N hydrochloric acid containing a strong reducing agent such as stannous chloride, oxidising agents are reduced and the formation of a turbidity becomes selective for arsenic^{V} (reduced in the reaction to arsenic^{III}) and germanium. Tungstic acid and some of the less common elements interfere. Used as a test for arsenic, the reaction has the advantages of high sensitivity, simplicity and speed.

WHEN the zinc complex of toluene-3:4-dithiol (zinc dithiol[1]) is sprinkled into hot hydrochloric acid of concentrations up to 7 to 8 N and the solution is boiled, the liquid remains clear and toluene-3:4-dithiol (dithiol), which separates at first as small droplets on the surface, rapidly escapes in the steam. If incompletely removed by boiling, dithiol may separate to give a slightly turbid solution on cooling, but the solution instantly clears on being re-heated.

If cations are present they may be precipitated, but the precipitates usually coagulate almost instantly, the excess of dithiol escapes and a clear supernatant liquid is produced in 20 to 30 seconds or less. When, however, oxidising agents, or cations that can act as such, are present, they give rise to a white suspension of dithioldisulphide, which does not immediately coagulate.

In the presence of an excess of hydroxylamine in 3 to 4 N acid, a white turbidity is instantly produced in the presence of copper, uranium, germanium, vanadium^{V}, arsenic^{III}, arsenic^{V} and antimony^{V}. With osmium^{VIII} a similar turbidity develops rather slowly. Dark or coloured turbidities also develop rather slowly in the presence of molybdenum^{VI}, tungsten, rhenium^{VII}, ruthenium^{III}, rhodium and platinum. The turbidity with arsenic is well marked at concentrations of 10^{-5} M and with germanium at concentrations of 10^{-4} M.

When a hot solution of constant-boiling hydrochloric acid containing, in turn, copper, uranium, germanium, vanadium, arsenic or antimony was treated with zinc dithiol and the solution was extracted with ethylene dichloride, it was found that arsenic and germanium, but not copper, uranium, vanadium or antimony, were removed from the aqueous layer. Copper^{II}, vanadium^{V} and antimony^{V} were reduced to copper^{I}, vanadium^{IV} and antimony^{III},

* For particulars of Part IV of this series, see reference list, p. 763.

but the final valency state of the uranium in the almost colourless solution was not ascertained. Nevertheless, it appears that these four cations function as oxidising agents and produce a suspension of dithioldisulphide.

With arsenic and germanium, colourless or nearly colourless compounds are produced. The arsenic compound is evidently produced from arsenic^{III}, since in dilute acid solutions the turbidity is produced immediately with arsenic^{III}, but only after the lapse of a few seconds with arsenic^{V}. Arsenic^{V} does not appear to react, since in experiments in which dithiol was added gradually to a mixture of arsenate and cations such as tin^{II}, lead and iron^{II} in weakly acid solutions, no interference with the colours given by the cations was observed. Preliminary work has indicated that the formulae of the sparingly soluble compounds formed are $H[As(C_7H_5S_2)_2]$ and, probably, $Ge(C_7H_5S_2)_2$. Arsenic and germanium are the only metallic cations that form stable colourless or nearly colourless complexes with dithiol in strongly acid solution.

Application of zinc dithiol for detecting arsenic and germanium

Arsenic—

When zinc dithiol is added to strongly acid solutions containing arsenic^{III} in moderate or large amounts, the arsenic complex separates as a solid of low melting-point that collects as an almost colourless film on the surface of the hot liquid. Cooling to about 80° C causes it to solidify in the form of particles that are visible in the presence of 10 to 20 μg of arsenic per ml. The addition of 1 drop per ml of a 0·1 per cent. solution of Sudan IV in benzene and then boiling the solution renders these particles readily visible with 4 μg of arsenic per ml. Turbidity in the presence of a reducing agent is readily visible, however, with 0·2 μg per ml and is strongly marked with 0·5 to 1 μg per ml. Since this reaction appears to offer a highly sensitive and almost instantaneous test for arsenic, further work was directed towards improving its selectivity.

Four lines of approach were investigated, *viz.*, (*a*) the use of a reducing agent to reduce the interfering cations to lower valency states in which they will not oxidise dithiol to dithioldisulphide, (*b*) the reduction of dithioldisulphide by the addition of a metal, although leaving the arsenic and germanium complexes unchanged, (*c*) the addition of organic solvents to dissolve the dithioldisulphide and leave the arsenic and germanium complexes undissolved, and (*d*) the removal, by distillation, of arsenious chloride from the acid solution before testing. Although methods (*c*) and (*d*) may be of occasional value, they rarely showed improvement over method (*a*) in detecting arsenic. Method (*b*) was not feasible and (*c*) greatly reduced the sensitivity. Method (*d*) may be of use with solutions containing tungsten or insoluble substances.

Of the reducing agents investigated, thioglycollic acid, conveniently added as its calcium salt, and stannous chloride both keep interference to a minimum, but stannous chloride is preferred. In the absence of uranium^{VI}, antimony^{V}, vanadium^{V}, tungsten^{VI} and some platinum metals, hypophosphorous acid also gives reliable results; if a small amount of precipitated copper is added, antimony^{V} is removed and there is then no interference from any of the more common cations. For all tests the concentration of the hydrochloric acid was kept to about 3 to 4 *N* or less, since in more concentrated acid arsenic ^{V} is rapidly reduced. To avoid reduction to metallic arsenic, it was necessary for the element to be present as arsenic^{V}.

The presence of rarer elements, other than germanium, that may interfere with the test is indicated by the formation of a coloured colloidal solution on adding zinc dithiol. In the presence of a very large excess of arsenic^{V}, however, the white milky suspension first formed may slowly darken, passing first through a red colour, owing to the reduction of arsenic^{V} to the metal by the reducing agent present.

Detection of arsenic in the presence of various cations—The presence of 0·5 to 1 μg of arsenic per ml (as arsenic^{V}) was immediately detected in the following—

(*a*) Solutions of all the more common cations in large excess either singly or in various combinations.

(*b*) A solution prepared by mixing equal volumes of 0·01 *M* solutions of all the more common cations except arsenic, but with a large excess of antimony^{V}.

(*c*) A solution prepared by mixing equal volumes of 0·01 *M* solutions of gold, mercury, cerium, gallium, indium, thallium, uranium^{VI}, vanadium^{V}, selenium^{IV}, tellurium^{IV}, molybdenum^{VI}, tungsten^{VI}, rhenium^{VII} and the six platinum metals.

(*d*) A solution prepared by mixing 0·01 *M* solutions of the six platinum metals.

(*e*) Either singly or in various combinations, 0·01 *M* solutions of each of the cations in (*c*), except tungsten^{VI} and rhenium^{VII}.

(*f*) A 0·01 *M* solution of germanium (as the chloride), after reduction in the volume of solution as described later, p. 763.

Interference was encountered only with germanium, tungsten, rhenium and osmium. Partial evaporation, as described later, removed germanium, osmium^{VIII} and sufficient rhenium for the test to be applied at concentrations of 0·01 *M* and then only tungsten interfered. This interference was due to the slow rate of formation of the green tungsten complex and its only slight ability to coagulate. With up to 50 to 100 μg of tungsten per ml a clear green solution was at first obtained in which the cloudiness caused by arsenic was clearly visible. The molybdenum complex and platinum metals in the colloidal state usually coagulated sufficiently rapidly and did not interfere with the test, but it was occasionally necessary to keep the mixture hot at the final stage for as long as 3 to 5 minutes.

Detection of arsenic in the presence of various anions—The presence of 0·5 to 1 μg of arsenic per ml (as arsenic^{V}) was immediately detected in solutions containing large excesses of borate, bromide, fluoride, iodide, perchlorate, phosphate, sulphate and thiocyanate.

Detection of arsenic in the presence of various organic compounds—The presence of 0·5 to 1 μg of arsenic^{V} per ml was immediately detected in hot saturated or 5 to 15 per cent. solutions in water of many soluble organic compounds. With carbohydrates it was found advisable to use a more dilute acid; about 1·5 *N* proved suitable. In most of the tests, sodium hypophosphite was used as the reducing agent. The test could rarely be applied in the presence of organic solvents, except in very dilute solutions, since these greatly reduced its sensitivity, presumably by increasing the solubility of the arsenic complex: the lowered sensitivity in the presence of a large amount of acetic acid is ascribed to this cause. Similarly, if droplets of solids of low melting-points began to separate from the hot solution, they dissolved the arsenic complex and no turbidity was observed. In no instance, however, was evidence of the prevention of the formation of the arsenic complex encountered.

The compounds tested were acetic acid,* acetamide, acetanilide, acetoxime, acetylsalicylic acid, adipamide, adipic acid, aminomethanesulphonic acid, aniline hydrochloride, benzenesulphonamide, brucine, caffeine citrate, catechol, cinnamic acid, ethylenediaminetetraacetic acid, fructose, galactose, glucose, guanidine carbonate, 8-hydroxyquinoline, maltose, mannitol, *p*-methylaminophenol sulphate (Metol), nicotinamide, *p*-nitrophenol, oxalic acid, oxanilic acid, phenol, phenylacetic acid, phenylurea, piperazine hydrate, resorcinol, quinine, quinol, salicylic acid, sebacamide, sodium sulphacetamide, sorbic acid, strychnine hydrochloride, succinic acid, sucrose, sulphanilic acid, tartaric acid,* thiourea, toluene-*p*-sulphonic acid (calcium salt), trichloroacetic acid,* trimethylamine hydrochloride and urea. The test was less sensitive in rather concentrated solutions of the compounds marked with an asterisk, but the turbidity was instantly visible when the concentration of arsenic was increased to 5 to 10 μg per ml.

Since the test is sensitive, simple and exceedingly rapid, it appears to be of value for detecting the presence of arsenic in a wide range of compounds, both organic and inorganic. Moreover, the comparison of the turbidities with standards would appear to enable approximations of the concentration of arsenic to be measured. For the preparation of synthetic standards, a suitably diluted 0·1 to 0·5 per cent. suspension of bentonite appears to be suitable. It was found that a solution containing 3 to 4 μg of arsenic per ml gave a turbidity approximately equal to that of a 0·1 per cent. suspension of bentonite.

Test for arsenic^{V}—To 1 ml of the test solution add 0·5 ml of concentrated hydrochloric acid and 50 to 150 mg of analytical-reagent grade stannous chloride. Boil gently for 3 to 5 seconds then sprinkle zinc dithiol on the surface of the liquid, shake and boil again for 10 to 20 seconds. A permanent white turbidity in the boiling solution indicates arsenic.

If the tin compound of dithiol is formed, as shown by the formation of a red precipitate or by a yellow solution, the concentration of hydrochloric acid is too low and another 1 to 5 drops should be added.

When germanium, rhenium or osmium is present, add a trace of chlorate to the acidified liquid and evaporate to reduce the bulk to one-third. Add diluted hydrochloric acid (1 + 2) and again reduce the bulk to one-third. Then test as described above.

If uranium, vanadium^{V} and antimony^{V} are known to be absent, 100 to 200 mg of sodium hypophosphite can be used in place of the stannous chloride. The use of a more dilute acid is also possible. The presence of antimony is shown by the transient formation of its oily bright-yellow complex on the surface of the liquid. If antimony^{V}, but not uranium or vanadium^{V}, is present, add 100 to 200 mg of precipitated copper and 100 to 200 mg of sodium hypophosphite and boil for 30 to 40 seconds before adding zinc dithiol.

GERMANIUM—

Germanium is easily detected by removing the vapour with a large teat pipette, a long glass tube fitted with a 25 or 50-ml collapsible rubber ball is suitable, from the surface of the hot test solution acidified with hydrochloric acid. Interference by arsenic is prevented by prior oxidation with chlorate to arsenic^{V} in acid less than 3 to 4 *N*. Any chlorine that distils over is rapidly removed by the presence of a reducing agent.

Detection of germanium—The presence of 7 μg of germanium per ml was readily detected in 2 ml of a solution prepared by mixing equal volumes of 0·01 *M* solutions of gold, cerium, gallium, indium, thallium, uranium, vanadium^{V}, selenium^{IV}, tellurium^{IV}, molybdenum^{VI}, tungsten^{VI}, ruthenium^{III}, rhodium^{II}, rhodium^{IV}, palladium, iridium^{IV}, platinum^{II} and all the more common cations, including silver, and with arsenic^{V} added in large excess. In simple mixtures, not containing arsenic, distillation was often unnecessary and, as with arsenic, sodium hypophosphite could be used in place of stannous chloride. On account of their slow reduction to the elements with resultant formation of colloids, osmium^{VIII} and rhenium^{VII} reduced the sensitivity and interfered with the rapidity of the test for germanium. Nevertheless, germanium was easily detected in 20-fold excesses of 0·005 *M* solutions of rhenium^{VII} and osmium^{VIII}, after the solutions had been kept hot for 4 to 5 minutes in order to coagulate the metallic colloids.

Test for germanium—Adjust the acidity of the test solution to between 2·5 and 3·5 *N* with hydrochloric acid and add just enough potassium chlorate to produce a smell of chlorine. Place the solution in a small flask with some porous pot. Boil gently and remove 50 ml of vapour with the aid of a teat pipette. To the condensate add half its volume of concentrated hydrochloric acid and 50 to 100 mg of analytical-reagent grade stannous chloride. Boil the solution, sprinkle zinc dithiol on the surface and re-heat. A white turbidity indicates germanium.

I express my gratitude to Mr. P. S. Jewell for his encouragement, and to Hopkin and Williams Ltd. for a gift of dithiol.

REFERENCE

1. Clark, R. E. D., *Analyst*, 1957, **82**, 182.

NOTE—Reference 1 is to Part IV of this series.

DEPARTMENT OF SCIENCE AND TECHNOLOGY
CAMBRIDGESHIRE TECHNICAL COLLEGE AND SCHOOL OF ART
CAMBRIDGE

June 6th, 1957

Recommended Methods for the Analysis of Trade Effluents

PREPARED BY THE JOINT A.B.C.M. - S.A.C. COMMITTEE ON METHODS FOR THE ANALYSIS OF TRADE EFFLUENTS

Methods for the Determination of Antimony, Barium Soluble in Dilute Hydrochloric Acid and Cadmium

Antimony

PRINCIPLE OF METHOD—

In this method,[1] after destruction of the organic matter, arsenic is removed as the element after reduction with hypophosphite; antimony is then separated by precipitation as the sulphide, brought into solution by a mixture of hydrobromic acid and bromine, reduced by sulphurous acid and determined by titration with potassium bromate.

RANGE—

For antimony contents up to 50 mg.

REAGENTS—

Hydrobromic acid - bromine solution—Dissolve 10 ml of bromine in 100 ml of hydrobromic acid, sp.gr. 1·48. Warm the mixture slightly to dissolve the bromine completely.

Hydrochloric acid, sp.gr. 1·18.

Ammonium hydroxide, sp.gr. 0·880.

Hydrogen peroxide solution, 6 per cent. v/v (20-volume).

Bromine.

Bromine water, saturated.

Hydrogen sulphide gas.

Sulphurous acid—Distilled water saturated with sulphur dioxide.

Potassium bromate solution, 0·05 N—Dissolve 1·392 g of potassium bromate in distilled water, dilute to 1 litre and standardise with arsenious oxide.

Potassium bromate solution, 0·01 N—Make an appropriate dilution of the 0·05 *N* solution.

Methyl orange indicator solution—A 0·1 per cent. aqueous solution.

PROCEDURE—

Carry out a digestion of a suitable volume of the effluent sample as described under "Destruction of Organic Matter." To the residue in the flask add 20 ml of hydrobromic acid - bromine solution, gently warm the mixture and transfer the solution to a conical flask. Add 10 ml of hydrochloric acid, dilute to 100 ml and boil the solution to remove bromine and until the volume is approximately 75 ml. Cool the solution. If any insoluble matter is present, filter, and wash the filter-paper with distilled water. Collect the filtrate and washings in a 600-ml conical flask and evaporate to a volume of 75 ml. Cool the solution. Add 50 ml of hydrochloric acid; precipitate any arsenic present by means of sodium hypophosphite and filter, as described under "Determination of Arsenic: Hypophosphite Method."

Adjust the volume of the filtrate to about 250 ml, add 50 ml of ammonium hydroxide to neutralise most of the acid present, cool, and precipitate the antimony by passing hydrogen sulphide through the solution for about 15 minutes. Immediately filter off the precipitate of antimony sulphide and wash it thoroughly with distilled water saturated with hydrogen sulphide. Place a beaker under the filter funnel, pierce a small hole in the bottom of the filter-paper and wash the precipitate through with distilled water delivered from the jet of a wash-bottle, using only about 25 to 30 ml of water. Dissolve any trace of precipitate remaining on the filter-paper

by pouring on to it a hot solution consisting of 20 ml of hydrochloric acid, 4 ml of hydrogen peroxide and 10 ml of saturated bromine water. Wash the filter a few times with distilled water and adjust the final volume to about 100 ml.

To the mixture add 10 drops of bromine and heat to boiling to complete solution of the antimony sulphide. Continue boiling the solution for a few minutes to remove most of the bromine, cool, add 150 ml of sulphurous acid and allow the solution to stand for 1 hour. Boil the solution to remove sulphur dioxide. If sulphur dioxide has not been completely removed when the volume of the solution has been reduced to 150 ml, add 100 ml of distilled water and continue boiling.

When the solution is free from sulphur dioxide, add 1 drop of methyl orange indicator solution and titrate the hot solution with potassium bromate solution (0·01 *N* or 0·05 *N*, according to the amount of antimony present).

1 ml of 0·01 *N* potassium bromate solution ≡ 0·000609 g of antimony.

Express the result as milligrams of antimony per litre of sample.

REFERENCE

1. Wilkinson, N. T., *Analyst*, 1953, **78**, 165.

Barium Soluble in Dilute Hydrochloric Acid

PRINCIPLE OF METHOD—

After the masking of any lead or strontium present, barium is precipitated under controlled conditions as the sulphate, which is measured turbidimetrically.

RANGE—

For barium contents up to 1·5 mg.

It is possible to measure barium contents greater than 1·5 mg by this method, but it has been found that different batches of sodium sulphate (although of the same mesh size) give rise to irregularities of the calibration graph in the higher range. If it is desired, therefore, to determine barium above the recommended range, a fresh calibration graph should be made for each batch of sodium sulphate.

APPLICABILITY—

The method is generally applicable. The possible interference of lead and strontium is avoided by the addition of ethylenediaminetetra-acetic acid.

REAGENTS—

Hydrochloric acid, N.

Sodium hydroxide solution, N.

Acid sodium chloride solution—Dissolve 58·5 g of sodium chloride in distilled water, add 200 ml of *N* hydrochloric acid and dilute to 1 litre with distilled water.

EDTA solution.—Dissolve 37·23 g of disodium ethylenediaminetetra-acetate dihydrate (EDTA) in distilled water and dilute to 1 litre.

Glycerol solution—A 40 per cent. v/v solution in distilled water.

Sodium sulphate, anhydrous—Graded, passing a B.S. 18-mesh sieve and retained on a B.S. 30-mesh sieve.

Standard barium solution—Dissolve 0·1778 g of barium chloride, $BaCl_2.2H_2O$, in distilled water and dilute to 1 litre.

1 ml ≡ 100 mg of barium.

Methyl orange indicator solution—A 0·1 per cent. w/v aqueous solution.

PROCEDURE—

(The test and calibration must be carried out at the same temperature, *i.e.*, 20° ± 2° C.)

First determine the amount of acid or alkali (using methyl orange as indicator) required to neutralise a suitable aliquot of the well shaken effluent.

Then measure two portions of the well shaken effluent, containing not more than 1·5 mg of barium, and neutralise each accordingly. To each solution add 1 ml of *N* hydrochloric acid and boil. If necessary, continue boiling until the

volume is reduced to about 35 ml. Filter each solution into a separate 100-ml calibrated flask, washing the filter-paper with two 5-ml portions of distilled water; add 1 ml of *N* sodium hydroxide solution and 5 ml of EDTA solution and then 15 ml of acid sodium chloride solution and 25 ml of glycerol solution, mixing after the addition of each reagent. Dilute each solution to the mark with distilled water and mix well.

Pour one of the solutions into a 150-ml stoppered conical flask, add 0·3 g of anhydrous sodium sulphate, replace the stopper and gently swirl the mixture for 2 minutes. Allow to stand for 5 minutes and then compare the turbidities by measuring the optical densities of both solutions (the untreated solution being used as a blank) absorptiometrically, with neutral filters and using distilled water in the comparison cell. Read the number of milligrams of barium equivalent to the observed optical densities of the test and blank solutions from a previously prepared calibration graph, and so obtain the net measure of barium in the sample.

Establish the calibration graph as follows—

Measure appropriate amounts of standard barium solution, covering the range 0 to 1·5 mg, into a series of 100-ml calibrated flasks. Dilute each to 50 ml with distilled water, add 5 ml of EDTA solution and proceed as described for the test solution. Measure the optical densities and, after correcting the readings for a blank with no added barium, construct a graph relating the optical densities to the number of milligrams of barium.

Express the result as milligrams of barium per litre of sample.

Cadmium

PRINCIPLE OF METHOD—

In this method,[1] after destruction of the organic matter, cadmium is determined colorimetrically as its red complex with dithizone.

RANGE—

For cadmium contents of (*a*) up to 50 μg (instrumental method)
or (*b*) up to 25 μg (visual colour-comparison method).

APPLICABILITY—

The method is generally applicable, but copper and nickel interfere unless complexed with cyanide.

REAGENTS—

Sodium hydroxide solution, 20 per cent. w/v.

Potassium sodium tartrate solution—Dissolve 25 g of potassium sodium tartrate, $C_4H_4O_6KNa.4H_2O$, in 100 ml of distilled water.

Sodium hydroxide - potassium cyanide solution A—Dissolve 40 g of sodium hydroxide and 1·0 g of potassium cyanide in 100 ml of distilled water.

Sodium hydroxide - potassium cyanide solution B—Dissolve 40 g of sodium hydroxide and 0·05 g of potassium cyanide in 100 ml of distilled water.

Hydroxylamine hydrochloride solution—Dissolve 20 g of hydroxylamine hydrochloride in 100 ml of distilled water. Transfer the solution to a separating funnel and extract with a 0·01 per cent. w/v solution of dithizone in carbon tetrachloride, using 5-ml portions until the last extract remains green; then wash the solution free from excess of dithizone by repeated extraction with 10-ml portions of carbon tetrachloride. Transfer the solution to a beaker, warm to remove excess of carbon tetrachloride, cool and filter into a 100-ml flask.

Carbon tetrachloride, redistilled.

Dithizone stock solution—Dissolved 0·1 g of diphenylthiocarbazone (dithizone) in 100 ml of redistilled carbon tetrachloride.

Dithizone extraction solution—Extract 15 ml of the dithizone stock solution with two 50-ml portions of dilute ammonium hydroxide (50 ml of distilled water containing 2 ml of 10 *M* ammonium hydroxide) and reject the extracted carbon tetrachloride layer. Filter the combined ammoniacal extracts if necessary. Acidify the extract with dilute hydrochloric acid (about 1 per cent.) and extract the precipitated dithizone

with 100 ml of carbon tetrachloride. Wash the extract with two 10-ml portions of distilled water and filter it through a dry filter-paper.

Tartaric acid solution—Dissolve 2 g of tartaric acid in 100 ml of distilled water.

Standard cadmium solution—Dissolve 0·100 g of cadmium metal in 50 ml of 10 per cent. nitric acid. Boil the solution to expel oxides of nitrogen and dilute to 1 litre with distilled water. Dilute 10 ml of this solution to 100 ml. This dilute solution should be freshly prepared as required.

$$1 \text{ ml} \equiv 10 \ \mu g \text{ of cadmium.}$$

PROCEDURE—

Measure into a separating funnel a suitable aliquot of the acid solution prepared as described under "Destruction of Organic Matter," and containing not more than 50 μg of cadmium. Neutralise with sodium hydroxide solution and adjust the volume to 25 ml.

Add 1 ml of potassium sodium tartrate solution, 5 ml of sodium hydroxide - potassium cyanide solution A and 1 ml of hydroxylamine hydrochloride solution, mixing after each addition. Add 10 ml of dithizone extraction solution, shake the mixture for 1 minute, allow the layers to separate and run the lower layer into a second separating funnel containing 25 ml of tartaric acid solution.

Repeat the extraction with further 10-ml portions of dithizone extraction solution, adding the lower layer to the tartaric acid solution contained in the second separating funnel, until no pink colour appears in the last extract.

Shake the combined extracts with the tartaric acid solution in the second separating funnel for 2 minutes. Allow to separate and discard the lower layer. Add 5 ml of carbon tetrachloride, shake for 1 minute, allow to separate and discard the lower layer.

Add 0·25 ml of hydroxylamine hydrochloride solution, 10 ml of dithizone extraction solution followed by 5 ml of sodium hydroxide - potassium cyanide solution B, and shake the mixture for 1 minute. Allow the layers to separate and filter the carbon tetrachloride layer through a toughened filter-paper (Whatman No. 541 is suitable) into a 50-ml calibrated flask. Continue to extract the aqueous layer remaining in the funnel with further 10-ml portions of dithizone extraction solution until no pink colour appears in the final extract, filtering each successive extract through the filter-paper into the calibrated flask. Dilute the bulked extract to the mark with carbon tetrachloride and mix.

Carry out a blank procedure on all reagents used.

Measure the optical densities of the test and blank solutions in a spectrophotometer or in an absorptiometer, using a 4-cm or a 1-cm cell according to the depth of colour, and using a wavelength of 5250 A in a spectrophotometer or a suitable green filter in an absorptiometer. Use carbon tetrachloride in the comparison cell. Read the number of micrograms of cadmium equivalent to the observed optical densities of the test and blank solutions from a previously prepared calibration graph, and so obtain the net measure of cadmium in the sample.

Establish the calibration graph as follows—

> Measure appropriate amounts of standard cadmium solution covering the range 0 to 50 μg into a series of separating funnels. Neutralise as necessary with 20 per cent. sodium hydroxide solution and adjust the volume to 25 ml. Proceed as described above, beginning at "Add 1 ml of potassium sodium tartrate solution . . .," in the second paragraph. Measure the optical densities and construct a graph relating the optical densities to the number of micrograms of cadmium.

If an instrument is not available, colours may be matched visually in 50-ml Nessler cylinders, a series of standards covering the range 0 to 25 μg of cadmium being used.

Express the result as milligrams of cadmium per litre of sample.

REFERENCE

1. Saltzmann, B. E., *Anal. Chem.*, 1953, **25**, 493.

Notes

A QUALITATIVE TEST FOR NITROGLYCERINE

BOTH in the explosives factory and in the explosives laboratory it is frequently necessary to test for the presence of nitroglycerine, and to be able to distinguish it (which the indeterminate tests previously proposed largely fail to do) from such substances as nitrocellulose, nitroglycol, nitrates, glycerol, phthalic acid esters, lubricating oils and, in ethereal extracts, from carbamite, diphenylamine and other constituents of explosives.

It has been observed that, whereas in aqueous media nitroglycerine (and nitroglycol) reacts slowly with Nessler's reagent to produce a reddish brown precipitate similar to that produced by ammonia (thereby offering apparent confirmation of the statement[1] that ammonia may figure among the hydrolysis products of nitroglycerine), in alcoholic solution nitroglycerine (but not nitroglycol) produces a black precipitate of metallic mercury. Further investigation leads to the conclusion that both in aqueous and alcoholic media, the active reducing agent is aldehyde formed during the reaction, this at great dilution reacting with Nessler's reagent in a manner closely resembling ammonia and at higher concentrations precipitating mercury therefrom. In fact, the presence of aldehyde in a reaction mixture of nitroglycerine and cold 0·5 *N* alcoholic potassium hydroxide may be shown by means of 2:4-dinitrophenylhydrazine.

The reaction described above has been developed in this laboratory as a test for nitroglycerine, which consists in adding freshly prepared Nessler's solution[2] to an equal volume of an ethanolic solution of extract of the sample under test. Provided that the concentration of nitroglycerine in the ethanol is not less than 1 per cent., a black precipitate is rapidly produced; as mentioned below, other nitro compounds that may be present produce colours or coloured precipitates, which can be ignored. With a 0·1 per cent. solution of nitroglycerine a yellow colour forms and it slowly becomes brown; a minimum concentration of 1 per cent. is necessary for production of a black precipitate.

In applying the test to propellant compositions, it is generally desirable to carry out a preliminary partial separation of the constituents of the compositions so as to minimise interference by nitrocellulose (reddish brown precipitate), nitroguanidine (a white precipitate, which diminishes the strength of the reagent) and dinitrotoluene (a dark reddish brown colour). The ingredients that may be expected to contribute to the result of the test occur in propellent compositions in the following approximate proportions: nitroglycerine, 20 to 40 per cent.; dinitrotoluene, 10 per cent.; 2-nitrodiphenylamine, 2 per cent.; nitroguanidine, 50 per cent.; and rough extraction of the finely ground propellant (say, 2 to 5 g) with 80 per cent. v/v aqueous ethanol (5 to 10 ml) produces sufficient segregation of the nitroglycerine to render the test specific. If no insoluble interfering substances are expected, as, for example, when the test is used in the explosives plant, it is best to use pure ethanol in the test.

Methanol may be used in place of ethanol, but it generally produces a slight reaction itself, presumably owing to the presence of aldehyde (see below). The test reaction proceeds also in *iso*-propanol and, slowly, in ethylene glycol, but does not occur in acetone (brown coloration) or dioxan (slight darkening).

Nessler's reagent alone produces little colour when treated with an equal volume of glycol, but a reddish colour develops in the cold with glycerol.

The colours produced by other ingredients of propellants with ethanol and Nessler's reagent are as follows—

Substance	Result
Dinitrotoluene	Deep reddish brown colour
2-Nitrodiphenylamine	Orange opaque solution
Methyl centralite (dimethyldiphenylurea)	White opaque solution
Carbamite (diethyldiphenylurea)	White opaque solution
Nitroguanidine	White opaque solution

Chemical reactions occur with dinitrotoluene (owing to the alkali present) and nitroguanidine, but in other instances the observed result is due to partial precipitation of the substance itself.

Some supplies of ethanol may give a yellow precipitate with Nessler's reagent, presumably owing to aldehydic impurity. This impurity can be removed by boiling the ethanol vigorously to about one-half its bulk, the vapours being allowed to escape freely.

REFERENCES

1. Davis, T. L., "The Chemistry of Powder and Explosives," J. Wiley & Sons Inc., New York, 1943, Volume II, p. 208.
2. Vogel, A. I., "Quantitative Inorganic Analysis," Second Edition, Longmans, Green & Co. Ltd., London, 1951, p. 643.

CHEMICAL INSPECTORATE
STATION APPROACH BUILDINGS
KIDBROOKE, LONDON, S.E.3

J. LAMOND
April 1st, 1957

THE DETERMINATION OF CARBON IN TITANIUM AND ZIRCONIUM

PUBLICATIONS are made periodically on the use of various fluxes and conditions of oxidation to be used in the determination of carbon in titanium and its alloys. This determination has been extensively examined in these laboratories, and it has been shown that oxidation of the prepared sample can be easily controlled, provided that the combustion tube is first filled with argon. With this essential difference, the determination of carbon follows conventional lines and the evolved carbon dioxide may be determined either gravimetrically or conductimetrically.

In the modified gravimetric determination, a large sample weight can be examined, which would otherwise present a problem during oxidation.

The proposed modification has been successfully applied to the examination of samples of titanium and its alloys in the presence of a lead flux.

In the examination of zirconium and its alloys, the proposed modification can be satisfactorily applied without the use of a flux.

ANALYTICAL SECTION
RESEARCH DEPARTMENT
IMPERIAL CHEMICAL INDUSTRIES LIMITED
METALS DIVISION
KYNOCH WORKS, WITTON, BIRMINGHAM

W. T. ELWELL
D. F. WOOD
July 3rd, 1957

Apparatus

A DIRECT-READING FLUORIMETER

(Presented at the meeting of the Society on Wednesday, April 3rd, 1957)

FLUORIMETRIC methods of assay can be applied to an extensive range of materials, but they have never been used as widely as they might be. In fact, during the last decade there has been a tendency to a reduction in their use. For example, the 1949 British Pharmaceutical Codex recommended a fluorimetric assay for riboflavin in Capsules of Vitamins, B.P.C., but the 1954 B.P.C. dropped the method in favour of a microbiological assay. Likewise, in 1948 the British Pharmacopoeia gave a fluorimetric method for the determination of thiamine (aneurin) in tablets, but in 1953 preferred a gravimetric method. One reason for these changes was that no commercial instrument capable of measuring accurately the fluorescent intensity of very dilute solutions was available.

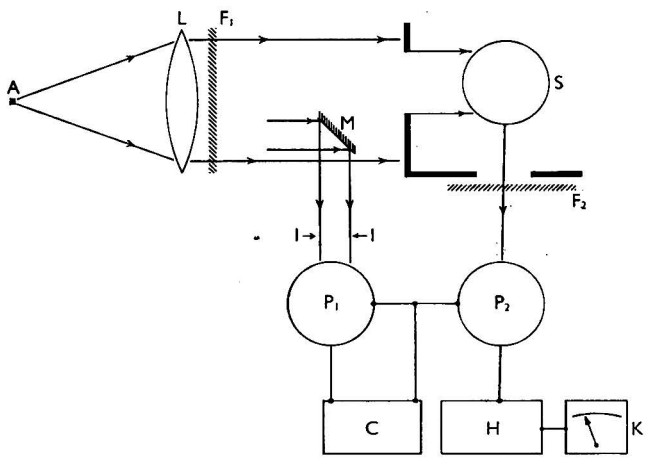
Analytical methods based upon fluorescence measurements possess the advantages of simplicity, great speed and high sensitivity, and in routine laboratories dealing with large numbers of samples these are very desirable features. Consequently some people have built their own apparatus capable of measuring accurately very feeble fluorescence.[1,2,3,4,5,6] Most of these instruments have been of the null-point type, some have been adaptations of commercial equipment, and one British manufacturer now offers a photomultiplier accessory to increase the sensitivity of his instrument.

Four years ago we had the problem of assaying upwards of 120 samples per day by a fluorimetric method. It was necessary to work at dilutions such that our commercial instrument was not capable of giving very accurate readings because of low sensitivity and for each sample a blank reading was required. It was considered at first that we could make our existing instrument sufficiently sensitive by replacing the barrier-layer photocell on the reading side with a photomultiplier, as Wokes had done previously,[2] but it became obvious that we could not hope to deal with all our samples on one such instrument, mainly because of the time taken in filling and cleaning the cuvettes. The use of a run-through cuvette was considered, but was found impossible to fit into our instrument. We therefore began to develop a fluorimeter with the following aims in mind—

(*a*) it must be highly sensitive,

(*b*) it must be very simple to use,
(*c*) instrument readings should be directly proportional to fluorescent intensity, so that a minimum of mathematical treatment is required,
(*d*) it must be of such a design as to permit the fitting of a run-through cuvette when large numbers of sample have to be assayed,
(*e*) it should have good long-term and short-term stability, so that when samples are being measured over considerable periods of time it should not be necessary to re-calibrate the equipment too frequently, and
(*f*) it should be mains operated and highly robust.

It was obvious that the ideal instrument for our purpose would be direct reading and so designed that, after being initially set up and calibrated, samples could be run through one after the other and a meter reading noted. This would at first sight seem to be a simple problem, the logical approach being a stabilised illuminating lamp together with a highly stable and sensitive measuring device. The latter is quite feasible when based upon the use of a photomultiplier, but, like others who have tried, we found it impossible to stabilise completely the mercury-vapour arc lamp used for illuminating the sample. Even when the applied voltage was kept constant, variations in the temperature of the lamp and the wandering of the arc around the electrodes resulted in variations in the intensity of the light. A different approach to the problem was therefore made, and the instrument that was finally developed is shown diagrammatically in Fig. 1.



A = 125-watt high-pressure mercury-vapour arc lamp
C = Control unit
F_1 and F_2 = Filters
H = Amplifier
I = Variable aperture
K = Meter
L = Lens
M = Mirror
P_1 and P_2 = Photomultiplier tubes
S = Cuvette

Fig. 1. Diagrammatic layout of fluorimeter

Light from the lamp, A, a 125-watt high-pressure mercury-arc type MB/D, is made reasonably parallel by the lens, L, and passes through the primary filter, F_1. The sample, S, is illuminated by this light and the fluorescent output, viewed at right angles to the incident light, passes through the secondary filter, F_2, and falls upon the photomultiplier, P_2. The output from P_2 is amplified and read directly on the meter, K.

The output from a photomultiplier rises sharply with an increase in applied dynode voltage, so that in most applications this applied voltage is highly stabilised. This feature can, however, be utilised in a different manner.

A small portion of the light transmitted by the primary filter, F_1, is reflected by a mirror, M, through the variable aperture, I, on to a second photomultiplier, P_1. The output from this is

fed into a controlling unit, which supplies the voltage applied across both photomultipliers. This voltage is made to fluctuate in such a way that the output of P_1 is kept constant, so that, if the intensity of the lamp increases, the applied voltage decreases and *vice versa*.

As both P_1 and P_2 are supplied from the same voltage supply, and as they have similar characteristics, the output from P_2 will also be independent of fluctuations in the intensity of the lamp and will be a function only of the concentration of the sample. The results on p. 773 show how the final sample reading remains constant when the voltage supply to the whole equipment, including the lamp, varies from 190 to 250 volts. The variation in the applied voltage to the photomultipliers from the controlling unit is also shown.

This arrangement is of course completely effective only if the characteristics of the two photomultipliers are the same, and this would imply a necessity to provide matched pairs. In practice, it has been found that perfect matching under all conditions is not essential, so long as the optimum conditions for using any particular pair are found. The mean voltage applied to the photomultipliers from the controlling unit can be changed by adjusting the amount of light falling on P_1 with the variable aperture, I. With any fluorescing sample in the instrument, the mains voltage is varied with a Variac autotransformer and aperture I is adjusted until a swing of 190 to 250 volts results in the least possible change in the meter reading. The instrument is then ready for use.

THE AMPLIFIER—

The output current from the photomultiplier monitoring the sample, P_2, is fed through one of a series of load resistors and the voltage developed is applied to the input grid of a conventional balanced cathode-follower degenerative circuit. The sensitivity is controlled by means of a variable shunt across the meter and the parameters of this shunt are so arranged that full-scale deflection can be obtained for input voltages between 0·3 and 1·2 volts. The amplifier is linear within this range. This gives a ratio of 4 to 1 on the sensitivity control and the range load resistors are selected to the ratio 0 : 1 : 3 : 10 : 30 : 100 : 300 : 1000. The zero range is included so that the amplifier zero can be adjusted before any readings are taken. Facility is provided for backing-off any signal at the input so that the instrument may be used for differential measurements. For example, if a solution containing about 0·5 p.p.m. of a material were being assayed, then the instrument could be made to read zero with a standard containing, say, 0·45 p.p.m. and to full scale with a standard containing 0·55 p.p.m.; this gives an effective scale 10 times longer than if the more conventional technique of setting up to zero with solvent and to full scale with a standard containing 1·0 p.p.m. were used.

The wide over-all ratio of input resistors results in a very large sensitivity range and, as the ratios are chosen to be accurate to within 1 per cent., it is possible to change the range for readings within a group of samples. If, therefore, the instrument has been standardised and a sample gives a reading that is over the top of the scale, then instead of preparing another sample or diluting that in the instrument, one may switch directly to a lower range and multiply the reading by the appropriate factor.

If the solvent being used has a small fluorescence, or if the optics or filters fluoresce or give rise to light scattering, the unwanted constant blank can be backed-off so that a zero reading is obtained. When this has been done, any further adjustment of the range switch or sensitivity control will not effect this zero reading. In fact, the backing-off is usually performed with the range switch at the most sensitive position so that it is done with greatest accuracy.

THE CUVETTES—

The cuvette, together with its associated optics, is housed in the instrument in a substantial brass block large enough to permit the inclusion of water circulation for thermostatic control if required. This whole block can be withdrawn and changed very easily, so that different types of cuvette can be used. An exploded diagram of the arrangement is shown in Fig. 2. For the sake of compactness it is made so that the incident and fluorescent light are on the same optical axis. After passing through the primary filter, a small portion of the incident light is reflected by mirror M through aperture G and eventually reaches photomultiplier P_1 (see Fig. 1). The remainder of the light falls on plate X, and that portion passed by apertures D_1 and D_2 is reflected by mirrors B_1 and B_2 to illuminate the sample in the cuvette, S. The fluorescent radiation passes through aperture E. The run-through cuvette is very simple and is made from thin-walled glass tubing of about $\frac{1}{2}$ inch outside diameter and $1\frac{1}{2}$ inches long. The central 1 inch is illuminated and to cut down scattering the ends and connecting tubing are painted black. The filling and

emptying are done by means of a vacuum—conveniently a water pump is used so that the discarded sample is disposed of immediately. The arrangement is shown in Fig. 3; with the tap in the FILL position, the sample from the funnel is connected to the bottom of the cuvette and the vacuum line to the top. In the EMPTY position this is reversed so that complete emptying is effected. The tap is made of Perspex with a polytetrafluoroethylene core; these materials would not be suitable for some samples, but the combination does have the advantage that no lubrication is required.

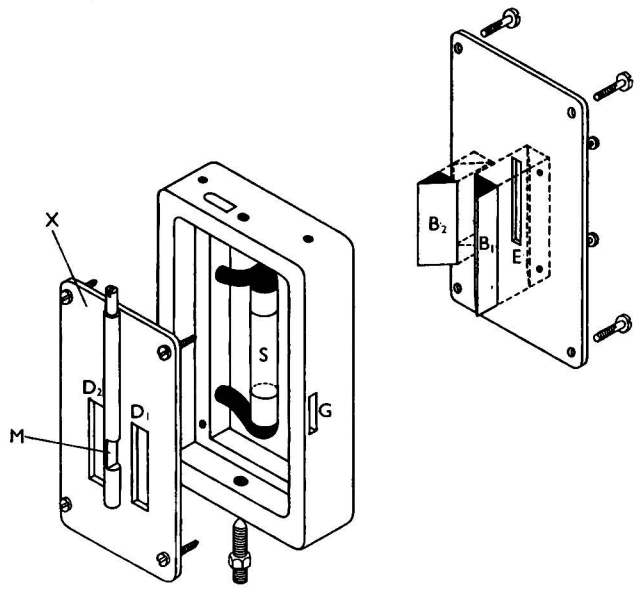


Fig. 2. The cuvette housing, with a run-through cuvette fitted

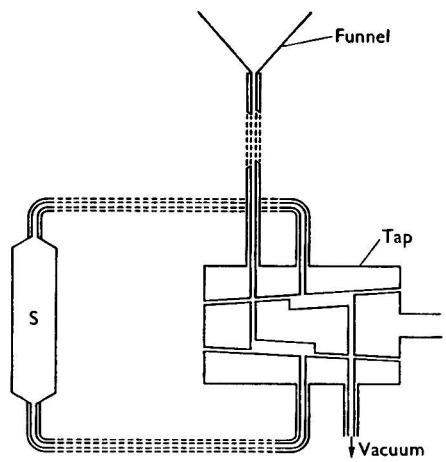


Fig. 3. Filling arrangement for cuvette, with tap in FILL position

The run-through cuvette and associated system requires only 20 to 25 ml of solution for effective washing and filling, but, if the sample is limited, it is possible to fit an alternative block to accommodate a replaceable tube requiring only about 4 ml of solution.

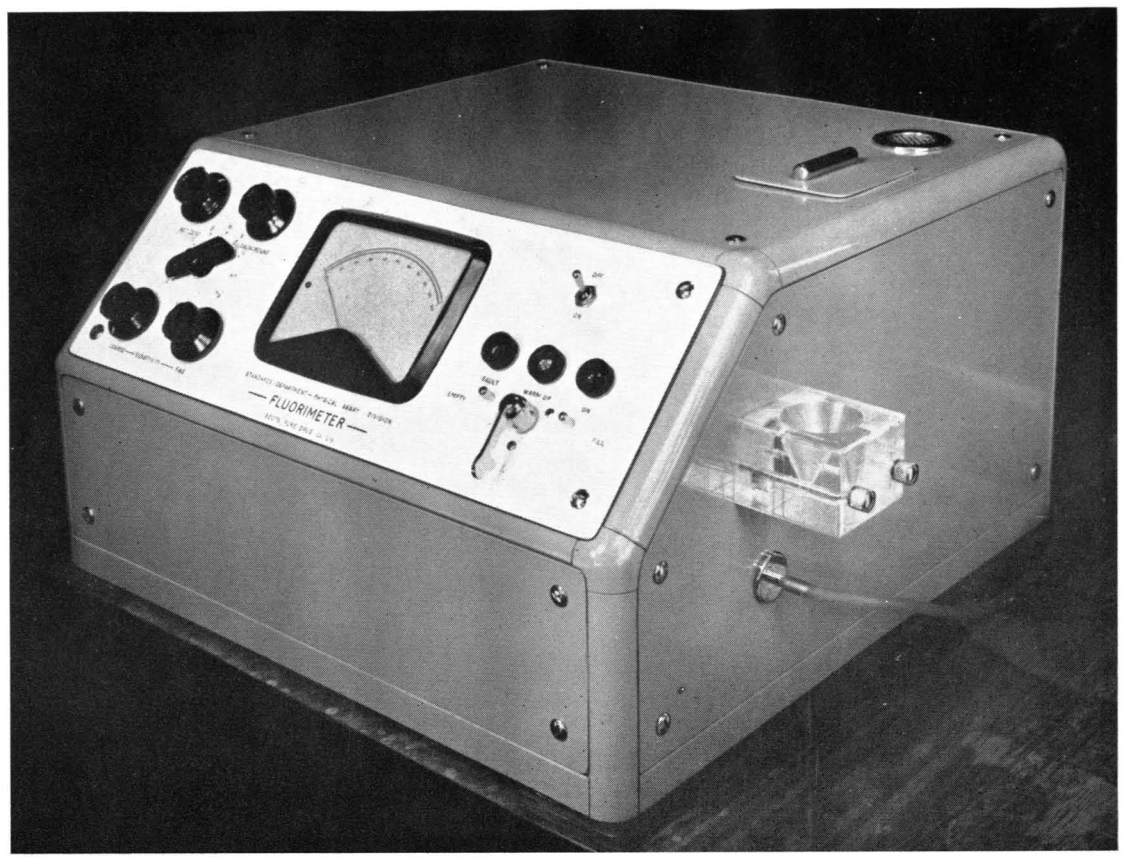


Fig. 4. The complete fluorimeter

[To face p. 772

USE OF THE INSTRUMENT—

The complete instrument is shown in Fig. 4. It is so arranged that the electronic circuits switch on automatically when the lamp has reached full brilliance and off if the lamp fails. This automatic switching is indicated by lamps on the front panel.

After a suitable warm-up period has been allowed, the RANGE switch is turned to the 0 position and the meter is made to read zero by means of the SET ZERO control. The cuvette is filled with water or solvent and, with the RANGE switch turned to the most sensitive position, the meter is again made to read zero by means of the BACKGROUND control. A suitable standard is then put into the cuvette and the RANGE switch and SENSITIVITY control are adjusted to give a suitable reading.

Samples can then be run through the equipment in succession and the meter readings noted. The method used for preparing standard curves is in accordance with well known fluorimetric techniques.

When very dilute solutions are used, photo-decomposition sometimes occurs, with a resulting decay of fluorescence. This decomposition is readily observed on the direct-reading instrument and it is possible either to take the readings very soon after filling the cuvette or to keep the sample running slowly through at such a rate that decomposition is negligible.

PERFORMANCE—

Both short-term and long-term stability are very good. The following results show how little effect mains fluctuation will have and how the voltage applied to the photomultipliers is automatically adjusted to ensure constant output—

Mains voltage	190	200	210	220	230	240	250
Fluorescent intensity as read	50·0	50·0	50·0	50·0	50·0	50·2	50·3
Voltage applied automatically to photomultipliers	580	570	560	550	540	535	530

The results in Table I give some idea of the long-term stability. When large numbers of samples are being examined, it is not necessary to check with standards very often during the day. It is usual to put through a quinine sulphate solution at intervals to see that no drift has occurred, but it is very seldom that adjustments are necessary.

The sensitivity is extremely high and, in fact, is limited only by the efficiency of the filter arrangement and fluorescence or scattering of filters or optics. The loads on the amplifier in our instrument have been so chosen that it is easily possible to obtain full-scale deflection with 0·01 μg of riboflavin per ml after the zero has been set with the buffer solution used for the solution, and with a combined OY13 + OGr1 as secondary filter the blank fluorescence is equivalent to 0·0004 μg of riboflavin per ml. This blank is very stable and 0·0002 μg of riboflavin per ml is easily detected.

TABLE I

LONG-TERM STABILITY OF THE FLUORIMETER

Time, hours	Zero reading	Reading for water	Reading for a solution of 0·1 μg of quinine sulphate per ml
0·0	0	0	50·0
0·5	0	0	50·5
2·0	−1·0	−1·0	50·0
2·5	0	0	50·5
3·0	0	0	50·5

Two of these instruments have now been in daily use for 1½ years and one for 3 years, and they have proved very reliable for routine use.

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3. Alford, W. C., and Daniel, J. H., *Anal. Chem.*, 1951, **23**, 1130.
4. Fletcher, Mary H., and Warner, E. R., *U.S. Geol. Survey Circ. No.* 311.
5. Field, W. E., and Drage, O., *Photoelect. Spectr. Gr. Bull.*, 1953, No. **6**, 128.
6. Dowdall, J. P., and Stretch, H., *Analyst*, 1954, **79**, 651.

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AMENDMENT SLIPS*

PRINTED slips bearing amendments to British Standards have been issued by the Institution, as follows—

PD 2872—Amendment No. 2 (August, 1957) to B.S. 700:1952. Graduated Pipettes and One-mark Cylindrical Pipettes.

PD 2887—Amendment No. 6 (September, 1957) to B.S. 1425:1954. Cleanliness of Fillings and Stuffings for Bedding, Upholstery, Toys and Other Domestic Articles.

PD 2896—Amendment No. 1 (October, 1957) to B.S. 734:1955. Density Hydrometers for Use in Milk.

Book Reviews

COLORIMETRIC ANALYSIS. Volume I. By NOEL L. ALLPORT, F.R.I.C., and J. W. KEYSER, M.Sc., Ph.D., A.R.I.C. Second Edition. Pp. xii + 424. London: Chapman & Hall Ltd. 1957. Price 50s.

The first edition of this book was published in one volume, but, owing to the rapid increase in the publication of analytical methods and consequently in the use of colorimetric methods, it has been found inconvenient to compress even the critically chosen selection into the limits of one volume.

Since colorimetric methods have been particularly useful in clinical and biochemical analysis, the authors decided to devote Volume I to this field, and they have had no difficulty in finding enough material for its 424 pages. In fact, when reading through the text, one is struck by the lengths to which analysts in this field will go in order to complete an assay by colorimetric measurement.

As it is hoped that Volume II will describe colorimetric methods applicable to metals, foods, pharmaceutical products and so on, the work cannot be considered a treatise on colorimetry and, from the authors' known field of activity, this would not be expected. However, they have used their wide knowledge and experience to select and criticise the methods given, and it is this that makes the volume under review so valuable.

The subject matter is alphabetically arranged in monographs for the particular substance being determined. For each there is an introduction, a method, its application and a discussion; each monograph is fully referenced. The authors have introduced supplementary information, such as preparation of reagents, alternative procedures, modifications for easy manipulation and so on, by means of footnotes, but, since these occur on nearly every page and are often lengthy, they can be a source of irritation to a busy analyst.

In order that the volume might be kept within convenient proportions no attempt has been made to describe every known colorimetric test even within the limited orbit of clinical and biochemical analysis. However, in the hope of extending the usefulness of the work, determinations less frequently required in general use have been enumerated in a table wherein the principles of the procedures employed are outlined and the references to the original literature are cited.

The approach has been critical throughout and, to quote the authors, as far as possible the limits of the methods have been indicated in the hope that users of the book may be spared the annoyance and waste of time occasioned by attempts to conduct colorimetric determinations under conditions to which they are not applicable.

Since 1945, when this book was first published, there has been a radical change in the concept of colour measurement, and instrumentation has both widened the field of application and improved the accuracy of measurement, so that many of the earlier colorimetric methods could well be re-assessed critically against other techniques now made possible by instrumentation. Sodium and potassium can now be determined so easily by flame photometry that the authors might have had the courage to delete methods based on chemical precipitation.

Frequently the authors only briefly point out the advantages of modifications introduced by later workers; the book would be even more useful if the modifications were inserted in the text of the method to be finally recommended. The graphic formulae on pages 107 and 116 are inconsistent with the style of the book and quite unnecessary. However, these are minor defects, which do not belittle the general excellence of the book.

* Obtainable from the British Standards Institution, Sales Department, 2 Park Street, London, W.1.

The book is quite up-to-date and, as far as can be judged from a cursory examination, remarkably free from errors; the type is very clear; the book is robust and stays open on the bench. Both the authors and the publishers may be congratulated on the production of a very useful book for the practising analyst. D. C. GARRATT

VAPOUR PHASE CHROMATOGRAPHY. Edited by D. H. DESTY. Pp. xvi + 436. London: Butterworths Scientific Publications; New York: Academic Press Inc. 1957. Price 70s.; $12.00.

This book contains a collection of the papers that were presented at the first full-scale Symposium on vapour-phase chromatography, sponsored by the Hydrocarbon Research Group of the Institute of Petroleum and held in this country in May, 1956. It is unique in that, in addition to giving first hand experiences of workers in this field, it can also be described as a textbook in gas chromatography; indeed, up to the time of its publication only one other textbook was available, namely that written by C. S. G. Phillips.

The 36 papers, given in the order in which they were presented at the Symposium, are divided into two sections. In the first section, the theoretical aspects are emphasised and fittingly the first paper is contributed by Dr. A. J. P. Martin, who with Dr. A. T. James introduced the technique in 1952. The second section contains a collection of papers that cover methods of detection of the constituents of the emergent gas stream, and there are excellent descriptions of the different detectors available at the time of the Symposium, together with workers' experiences of their use. In addition to the more usual analytical applications to small samples, there are descriptions of larger scale separations. There are papers describing conditions for the application of gas chromatography to widely different groups of substances such as petroleum hydrocarbons, chlorinated hydrocarbons, fatty acids and miscellaneous solvents.

A valuable feature of the book, the detailed discussion given at the end of each paper, has added considerably to its usefulness.

At the beginning of the book, an opportunity has been taken to include recommendations on nomenclature put forward by a group of well known early workers in the field. For a young, rapidly expanding subject, this move seems most timely and appropriate.

The Editor and publishers are to be congratulated on the production of the book and the quality of paper. It is a pity that an index has not been made available, but with a book of this type the omission can be excused. A. F. WILLIAMS

SOLVENTS. By THOMAS H. DURRANS, D.Sc., F.R.I.C. Seventh Edition. Pp. xvi + 244. London: Chapman & Hall Ltd. 1957. Price 30s.

Seven editions of "Durrans" have been published during the past 25 years. The seventh edition, although on the general lines of previous editions, has been extended considerably. In particular a great deal of attention has been paid to solvents and plasticisers for many new plastics. Analysts and chemists in the plastics industry will find this book of particular value, because it contains a great deal of valuable information about solvents and plasticisers and their trade names. Moreover, there is a refreshing British bias about a great deal of the information.

Points of criticism of a book of this kind must be few. Nevertheless, it does appear that the initial chapters, which are about such topics as "Solvent Action" and "Solvent Power," are perhaps in these days too much concerned with the cellulose plastics, and it is surprising, when so much ground is covered, that polymeric plasticisers such as polypropylene adipate and sebacate are hardly mentioned. J. HASLAM

Publications Received

STABILITY CONSTANTS OF METAL-ION COMPLEXES, WITH SOLUBILITY PRODUCTS OF INORGANIC SUBSTANCES. Part I: ORGANIC LIGANDS. Compiled from publications earlier than January 1st, 1956, by JANNIK BJERRUM, GEROLD SCHWARZENBACH and LARS GUNNAR SILLÉN, with collaboration by G. ANDEREGG and S. E. RASMUSSEN. Special Publication No. 6. Pp. xvi + 105. London: The Chemical Society, under the auspices of The International Union of Pure and Applied Chemistry. Price 60s.

CHEMICAL METHODS IN CLINICAL MEDICINE. By G. A. HARRISON, B.A., M.D., B.Ch., M.R.C.S., L.R.C.P., F.R.I.C. Fourth Edition. Pp. xii + 667. London: J. & A. Churchill Ltd. 1957. Price 65s.

EXPERIMENTS IN BIOCHEMICAL RESEARCH TECHNIQUES. By R. W. COWGILL and A. B. PARDEE. Pp. x + 189. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1957. Price $3.50; 28s.

QUANTITATIVE ORGANIC ANALYSIS. By JAMES S. FRITZ and GEORGE S. HAMMOND. Pp. xiv + 303. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1957. Price $6.50; 52s.

DAIRY BACTERIOLOGY. By BERNARD W. HAMMER, Ph.D., D.Agr., and FREDERICK J. BABEL, Ph.D. Fourth Edition. Pp. x + 614. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1957. Price $9.00; 72s.

ORGANIC REACTIONS. Volume IX. Editor-in-Chief: ROGER ADAMS. Pp. viii + 468. New York: John Wiley & Sons Inc.; London: Chapman & Hall Ltd. 1957. Price $12.00; 96s.

RECOMMENDED METHODS FOR THE ANALYSIS OF TRADE EFFLUENTS

REPRINTS of the Recommended Methods prepared by the Joint A.B.C.M. - S.A.C. Committee on Methods for the Analysis of Trade Effluents are now available from the Secretary, The Society for Analytical Chemistry, 14 Belgrave Square, London, S.W.1; price to members 1s. 6d., or to non-members 2s. 6d., each. Remittances made out to The Society for Analytical Chemistry must accompany orders, and these reprints are not available through Trade Agents.

Each reprint contains the methods published in one issue of *The Analyst*; the date of original publication is given in brackets after each title. The subjects covered so far are as follows—

Reprint No. 1. Preparation of Sample *and* Determination of Arsenic and Copper (January, 1956).
Reprint No. 2. Determination of Iron, Mercury and Nickel (March, 1956).
Reprint No. 3. Sampling and Physical Examination of the Sample (including also Determination of Settleable Solids, Total Suspended Solids, Residue on Evaporation and Dissolved Solids) (August, 1956).
Reprint No. 4. Determination of Chromium, Lead and Selenium (October, 1956).
Reprint No. 5. Determination of Organic Carbon, Chloride (Chlorion), Acidity, Alkalinity and Manganese (December, 1956).
Reprint No. 6. Determination of Non-volatile Matter Extractable by Light Petroleum and Determination of Volatile Immiscible Liquids (February, 1957).
Reprint No. 7. Determination of Hardness, Calcium and Magnesium (March, 1957).
Reprint No. 8. Determination of Combined Nitrogen (April, 1957).
Reprint No. 9. Determination of Aluminium and Zinc (June, 1957).
Reprint No. 10. Determination of Phenols and Sulphide (July, 1957).

Reprints Nos. 1 to 8 have already been announced; the availability of reprints Nos. 9 and 10 is announced here for the first time.

REPORT OF THE ANALYTICAL METHODS COMMITTEE: ORGANIC CHLORINE

THE Report prepared by the Pesticides Residues in Foodstuffs Sub-Committee, "The Determination of Small Amounts of Total Organic Chlorine in Solvent Extracts of Vegetable Material," reprinted from *The Analyst*, June 1957, 82, 378–382, is now available from the Secretary, The Society for Analytical Chemistry, 14 Belgrave Square, London, S.W.1; price to members, 1s. 6d.; to non-members, 2s. 6d. Reports of the Analytical Methods Committee are only available from the Secretary (not through Trade Agents) and remittances must accompany orders.

Errata

SEPTEMBER (1957) ISSUE, p. 651, 16th line. *For* "absorption" *read* "adsorption."

IBID., p. 651, 3rd line under "REAGENTS." *For* "10 per cent. sodium hydroxide" *read* "20 per cent. sodium hydroxide."

OCTOBER (1957) ISSUE, p. 677, 7th line of the main text. *For* "Muer" *read* "Muers."

IBID., p. 679, 9th line under Table III. *For* "valid" *read* "void."

IBID., p. 682, reference 3. *For* "Muer" *read* "Muers"; *for* "717" *read* "71T."

IBID., p. 708, reference 32. *For "Chem. & Ind." read "J. Soc. Chem. Ind."; for* "717" *read* "71T."